RESULTS & DISCUSSION
SECTION I:

PURIFIED PROLINE PERMEASE AS FUNCTIONAL UNIT.
3 RESULT AND DISCUSSION

3.1 SECTION I: PURIFIED PROLINE PERMEASE AS FUNCTIONAL UNIT.

3.1.1 INTRODUCTION

Amino acids are the chief source of nitrogen, which enters the yeast cells via different permeases. In *Saccharomyces cerevisiae*, there exist at least 23 genes, which encode amino acid permeases (*Saccharomyces* Genome Database; ANDRE, 1995; CHERRY *et al.*, 1997). These amino acid permeases can be divided into two different classes according to their function and regulation (COOPER, 1982a; COOPER, 1982b). One class of permease is active for transport of specific amino acids or chemically related set of amino acids [like, proline permease (VANDENBOL *et al.*, 1989); arginine permease (SYCHROVA & SOUCIET, 1994); histidine permease (TANAKA & FINK, 1985); lysine permease (SYCHROVA & CHEVALLIER, 1993a)], whereas other class of permeases is general and broad category of amino acids [general amino acid permease (JAUNIAUX & GRENSON, 1990) and asparagine and glutamine permease (IRAQUI *et al.*, 1999)]. Most of the advancement towards understanding the mechanism and uptake regulation of amino acids in yeast was made at genetic level. This approach had been extremely informative for haploid yeast like *S. cerevisiae*, but not so successful with diploid yeast *C. albicans* (CHRISTINA *et al.*, 1999; GOW *et al.*, 2000; HULL *et al.*, 2000; MAGEE & MAGEE, 2000).

There are almost no reports where transport of amino acids has been characterized by using purified permease protein. This has been mainly due to poor amount of permease protein in the membrane and lack of rapid and reproducible *in vitro* transport assay system. The role of L-proline as one of the strongest germ tube inducer in *C. albicans* (DABROWA *et al.*, 1976; CHO *et al.*, 1983; HOLMES & SHEPHERD, 1987; HAYASHI *et al.*, 1987) and possible involvement of its transporter in some drugs/toxic substances (CAPOBIANCO *et al.*, 1993; JETHWANEY *et al.*, 1997) have interested our group to characterize proline transport of *C. albicans* in greater detail. We could purify a proline permease from plasma membrane of *C. albicans* by using affinity chromatography and demonstrate that purified proline permease could bind and translocate L-proline (JETHWANEY *et al.*, 1997). Although, some primary and basic ligand-binding studies with purified proline permease
could be done in that study, several aspects required modifications in the purification and stabilization protocols. In the present study we have optimized the purification and subsequent stabilization of proline permease protein. As a result we now demonstrate high specific binding activity of purified proline permease to L-proline, with all previously described features retained (Jethwaney et al., 1997). By functional reconstitution of purified proline permease into proteoliposome, we could demonstrate it as a specific functional transporter of L-proline. We have also purified another permease from C. albicans, the arginine permease (Mukherjee & Prasad, 1998) and co-reconstituted into liposomes with the purified proline permease, to demonstrate the interplay of two purified permease proteins.

3.1.2 Results

3.1.2.1 Kinetics of Purified Proline Permease in Solution

In earlier studies, during course of purification of proline permease two non-ionic detergents, Tween40™ [(Polyoxyethylene (20) sorbitol monopalmitate), 0.8%, v/v] and β-OG [n-octyl-β-D-glucopyranoside (2% w/v)] were tested for solubilization of plasma membrane proteins. As, both of the detergents were found to solubilise more than 80% of total plasma membrane proteins, Tween40™ was earlier selected for solubilization procedure. Although, both Tween40™ and β-OG are non-ionic detergents, they have quite different physical properties. Table 7 shows the comparative data, which demarcate the advantage and disadvantage of using Tween40™ and β-OG for solubilization. It is clear from the Table 7 that proline permease purified using Tween40™ gave high specific activity as compared to that obtained when β-OG used during proline permease purification. The difference was found to be mainly because of presence or absence of the detergent during activity assays (discussed below). It was observed that even after subjecting prolonged dialysis, complete removal of Tween40 from purified protein was not ensured, which was reflected in high binding activity of permease. On the other hand, because of higher CMC of β-OG, it could be removed almost completely, which resulted
in loss in binding activity. In case of membrane transporter proteins, the presence of lipid or non-ionic detergent is essential to shield their hydrophobic membrane spanning regions from aqueous environment, therefore, absence of non-ionic detergent or lipids leads to loss of protein activity.

**TABLE 7. EFFECT OF TWO NON-IONIC DETERGENTS ON THE ACTIVITY OF PROLINE PERMEASE, AT VARIOUS STEPS OF PURIFICATION.**

<table>
<thead>
<tr>
<th>DIFFERENT STAGES DURING PROTEIN PURIFICATION</th>
<th>DETERTGENT USED (W/V)</th>
<th>SPECIFIC ACTIVITY (NMOLS/MG PROTEIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-OG (0.8%)</td>
<td>Tween40 (0.1%)</td>
</tr>
<tr>
<td>1 Solubilized PM (before dialysis)</td>
<td>12.42</td>
<td>10.2</td>
</tr>
<tr>
<td>2 Solubilized PM (after dialysis)</td>
<td>0</td>
<td>14.6</td>
</tr>
<tr>
<td>3 Purified Permease (0 Hr. dialysis)</td>
<td>300</td>
<td>296.2</td>
</tr>
<tr>
<td>4 Purified Permease (4 Hr. dialysis)</td>
<td>54.0</td>
<td>302.5</td>
</tr>
<tr>
<td>5 Purified Permease (10 Hr. dialysis)</td>
<td>49.3</td>
<td>312.5</td>
</tr>
</tbody>
</table>

1 Specific Activity of Proline binding to proline permease was measured as described in Section "Material and Methods". Where L-[14C] proline concentration used was 10 μM (Sp. Activity = 1 μCi /μmole) and protein estimation was done using modified Bradford microassay method.

The concentration of Tween40™ (i.e., 0.8%, v/v) used during earlier experiments was much higher than its CMC value and hence could not be dialyzed out completely. Thus sufficient concentration of the detergent was always present in the protein solution. In order to minimize the detergent concentration, it was reduced to 0.1% (v/v) in subsequent experiments, which could still solubilise 80 to 90% of plasma membrane proteins.

We observed that not only the removal of even low concentration of Tween40 detergent was difficult; its presence interfered with the protein estimation too. The presence of the detergent was found to interfere with protein estimation methods e.g., Bradford method and Warburg & Christian method (Warburg & Christian, 1941; Bradford, 1976; Compton & Jones, 1985; Caroll & O'Kennedy, 1988; Boccauccio & Quisada-Allue, 1989; Friedenauer & Berlet, 1989). As a result, in presence of up to 0.7% (v/v) Tween40 the "Bradford method" resulted up to 5-fold overestimation of protein, while a 2-

1 Tween40 has a very low CMC value (0.003 %w/v) with high membrane solubilizing property, but is very difficult to dialyze. While, β-OG with comparatively higher CMC (0.7 %w/v) allows it to get dialyzed easily, with mild solubilization property.
fold overestimation of protein was found with Warburg & Christian method (Figure 16). This problem of erroneous protein estimation was minimized by using a "Modified Bradford Micro-assay" for protein estimation, which showed minimal interference with many detergents (Figure 16C). The Modified Bradford Microassay showed almost no interference (Figure 16C), since in Modified Bradford Micro-method, proteins are co-precipitated quantitatively with calcium phosphate and the interfering substances are washed away with 80% ethanol, prior to the addition of the Coomassie dye reagent (PANDE & MURTHY, 1994).

As mentioned, Tween40 used earlier for the solubilization was much above its CMC and was therefore always present with purified protein, even after prolonged dialysis. Under these conditions there existed two kind of detergent populations at the time of performing binding-experiments: (1) permease-detergent complex and (2) detergent micelles (REYNOLDS, 1979). Consequently, there was fair chance that in addition to true permease-ligand complex, there existed detergent micelles, which might entrap significant amount of ligands and in turn leading to erroneous higher specific activity (REYNOLDS, 1979). The Scatchard Plot of the purified permease: L-proline binding shown that about 300 L-proline molecule binds per permease molecule with dissociation constant \( \left( K_{d} \right) \) of \( 10.45 \times 10^{-3} \) M, which in no case could be the actual stoichiometry of the permease protein. It was apparent that in addition to true ligand-permease binding, the ligand binding also represented by non-specific binding or ligand entrapment in detergent micelles (Figure 17A). When the excess of detergent was removed using hydrophobic SM2 bio-beads, the protein estimation became realistic and resulted in binding stoichiometry with only nine molecules of L-proline binding per permease and \( K_{d} \) of \( 58 \times 10^{-6} \) M (Figure 17B).

To verify if the modifications made in detergent concentration and its subsequent removal did not affect binding properties of permease; amino acid competitive binding experiments were performed. Out of the tested amino acids only unlabeled L-proline and its structural analogue azetidine-2-carboxylic acid could efficiently compete for L-[\( \text{C}^{14} \)]proline binding to proline permease (with an exception of L-citrulline, which could also compete for binding site but to lesser extent). Purified proline permease remained stereo-specific for L-proline, as D-proline could not compete for L-proline binding (Figure 18A & B).
Figure 16. Comparative study between protein estimation methods in presence of detergent Tween40™.

Protein estimations were made in presence of different concentrations of Tween40™ (% w/v) using (A) Bradford, (B) Warburg & Christian, and (C) Modified Bradford Micro assay methods.
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FIGURE 17. SCATCHARD PLOT OF THE BINDING OF L-[14C]PROLINE TO PROLINE PERMEASES.

(A) L-[14C]proline binding to purified proline permease using earlier un-modified method using 0.7% Tween40 as membrane solubilization agent.

(B) L-[14C]proline binding to purified proline permease using modified purification method at 0.1% Tween40 as solubilizing agent and SM2-biobeads to remove excess detergent from protein solution. Experiment performed as described in section “Material and Methods”. $S_b/E_t = \text{number of moles of L-[14C]proline bound per mole of the proline permease}$; $S_b = \text{bound L-[14C]proline}$; $S_f = \text{free L-[14C]proline}$ and $E_t = \text{total molar concentration of proline permease}$.
Figure 18. Comparison of proline transport and binding pattern through proline transport protein.

(A) L-[14C]proline binding to purified proline permease, using earlier used purification strategy. The 100% of control of L-[14C]proline (100 μM) binding corresponds to 800 nmoles/mg protein.

(B) L-[14C]proline binding to purified proline permease, using modified purification strategy. The 100% of control of L-[14C]proline (100 μM) binding corresponds to 330 nmoles/mg protein.

(C) L-[14C]proline uptake in presence of different competitive molecules, in intact cell. 100% of L-[14C]proline (100 μM) transport in cell corresponds to value 9.2 nmoles/mg dry wt of cell.
The inhibition pattern of azetidine-2-carboxylic acid has shown it to be a competitive inhibitor of L-proline binding to proline permease (Figure 19).

Results obtained distinctly show that the binding properties of purified protein remain unaltered, even after the removal of excess detergent. The basic features also matched with L-proline transport mediated by intact cells. L-proline uptake in intact \textit{C. albicans} cells have exhibited biphasic transport kinetics (Prasad, 1987; Prasad, 1991), with two kinetically distinct transport systems, one system with high-affinity ($K_T = 50$-$100$ $\mu$M) and other with lower affinity ($K_T = 1$ mM). The specificity of lower affinity transport system was found transporting broad range of amino acids, while the high-affinity system appeared as selective transporter of L-proline and structurally related analogues.

The binding specificity of purified proline permease was found in close resemblance with the reported transport properties of high-affinity proline transport system of \textit{C. albicans} cells (Figure 18C). For example, N-ethyl maleimide, a SH- group blocking agent could inhibit the L-proline binding to purified proline permease (Figure 18B), which was again consistent with the earlier reports where NEM was shown specifically to block high-affinity L-proline transport system. Interestingly the low-affinity L-proline transport system was found to be insensitive to NEM.

On comparing the transport properties of high-affinity system of intact cell with binding specificity of the purified permease, it appears that purified permease represents the high affinity L-proline transport system of \textit{C. albicans}.

Taken together, the results have shown that the modification made to protein estimation method and the removal of excess of detergent from purified protein, have not only corrected the specific activity of permease but also helped in attaining specific and more realistic L-proline-purified permease stoichiometry. In addition, the enhancement in binding specificity and activity of the purified permease as a result of modification made to protein estimation and binding assays, all the binding properties reported earlier were found retained by these modifications.
Figure 19. Woolf-Augustinsson-Hofstee Plot. Effect of L-azetidin-2-carboxylic acid on L-[14C]proline binding to purified proline permease.

1 μg of protein was added to one chamber of the equilibrium dialysis apparatus containing 100 μM L-[14C]proline, 100 mM Tris-citrate pH 5.5, and 0 μM (▲), 1 μM (●), and 10 μM (●) L-azetidin-2-carboxylic acid. The assay mixture was allowed to equilibrate for 14 hrs at 30°C and the amount of ligand bound per mg protein was calculated from the difference in radioactivity present at equilibrium in the two chambers. Binding assays were done as described in section “Materials and Methods”.
3.1.2.2 Functional reconstitution of purified Proline Permease

While working with the purified membrane permease protein, it would be ideal to functionally reconstitute it into liposomes and directly demonstrate the protein as a ligand-transporter across the liposomal membrane. As mentioned in the section “Introduction”, the main advantage of using liposomal reconstitution of purified protein is that, that it eliminates post-transport compartmentalization and metabolism. In addition it provides access to manipulate intra- or extra-vesicular solution composition, as required by the designed experiment. Therefore, as a next step towards characterization of the purified proline permease (prepared by modified protocol) as a functional membrane protein involved in L-proline transport, it was reconstituted into liposomes.

As mentioned that due to excess of detergent used earlier, its subsequent removal from purified protein was difficult. However, the removal of detergent from the purified permease is extremely important (as discussed above) and critical step while reconstitution of the permease in proteoliposomes, since even traces of detergent could result into leaky liposomes. In order to overcome the problem, we selected detergent n-octyl-β-D-glucopyranoside (β-OG) (instead of Tween40) for purification of the permease protein. β-OG due to its high CMC could easily be removed by dialysis as compared to other non-ionic detergents. As a result we could generate tight proteoliposomes impermeable to H' and consequently demonstrate that the proline transport across the proteoliposomal vesicle is also driven by H'-gradient (Figure 20B). Of note, it was observed that proline transport in cell and its binding to purified permease was optimal at lower pH (between pH 4 to pH 5), therefore it is expected that L-proline transport be mediated via H'-symport mechanism. But due to leakyness of the proteoliposomes prepared earlier, the H'-gradient driven uptake of proline was not demonstrated. As mentioned in Section- “Material and Methods”, the liposome was prepared at pH 4.5 where the entrapped inter-liposomal buffer maintained at pH 4.5. The H'-gradient across the membrane of reconstituted liposome was generated by adding a reaction buffer of pH 7.5, thus creating negative potential outside which in turn drove reconstituted purified permease mediated L-proline accumulation.

In addition, the uptake of L-proline could also be energized by artificially imposed K'-gradient, generated by the addition of valinomycin to K' pre-loaded proteoliposomes. As
shown in Figure 20A, the addition of 10 μM valinomycin to K⁺ preloaded proteoliposomes could enhance the uptake of L-[14C]proline by 10-fold, while there was almost no uptake in the absence of valinomycin induced K⁺-gradient (control). Effect similar to ‘control’ experiment was seen when outside concentration of K⁺ is kept equal to that of inside or when no K⁺ is entrapped in the vesicle. Of note, the H⁺-gradient dependent proline uptake level was less than what imposed K⁺-gradient could derive. It is possible that the vesicles still remained partially leaky to “proton ions”. For further routine uptake studies, we used K⁺-derived gradient.

The L-proline transport-kinetics in the proline permease reconstituted proteoliposomes was determined, where a half saturation constant (K₁) of 100 μM was observed. In these set of experiment, we could get much higher L-proline accumulation rate of 3944 nmoles/mg protein/min than that observed earlier by Jethwaney, D. et al. (i.e., 742.2 nmoles/mg protein/min) (Figure 21, legends). The possible explanation for 5-fold higher proline uptake rate observed this time is because the complete removal of detergent ensured more intact and non-leaky proteoliposome towards K⁺ or H⁺ ions by preventing non-productive dissipation of potential gradient.

The structural analogue (L-azetidine-2-carboxylic acid) and NH₂M, a SH-group modifying reactive agent, both could significantly reduce the L-proline uptake, while other amino acids including D-isomer of proline could not compete for transport of L-proline. These results mimic the transport specificity of a high-affinity proline transport system of intact C. albicans cells and L-proline binding specificity of purified proline permease (Figure 21). On the basis of these results, it was shown that the proteoliposome reconstituted purified permease mediates selective, stereo-specific and high-affinity transport of L-proline.

3.1.2.3 Functional co-reconstitution of the L-proline and L-arginine purified permease

To mimic the cellular condition where vast number of permeases co-exists and probably affect each-others’ functionality, we have co-reconstituted proline permease with another purified permease protein [arginine permease: Mukherjee & Prasad, 1998].
Figure 20. L-[\textsuperscript{14}C]proline uptake in proteoliposomes reconstituted with purified proline permease.

(A) L-[\textsuperscript{14}C]proline uptake was initiated by the addition of 10 \( \mu \)M valinomycin after 0 second \( (\blacklozenge) \), uptake in the absence of valinomycin imposed K\textsuperscript+}-gradient \( (\blacklozenge) \).

(B) L-[\textsuperscript{14}C]proline uptake in reconstituted proteoliposomes was initiated by H\textsuperscript+-gradient, generated by addition of 50 mM Tris-citrates pH 7.5 to transport buffer \( (\blacklozenge) \), effect of CCCP was checked by exogenously adding 25 \( \mu \)M CCCP at 25 second after start of uptake \( (\blacktriangle) \), L-[\textsuperscript{14}C]proline uptake in absence any imposed H\textsuperscript+-gradient \( (\blacklozenge) \).
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FIGURE 21. COMPARATIVE SPECIFICITY OF L-PROLINE TRANSPORT IN CELL, L-PROLINE BINDING TO PROLINE PERMEASE IN SOLUTION AND L-PROLINE ACCUMULATION IN PROTEOLIPosomes IN RECONSTITUTED PROLINE PERMEASE PROTEIN IN PRESENCE OF DIFFERENT ADDITIVES.

(A) L-[14C]proline transport in cell. (B) L-[14C]proline binding to purified proline permease and (C) L-[14C]proline accumulation in proteoliposomes. The 100% L-[14C]proline (100 μM) uptake in cell, binding of L-[14C]proline (100 μM) to purified proline permease and uptake in proteoliposome reconstituted proline permease in cell corresponds to values 9.2 nmoles/mg dry wt. of cell/sec., 330 nmoles/mg protein and 3944 nmoles/mg protein/min., respectively. The uptake rates were calculated by fitting initial uptake or binding values to simple linear regression, using least square method. The competition studies were made as described in Section “Material and Methods”.

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 Till date, co-reconstitution of two membrane proteins has been attempted only in cases, where one of them was used as source of potential gradient deriving force for the other protein (MALPARTIDA & SERRANO, 1981; MAIR & HÖFER, 1988; BISHOP et al., 1989; MALONEY & AMBUDKAR, 1989; AMBUDKAR et al., 1992; OPEKAROVÁ et al., 1993; WACH et al., 1993; OPEKAROVÁ et al., 1993; WACH et al., 1993; KNOL et al., 1996).

Wach et al. have purified plasma membrane H+-ATPase of S. cerevisiae and co-reconstituted it with bacteriorhodopsin into asolectin liposomes, where light-energized H+ pump bacteriorhodopsin allows the generation of stable transmembrane ΔpH by illumination. The pH-gradient generated was used to characterize PM-ATPase kinetics and the relation between the rate of ATP hydrolysis and the ΔpH was ascertained. Whereas, OPEKAROVÁ et al. have co-reconstituted arginine permease (Can1p) and Cytochrome-c oxidase in proteoliposomes, to study arginine transport property of former protein, where cytochrome oxidase was used to energize the arginine transport (OPEKAROVÁ et al., 1989; OPEKAROVÁ et al., 1993). The purified proline and arginine permeases have optimal pH 4.5 and 5.0 for their ligand translocation, respectively. The co-reconstitution experiments were performed at pH 4.5, since the arginine permease translocation properties did not show any significant variation between 4.5 and 5.0 pH.

Co-reconstitution of purified proline- and arginine-permease proteins were performed by adding equimolar concentration of these proteins during proteoliposome formation. Proteoliposomes were generated using reverse-phase evaporation method, where purified permease protein was added to preformed lipid-detergent film. Slowly purified permease protein got inserted into detergent-lipid micelle complexes, which upon dialysis resulted into intact lipid vesicles with inserted permease protein molecules. In the co-reconstituted proteoliposomes, K+-gradient could derive 8-fold L-[14C]proline accumulation mediated by proline permease (Figure 22A). Where, both NEM and L-azetidine-2-carboxylic could markedly and selectively inhibit proline accumulation in co-reconstituted proteoliposomes (Figure 22B), also 100-fold unlabeled (cold) L-proline could efficiently reduce L-[14C]proline accumulation because of its direct competition with L-[14C]proline for the proline permease (Figure 23B). Above results were similar to that obtained when only proline permease protein was reconstituted (Figure 21C). Thus, the transport properties of proline permease remain unaltered even in presence of co-reconstituted arginine permease.
Likewise, uptake of L-[\(^{14}\)C]arginine in co-reconstituted proteoliposomes resulted into 4-fold accumulation, as compared to non-energized proteoliposomes (Figure 24A). Expectedly, 100-fold cold (non-radiolabeled) L-arginine, L-lysine and L-ornithine competitively inhibited the L-[\(^{14}\)C]arginine accumulation mediated by arginine permease (Figure 25).

L-canavanine, an arginine analogue could inhibit the arginine permease mediated L-[\(^{14}\)C]arginine accumulation, whereas neither NEM nor L-azetidine-2-carboxylic acid could show any inhibitory effect on arginine permease (Figure 24B & 25B). So, the L-[\(^{14}\)C]arginine accumulation in co-reconstituted proteoliposomes were also found congenial with that seen when the arginine permease was singly reconstituted (Figure 27).

It was observed that upon co-reconstitution of purified proline- and arginine- permeases, there was about 15 percent decrease in fold-accumulation of both, L-[\(^{14}\)C]proline and L-[\(^{14}\)C]arginine (Figure 22A & 24A), as compared to when they were singly reconstituted (Figure 20A & 27).

The addition of L-canavanine (a toxic analogue of arginine and the selective inhibitor of the arginine transport via arginine permease; Mukherjee & Prasad, 1998) resulted in an increase in L-[\(^{14}\)C]proline uptake (Figure 22B), even higher to that obtained by L-[\(^{14}\)C]proline uptake in the absence of any competitive molecule (Figure 22A). Likewise, the specific inhibitors of the purified proline permease i.e., NEM and L-azetidine-2-carboxylic acid could enhance the L-[\(^{14}\)C]arginine in co-reconstituted liposomes (Figure 25A & B). That the increase observed in fold-accumulation of the substrate mediated by one permease upon selectively blocking the other permease was because the selective blockage of one permease provided total available gradient to the other permease.

The addition of L-proline significantly reduced the L-[\(^{14}\)C]arginine accumulation and L-[\(^{14}\)C]proline accumulation was diminished when arginine was added during the uptake experimentation (Figure 24B). This decrease in level of accumulation of L-[\(^{14}\)C]-labeled amino acid through one permease upon adding unlabeled substrate of the other permease was due to the sharing of the commonly available K\(^+\)-gradient by both the permeases (for transporting their respective substrates).
FIGURE 22. L-[14C]PROLINE UPTAKE IN CORECONSTITUTED PROLINE AND ARGinine PERMEASES IN EGG-PC PROTEOLIPOSOMES.

(A) Uptake in absence of energisation by valinomycin (●), and valinomycin energized (○); (B) competitive uptake in presence of 100-fold, L-Azetidine-2-carboxylic acid [an analogue of L-proline] ( ), NEM [600 μM] ( - ) and, L-caravanine [an analogue of arginine] (△). 1μg of each of the proline and arginine permeases was mixed together at the time of vesicle formation. L-[14C]proline (specific activity = 7.5μCi/μmole) uptake in reconstituted proteoliposomes was studied as described in Section "Material and Methods", initiated by the addition of 10 μM valinomycin after 10 s, indicated by the arrow. For competitive uptake experiment, proteoliposomes were pre-incubated with respective amino acids before the start of valinomycin induced uptake.
FIGURE 23. L-[14C]PROLINE UPTAKE IN CORECONSTITUTED PROLINE AND ARGinine PERMEASES IN EGG-PC PROTEOLIPOSOMES.

(A) Uptake in absence of energisation by valinomycin (●), and competitive uptake of in presence of 100-fold L-arginine ( ■ ); (B) competitive uptake in presence of 100-fold, L-proline ( ▼ ), L-canavanine + L-arginine (●) and, L-canavanine + NEM [600 μM] ( ▲ ). 1 μg of each of the proline and arginine permeases was mixed together at the time of vesicle formation. L-[14C]proline (specific activity = 7.5 μCi/μmole) uptake in reconstituted proteoliposomes was studied as described in Section "Material and Methods", initiated by the addition of 10 μM valinomycin after 10 s, indicated by the arrow. For competitive uptake experiment, proteoliposomes were pre-incubated with respective amino acids before the start of valinomycin induced uptake.
**Figure 24.** L-[14C]arginine uptake in coreconstituted proline and arginine permeases in EGG-PC proteoliposomes.

(A) Uptake in absence of energisation by valinomycin (●), and valinomycin energized (●); (B) competitive uptake in presence of 100-fold, L-proline (●), L-azetidine-2-carboxylic acid + L-proline (●) and, L-canavanine [an analogue of arginine] (●). 1 μg of each of the proline and arginine permeases were mixed together at the time of vesicle formation. L-[14C]-arginine (specific activity = 7.5μCi/μmole) uptake in reconstituted proteoliposomes was studied as described in Section “Material and Methods”, initiated by the addition of 10 μM valinomycin after 10 s, indicated by the arrow. For competitive uptake experiment, proteoliposomes were pre-incubated with respective amino acids before the start of valinomycin induced uptake.
**Figure 25. Competitive uptake L-[\textsuperscript{14}C]arginine in coreconstituted proline and arginine permeases in egg-PC proteoliposomes.**

(A) Uptake in absence of energisation by valinomycin (●), competitive uptake in presence of 100-fold, L-ornithine (○) and, NEM [600 μM] (●); (B) competitive uptake in presence of 100-fold, L-Azetidine-2-carboxylic acid (†), L-lysine (■) and, L- arginine (●). 1 μg of each of the proline and arginine permeases was mixed together at the time of vesicle formation. L-[\textsuperscript{14}C]-arginine (specific activity = 7.5μCi/μmole) uptake in reconstituted proteoliposomes was studied as described in Section "Material and Methods", initiated by the addition of 10 μM valinomycin after 10 s, indicated by the arrow. For competitive uptake experiment, proteoliposomes were pre-incubated with respective amino acids before the start of valinomycin induced uptake.
The addition of 100-fold L-arginine could significantly reduce the L-[14C]proline fold-accumulation (Figure 23A), since the $K^+$/gradient was then shared/utilized for both arginine and L-[14C]proline transport through their respective permeases. When L-canavanine was added together with L-arginine, proline permease could still retain unaffected L-[14C]proline uptake (Figure 23B), which further supported that even under co-reconstitution the L-proline accumulation was selectively through the purified proline permease.

Altogether, the results obtained have clearly shown that even after co-reconstitution both the permeases were found to retain their specificity, where NEM and azatidine-2-carboxylic acid specifically inhibited L-[14C]proline accumulation, while L-canavanine, L-lysine, and L-ornithine selectively competed with L-[14C]arginine accumulation through proline- and arginine-permease, respectively (Figure 26). Additionally, the transport features (i.e., both the specificity and the inhibition patterns) of proline and arginine through co-reconstituted proline and arginine permeases proteoliposome were found comparable to that obtained when proline and arginine permease were individually reconstituted permeases (Figure 21C and 27). Thus co-reconstitution of the two purified permease proteins can function independent to each other by sharing of the imposed gradients.
**Figure 26. Effect of different analogues and inhibitors on uptake rate of**

(A) L-[14C]proline; (B) L-[14C]arginine by the co-reconstituted proline- and arginine-permeases proteoliposomes. 100% of uptake rate of proline and arginine through their respective permeases corresponds to 3944 and 2626 nmoles/mg protein/min, respectively. Proteoliposomes were prepared as described in Section "Material and Methods". Proteoliposomes were pre-incubated with respective additive molecules before the start of uptake.
**Figure 27. Ligand Specificity of Arginine Permease Using Competitive L-[14C]Arginine Accumulation in Proteoliposomes in Reconstituted Arginine Permease Protein.**

L-[14C]Arginine accumulation in proteoliposomes, in presence of different competitive molecules. The 100% of control of L-[14C]arginine (10 μM) uptake rate in proteoliposome reconstituted arginine permease corresponds to values 2550 nmoles/ mg protein/ min. The competition studies were made as described in section "Material and Methods" (taken from Mukherjee, P.K. and Prasad, R. 1998).
The amino acid, L-proline is one of the major regulators of dimorphic transitions in yeast *C. albicans*, out of several conditions like, temperature, pH, and nitrogen source. In earlier studies, it was shown that L-proline transport in intact cell was driven by electrochemical gradient of protons generated by a plasma membrane bound ATPase (Jayakumar et al., 1981). The role of proline as a germ tube inducer in pathogenesis of *C. albicans* and the involvement of proline transporter proteins in transport of some drugs/toxic substances is well established (Prasad, 1987; Holmes & Shepherd, 1987; Eddy, 1988; Sychrova et al., 1989; Capobianco et al., 1993; Jethwaney et al., 1997).

Most of the amino acid transport studies made in yeasts were restricted to their kinetic in intact cells and to isolation of permease genes. The reason for this limitation was mainly difficulty in isolation and purification of amino acid permeases of yeast, due to their relatively poor amounts in the membrane. In addition, the genes coding for putative amino acid permeases of yeast have been cloned but remain to be characterized at the protein level.

Earlier we could successfully purify two amino acid transporters, proline and arginine permeases from plasma membrane of *Candida albicans* cells (Jethwaney et al., 1997; Mukherjee & Prasad, 1998). The proline- and arginine-permease were purified to homogeneity using affinity chromatography, where agarose cross-linked ligands were used. In that study Jethwaney et al. had demonstrated binding and translocation properties of purified proline permease. Recently, it was observed that the detergent used for the solubilization of the proline permease protein interfered with protein quantitation and functional assays. Therefore in the present study, the protein estimation assay was modified to minimize the interference of the detergent and subsequently the excess detergent was removed from protein to get an optimally high specific ligand binding activity of the purified proline permease.

While working with the purified proline permease, we found that the methods employed (i.e., Bradford and Warburg & Christian methods) for membrane protein and purified permease estimation strongly interfered by presence of detergents and lipids, showing up
to 2- to 5-fold overestimation of the protein. These over-estimations of protein in turn resulted into erroneous specific binding activity of the purified protein. Subsequently to avoid interference due to erroneous protein quantitation methods, we have adopted the Modified Bradford Micro-assay for protein estimation, which showed minimal interference in presence of detergent Tween40 (Figure 17C).

As mentioned in the results, Tween40 concentration used for solubilization of plasma membrane proteins was many-fold higher than critical micelle concentration (CMC) and using the conventional dialysis it could not be dialyzed out from purified permease, even below to its CMC. Therefore, the detergent was always present much above its CMC, with purified permease protein. Hence the detergent micelles were also invariably present in addition to ligand-purified permease complex, where the former could substantially mask the true binding kinetics by entrapping the free ligand molecules (REYNOLDS, 1979).

Consistently, the Scatchard Plot derived from L-proline-purified permease binding results obtained by Jethwane, et al., showed that on average 300 L-proline molecule bind per permease molecule, which could not be the actual stoichiometry of the permease-ligand binding. The binding kinetics performed after removing excess of detergent from the permease, have shown more realistic results with approximately 9 L-proline molecules binding to each permease protein. The results obtained thus far demonstrates that the higher specific binding activity (800 nmoles / mg protein) reported before was certainly attributed to non-specific entrapment of ligands within detergent micelles in addition to true permease-ligand complex.

The detergent (Tween40, 0.7% v/v) used earlier did not allow the complete removal of the detergent, which seriously hampered the functional reconstitution of purified proline permease. For example, the transport studies from intact cells suggested that the proline transport and H' motive force imposed across the liposome could not energize the uptake of L-proline when Tween40 was used (JETHWANEY, et al., 1997). Therefore we selected β-OG (0.8% w/v) as solubilizing agent (which could easily be dialyzed out while reconstituting protein into liposomal membrane). As a result we could demonstrate H'-gradient driven L-proline accumulation in liposome (Figure 21B).

After adopting the β-OG for reconstitution of purified proline permease, we could also get more than 5-fold higher accumulation rate of L-[14C]proline compared to that obtained
earlier, while the half saturation constant ($K_I$) of 100 μM and substrate specificity remained close to that been obtained earlier. The reason for this higher accumulation rate of L-[^14]C]proline was attained due to tightness and non-leakyness of proteoliposomes towards both $K^+$ and $H^+$ ions, as a result of complete detergent removal from the prepared liposome.

Further, in an attempt to mimic the cellular condition where several transporters resides together and may mutually interact and regulate each-others' function, we have purified another protein 'arginine permease' and co-reconstituted it with proline permease, to see if they have any affect on each other's functioning. The selectivity of the two permeases to different inhibitors was exploited in co-reconstitution experiments, where NEM and azetidine-2-carboxylic acid specifically blocks transport through the proline permease, while transport activity of arginine permease was reduced selectively by L-canavanine (MUKHERJEE & PRASAD, 1998). So, transport kinetics studies with one permease were made by selectively blocking the other permease and vise-versa.

For mutual interaction studies, the co-reconstituted system was exploited to manipulate the experimental conditions of competition studies with one permease at a time, where radiolabeled ligand was used for one and unlabeled ligand for the other permease (i.e., under condition when both of the permeases simultaneously transporting their respective ligands).

The results have shown that, even after co-reconstituting the two permeases, both could retain their respective transport properties independent of each other, comparable to that obtained when they were singly reconstituted in proteoliposomes. Interestingly, the presence of inhibitors of proline permease increased the transport activity of the other permease, and vise-versa. One possible reason for this could be that, they have been sharing the membrane potential driven by proton- or potassium-gradient and specific blockage of transport through one of the permease allows other permease to exploit total potential gradient present, exclusively for its substrate transport. The co-reconstitution studies have shown that both the binding and translocation specificities of proline- and arginine-permease remained the same and despite upon co-reconstitution both work independently of each other.
SECTION II:
DEVELOPMENT OF A RAPID FLUOROMETRIC METHOD FOR STUDYING BINDING KINETICS OF PURIFIED PERMEASES.
3.2 SECTION II: DEVELOPMENT OF A RAPID FLUORIMETRIC METHOD FOR STUDYING BINDING KINETICS OF PURIFIED PERMEASES.

3.2.1 INTRODUCTION

In recent years, spectroscopic techniques, like circular dichroism (CD), optical rotatory dispersion (ORD), nuclear magnetic resonance (NMR), including fluorescence spectroscopy have played an important role in studies of ligand interactions with macromolecules. One important advantage that spectroscopic techniques have over the more classical techniques, such as equilibrium dialysis, is that they can not only measure the amount of bound and free ligand in a given system but can also yield additional information on the nature of the protein-ligand interaction (CHIGNELL, 1973). These additional information includes, polarity of ligand binding site, group or parts of a ligand molecule are involved in the binding process, 3D-structure of binding sites.

Out of these methods, fluorescence spectroscopy is one of the most versatile and sensitive methods for the physical studies of the protein systems (CHIGNELL, 1973). While complementary to other physical and chemical methods, fluorescence methods often provide information not obtainable in other ways. It is been used to follow protein denaturation, conformational transitions, changes in subunit association, measuring the polarity of the binding sites, orientation and distribution of ligand (SCHULDINER et al., 1975a; SCHULDINER et al., 1975b; SCHULDINER et al., 1975c; OVERATH et al., 1979; BHATTACHARYA et al., 1996; LIU & SHAROM, 1996; LIU & SHAROM, 1997). Still, less attention has been given to the study of actual binding processes, for which fluorescence is also a powerful method. Fluorescence measurements can be made under a wide variety of conditions and large amounts of information can be obtained.

The fluorescence of a ligand molecule may be characterized by parameters like, wavelength of maximal activation and emission, quantum yield, fluorescence lifetime, and degree of polarization. Because these parameters are often exquisitely sensitive to changes in the macroenvironment of the fluorophore, it is perhaps not surprising that the
fluorescence of a ligand (or protein) molecule may be drastically altered when the molecule binds to a protein (or ligand) molecule. Studies of the mechanisms of protein interactions and conformational changes provide insight into the dynamics of protein action, and in turn contribution to the understanding many of the mechanisms underlying biological specificity.

The fluorescence techniques are been much used with cytosolic proteins, both using kinetic and equilibrium methods. But almost none of the membrane proteins are studied using the method for characterization, may be because of great difficulty to isolate membrane proteins as pure homogeneity entity and subsequent problems like, maintaining their stability in solution. As, fluorimetric method has numerous added advantages over the earlier conventional equilibrium dialysis method (BUSH & ALVIN, 1973; CHIGNELL, 1973; KLOTZ, 2000) (we have been using till now), we wanted to develop a fluorimetric method to kinetically characterize the purified proline permease. There are several disadvantages and limitations of equilibrium dialysis method, out of which few major ones are, use of bio-hazardous radiolabeled ligands and being much more time consuming. As, in our case it takes 10-12 hours for the equilibrium dialysis result to come and during this long incubation time, partial loss of protein activity becomes unavoidable (As, keeping membrane protein stable in buffer solution is very strenuous and time dependant).

The ability of many proteins to bind non-biological “artificial probes,” which are designed to mimic natural ligands or to bind to hydrophobic protein regions, has been utilized in studying protein binding. Here, we have used “dansyl-aminoc acids” as artificial probe (ligand) to study proline permease-ligand interactions. We have exploited the fluorescence intensity quenching of dansyl-L-proline (Dns-proline) after its binding to the active site of proline permease, for studying kinetic parameters of the permease-ligand interactions.

This new developed fluorescence method has not only surmounted the limitations of equilibrium dialysis method, but also has many added advantages spanning from resulting in increased protein activity to reusability of the experimentally used-protein. The reusability has main attraction for the protein biochemists (especially for the biologists working with purified membrane proteins, which amounts only < 0.5 % of total cellular protein under normal conditions), enabling them to reuse expensive proteins with minimal loss of activity.
3.2.2 RESULTS

To check if the fluorescence developed method could successfully adopted for characterization of other membrane proteins, we have purified another transporter protein, arginine permease from *C. albicans* (which earlier being purified in our laboratory; Mukherjee & Prasad, 1998) and used it along with proline permease for binding studies. Out of most of the dansylated amino acids used, only dansylated proline and arginine were able to show the quench in fluorescence intensity, after their binding to purified proline and arginine permeases, respectively.

3.2.2.1 Fluorescence properties of Dansyl-L-amino acids binding to the purified permeases

Dansyl-L-proline has excitation maxima at 330 nm and emission maxima at 535 nm. Binding of Dansyl-L-proline to the proline permease, resulted into quench in its fluorescence intensity accompanied by the shift of emission maxima towards longer wavelength by 20 nm. Whereas, dansyl-L-arginine binding to arginine permease, lead to slight blue shift in the emission maxima along with quench in fluorescence intensity (Figure 28). The above results shown that the Dansyl-L-proline binding site of proline permeases resides in the hydrophobic environment whereas, arginine binding occurs in the hydrophilic microenvironment of arginine permeases.

3.2.2.2 Dansyl-L-proline binding kinetics to proline permease

When the fluorescence of dansyl-L-proline was plotted as a function of the molar ratio between the dansyl-L-proline and the proline permease, the degree of quenching is seen to be dependent upon the concentration (Figure 29). The Scatchard plot derived from the fluorescence titration curve obtained (Fletcher & Ashbrook, 1973; Chignell, 1973), by addition of increments of dansylated ligand to a fixed amount of the purified permease (Figure 30). Plot indicated that on average there exists 1.5 ligand-binding site per proline permease molecule, with dissociation constant of $2.1 \times 10^{-7}$ M. The specificity of the permeases to their ligand(s) is tested using other different dansylated amino acids as ligand...
Experiments were carried out at 30 °C, with excitation at 330 nm. (A) Dansyl-L-proline (green line) alone, and in presence of 40 μg/ml purified proline permease (blue line). (B) Dansyl-L-arginine (green line) alone, and in presence of 40 μg/ml purified arginine permease (blue line). The experiment is performed as described in section "Material and Methods".
The quenching of the fluorescence of Dnasvi-L-proline after binding to the purified proline permease. The activation and the emission wavelength were 330 and 535 nm. The bandwidths were 10 nm. The concentration of purified proline permease were $2.521 \times 10^{-7}$ M ( ), $2.521 \times 10^{-6}$ M ( ). All solutions contained 0.1 M Tris-citrate buffer (pH 5.5).
FIGURE 30. SCATCHARD PLOT OF THE BINDING OF DANSYL-L-PROLINE TO PROLINE PERMEASE.
Results were obtained from the fluorescence titration curve in Figure 29. All solutions contained 0.1M Tris-citrate buffer, pH 5.5 for proline permeases. \( S_b/E_t \) = number of moles of Dns-L-proline bound per mole of the proline permease; \( S_b \) = bound Dns-L-proline; \( S_t \) = free Dns-L-proline and \( E_t \) = total molar concentration of proline permease.
and no binding found, only dansyl-L-proline and dansyl-L-arginine binds specifically to proline and arginine permeases, respectively. Half saturation constant ($K_m$) values of both the proline permease and arginine permease, correlate well with that found using equilibrium binding method (Figure 31 & 32). But, comparatively the specific activity and $V_{max}$ values were found higher for the new fluorescence-quench method (Table 8). The relatively diminished permease activity and $V_{max}$-value obtained using equilibrium dialysis method, was probably because of longer incubation time (i.e., 14~16 hrs) of equilibrium dialysis method, during which partial thermal denaturation of protein became inevitable. Whereas, rapidity of fluorescence method kept ensured the optimal binding activity of the permease.

### Table 8. Comparative kinetic features of purified proline and arginine permeases by two binding assay methods.

<table>
<thead>
<tr>
<th></th>
<th>Proline Permease</th>
<th>Arginine Permease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>S.A.$^*$</td>
</tr>
<tr>
<td>Equilibrium dialysis</td>
<td>100.0</td>
<td>50</td>
</tr>
<tr>
<td>Fluorescence method</td>
<td>104.17</td>
<td>250</td>
</tr>
</tbody>
</table>

$^*$S.A. (Specific activity, nmol/min/mg protein) of both permease proteins determined at 10μM ligand concentration. $K_m$ determined in μM and $V_{max}$ was determined in nmol bound per mg protein.

3.2.2.3 Specificity of proline- and arginine- permease for different amino acids: using fluorescence study

Subsequently, we have checked for binding specificity of different amino acids and their analogs to permeases, by adding 100-fold concentration of the competitive molecule(s) after binding of the dansylated ligands to their respective permeases. The relative quench in fluorescence intensity in presence or in absence of the competitive molecule, was plotted as the percentage change in binding activity of the dansyl-L-proline or dansyl-L-arginine to purified proline- and arginine-permeases, respectively. Similar competitive studies were made in parallel using equilibrium dialysis method for both the permeases, to see any variation obtained as a result of using two different methods.
FIGURE 31. LINEWEAVER-BURK PLOT OF PROLINE BINDING TO PURIFIED PROLINE PERMEASE IN SOLUTION.

(A) Binding kinetics of Proline permease to L-[C\textsuperscript{14}]proline using equilibrium dialysis method. L-[C\textsuperscript{14}]proline used in range of 10 μM to 3 mM, with specific activity of 1 μCi/μmoles.

(B) Binding kinetics of proline permease to Dns-L-proline. Binding was performed as described in section “Material and Methods”. Dansyl-L-proline used with in 1 to 10 μM concentration range.

The values are arithmetic mean ±S.E.M. of three determinations.
**FIGURE 32. LINeweaver-Burk Plot of arginine binding to purified arginine permease in solution.**

(A) Binding kinetics of arginine permease to L-[C$^{14}$]arginine using equilibrium dialysis method. L-[C$^{14}$]arginine used in range of 2 μM to 100 μM, with specific activity of 1 μCi/μmoles.

(B) Binding kinetics of arginine permease to Dns-L-arginine. Binding was performed as described in section “Material and Methods”. Dansyl-L-arginine used with in 1 to 10 μM concentration range.

The values are arithmetic mean ±S.E.M. of three determinations.
As, results clearly shown that though the fluorimetric method resulted in higher activity of both proline- and arginine-permeases, there was apparently no change seen in specificity of the respective permeases using both of the methods (Figure 33).

As discussed earlier, the presence of lipids is important for stabilization while purifying membrane permeases. So, to ascertain role of egg-phosphatidylcholine (egg-PC) in permease activity, the binding of Dns-L-amino acid(s) to purified permeases were done in presence of egg-PC (3:1). The addition of egg-PC resulted into slight increase in the ligand-binding activity of the permeases, due to their binding and shielding the hydrophobic stretches, and thus assisting the permease in the attainment of a more compact native conformation (Figure 33).

Although, it is been reported in earlier publications that the dansylated-ligand bind non-specifically to the membrane lipids, but they have made studies in intact cells or in isolated membrane sheets (SCHULDINER et al., 1975a; SCHULDINER et al., 1975b; SCHULDINER et al., 1975c; OVERATH et al., 1979). In contrast, we have been working with purified permease having only trace amount of lipids or detergents sufficient to stabilize the native permease conformation. That also taking proper account of effect caused (if any) by additives used during binding experiments, by keeping all additives used in buffer minus purified permease(s) in the reference cuvette.

3.2.2.4 Fluorescence method and reusability of purified permease

Our results till now, have shown that due to the rapidity of this fluorescence method, protein stability is ensured with significant rise in activity as compared to that obtained using equilibrium method. To see if this added advantage could be exploit in terms of reusability of the same protein for further experiments. To substantiate this possibility, we have selected ultra-filtration dialysis method to dialyze out both the bound and free fluorescent-tagged ligands from protein solution.

The ultra-filtration dialysis method was selected on the basis of its many-fold advantages, including its rapidity with almost complete removal of ligand molecules and other additives (more than 99%) while concentrating the protein, without any adsorption of protein to the membrane surface.
FIGURE 33. EFFECT OF DIFFERENT AMINO ACIDS, INHIBITORS AND EGG-PC ON BINDING OF PERMEASES.

A. Binding of 10 μM dns-L-proline or L-[14C]proline to proline permease. (a) control, (b-I) in presence of 100-fold concentration of L-proline, D-proline, L-arginine, azatidine, NEM (600μM), L-citrulline, GABA, egg-PC/permease (3:1), L-canavanine, L-ornithine, D-arginine respectively.

B. Binding of 10 μM dns-L-arginine or L-[14C]arginine to arginine permease (a) control, (b-I) in presence of 100-fold concentration of L-arginine, D-arginine, L-canavanine, L-proline, L-citrulline, GABA, L-ornithine L-tyrosine, L-aspartate, NEM(600 μM), egg-PC/permease (3:1), respectively. Where, (■) bars stands for L-[14C]amino acids binding using equilibrium dialysis and (□) bars corresponds to dansyl-L-amino acids binding using fluorescence method to their corresponding permeases.
The protein activity was rechecked, after applying rapid ultra-filtration dialysis to used permease (Table 9). As, results clearly shown that we could reuse the permease for further experiments, while retaining more than 80% of the permease activity.

**Table 9. Reuse of the purified permease proteins for binding to their dansylated ligands.**

<table>
<thead>
<tr>
<th></th>
<th>Proline Permease (Dansyl-L-Proline as ligand)</th>
<th>Arginine Permease (Dansyl-L-Arginine as ligand)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>Dialysed Protein</strong></td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Reused Protein</strong></td>
<td>81.8</td>
<td>87.02</td>
</tr>
</tbody>
</table>

1. The binding of the dansylated-ligands (10 μM) to the respective permeases.
2. The bound dansylated-ligand is dialyzed out from ligand-permease complex (1) by applying ultra-filtration and residual fluorescence left in resulted protein fraction was checked.
3. The dialyzed permease proteins (2) were reused for their binding with respective dansylated amino acids.
3.2.3 DISCUSSION

As mentioned in the Section-I, for characterization of purified permeases, we have been using equilibrium dialysis method. While studying binding kinetics of purified permeases, we realized that there are several disadvantages and limitations of equilibrium dialysis method, out of which few major ones are, use of bio-hazardous radiolabeled ligands and being much more time consuming. As, in our case it takes 10-12 hours for the equilibrium dialysis result to come and during this long incubation time, partial loss of protein activity becomes unavoidable.

Recently, to study ligand interactions with macromolecules (like, proteins and DNA), people started using spectroscopic techniques and fluorescence spectroscopy is one of the most versatile and sensitive methods for the physical studies of the protein systems (BUSH & ALVIN, 1973; KLOTZ, 2000; CHIGNELL, 1973). It has been used to characterize binding processes, and to measure the polarity of the ligands binding sites (SCHULDINER et al., 1975a; SCHULDINER et al., 1975b; SCHULDINER et al., 1975c; OVERATH et al., 1979; BHATTACHARYA et al., 1996; LIU & SHAROM, 1997; LIU & SHAROM, 1996). Another important advantage that fluorescence techniques have over the more classical techniques, such as equilibrium dialysis, is that they can yield additional information on the nature of the protein-ligand interaction in addition to measuring the amount of bound and free ligand in a given system. These additional information includes, polarity of ligand binding site, group or parts of a ligand molecule are involved in the binding process.

The fluorescence of a ligand molecule may be characterized by parameters like, wavelength of maximal activation and emission, quantum yield, fluorescence lifetime, and degree of polarization. Because these parameters are often exquisitely sensitive to changes in the microenvironment of the fluorophore, it is perhaps not surprising that the fluorescence of a ligand (or protein) molecule may be drastically altered when the molecule binds to a protein (or ligand) molecule (CHIGNELL, 1973; BUSH & ALVIN, 1973). As, fluorimetric method has numerous added advantages over the earlier conventional equilibrium dialysis method (we have been using till now), we wanted to develop a rapid fluorimetric method to kinetically characterize the purified proline permease. Here, we
have used "dansyl-amino acids" as artificial probe (ligand) to study proline permease-ligand interactions. We have exploited the fluorescence intensity quenching of dansyl-L-proline (Dns-proline) after its binding to the active site of proline permease for studying kinetic parameters of the permease-ligand interactions.

Binding of Dansyl-L-proline to the proline permease resulted into quenching in fluorescence with shift of emission maxima from 535 nm to 555 nm (Figure 28A), clearly indicates that the microenvironment of Dansyl-L-proline binding site is hydrophilic in nature. The above finding correlates very well with the previous finding that NEM could specifically block L-proline binding to purified proline permease, while DTNB could not inhibit the binding activity. Whereas, dansyl-L-arginine binding to arginine permease, lead to slight blue shift in the emission maxima (Figure 28B), which shows that the arginine binding site situates in hydrophobic environment.

Upon titration of the constant purified permease with varying concentrations of Dansyl-L-proline, the degree of fluorescence quenching was seen to be proportionately dependent upon the concentration (Figure 29). Further Scatchard plot derived from the same titration curve, indicated that on average there exists 1.5 ligand-binding site per proline permease molecule, with a dissociation constant of $2.1 \times 10^{-7}$ M (Figure 30). Lineweaver-Burk Plot has shown that, half saturation binding constant ($K_m$) values of both the proline permease and arginine permease correlate well with that found using equilibrium binding method (Figure 31 and 32). But, comparatively the $V_{max}$ values obtained from fluorimetric method were found much higher than that obtained by equilibrium dialysis method (Table 8).

As the equilibrium dialysis method takes longer incubation (i.e., 14-16 hrs) and during this experimental period the partial thermal denaturation of permease became unavoidable, which in turn resulted into lower protein activity. On the contrary, rapidity of fluorescence quenching method kept ensured the almost complete retention of protein activity during experimental process.

The binding specificity of different amino acids and their analogs to proline- or arginine-permeases, have shown that there were almost no change in features of respective permease(s), using both equilibrium dialysis method and fluorimetric methods (Figure 33). Upon evaluating and comparing kinetics and specificity of purified proline- and arginine-
permease proteins of C. albicans, using both equilibrium dialysis and fluorescence methods, evidently, all the tested parameters of binding activity, as determined by both the methods for the two-permease proteins were comparable. As the fluorimetric quenching being very rapid method compared to equilibrium dialysis method, we have exploited this method in combination with ultrafiltration for speedily removal of dansylated ligand from purified permease to extend the novel method for protein reusability for further experiments. The results distinctly shows that protein used in fluorescence binding experiments could successfully reused for further experiments, while retaining more than 80% of the permease activity (Table 9). The new developed fluorescence method, has not only surmounted the limitations of equilibrium dialysis method, but also resulted into many added advantages spanning from, increased protein activity to reusability of the experimentally used-protein. The reusability has main attraction for the protein biochemists (especially for those working with purified membrane proteins, which amounts only < 0.5 % of total cellular protein under normal conditions), enabling them to reuse expensive proteins with minimal loss of activity.

In future the applicability of this method can also be extended to the determination of active site conformation and amino acids involved (by selective amino acid labeling and cross-linking reagents), western blotting, fluorescence, circular dichroism, and also in thermodynamic studies in protein folding and stability. We anticipate that fluorescence-tagged ligands can be successfully used to monitor binding activity of purified permease proteins of different origin. By using fluorescent ligands this method can be extendible to monitor ligand protein interaction of any isolated proteins.
SECTION III:
MOLECULAR CLONING AND CHARACTERISATION OF GENES WHICH COMPLEMENT L-PROLINE TRANSPORT AND GROWTH DEFECT OF S. CEREVISIAE MUTANT.
3.3 SECTION III: MOLECULAR CLONING AND CHARACTERIZATION OF GENES WHICH COMPLEMENT L-PROLINE TRANSPORT AND GROWTH DEFECT OF S.CEREVISIAE MUTANT.

3.3.1 INTRODUCTION

Like other yeasts, Candida albicans also selectively uses wide variety of the nitrogenous compounds that they find in their rich habitat, where amino acids are the main nitrogen source. On the basis of the studies made with intact cells, there exists several different amino acid permeases in the C. albicans with overlapping specificity and affinity (Prasad, 1987; Prasad, 1991). In order to understand the molecular basis of their function, it would be useful to isolate and study the respective genes.

Accordingly, several amino acid transporter genes have been cloned and characterized in S. cerevisiae, which falls mainly under two groups; general and specific transport systems. The general amino acid permeases encoded by GAP1 and AGP1 genes are low-affinity and can transport broad range of amino acids, while specific amino acid permeases are high-affinity transporters selective for one or a few related amino acids. These permease genes are members of the conserved amino acid permease (AAP) gene family that includes about 20 members and are differentially expressed (Tanaka & Fink, 1985; Hoffmann, 1985; Jauniaux et al., 1987a; Jauniaux & Grenson, 1990; Andre et al., 1993; Sychrova & Chevallier, 1993a; Sychrova & Chevallier, 1994; Graulund et al., 1995; Andre, 1995; Cherry et al., 1997).

The nitrogen regulated permeases, the general AAP (Jauniaux & Grenson, 1990), and the proline specific permease (Vandenbol et al., 1989) are high-capacity systems that are induced by growth on low-quality nitrogen source, and their expression enables the cell to use amino acids as sole nitrogen source (Courchesne & Magasanik, 1983). Majorities of other AAP family members are low-capacity, high-affinity amino acid permeases, which are specific to one or a few related L-amino acids. However, unlike S. cerevisiae very few amino acid permease genes have been cloned and characterized in C. albicans. This has been mainly due to limited advancement in molecular genetics of the diploid C.
albicans. It has now become possible to clone and disrupt genes of this human pathogen. Accordingly, a few permease genes of C. albicans have recently been cloned, by functional complementing the heterologous host like S. cerevisiae.

A genomic library of C. albicans was used to functionally complement a S. cerevisiae lyp1 and can1 mutant, to clone LYP1 & CAN1 genes of C. albicans (SYCHROVA & CHEVALLIER, 1993a; SYCHROVA & CHEVALLIER, 1993b; SYCHROVA & SOUCIET, 1994). The putative C. albicans lysine permease (lyp1p) has 571 amino acids and a calculated molecular weight of 63.3 kDa, and is highly homologous to S. cerevisiae Can1p and Lyp1p. The transport mediated by Lyp1p was found specific for lysine, arginine, histidine and had a high-affinity for lysine and arginine, resembling the earlier reported S2 system for lysine (RAO et al., 1986; SYCHROVA et al., 1993). The CAN1 homologous gene in C. albicans found encoding a high-affinity permease protein, specific for arginine transport (Hoffmann, 1985; AHMAD & BUSSEY, 1986; SYCHROVA & SOUCIET, 1994).

Data available from Candida Genome Sequencing Project (where till date more than 90% of its genome have been sequenced), suggest that there exists more than 10 putative amino acid permeases in its genome (http://www.alces.med.umn.edu/bin/genelist). In preceding chapters, the purification of the proline permease and its properties therein were described. This section deals with the isolation of genes, which helped to restore proline transport defect of a S. cerevisiae mutant.

3.3.2 RESULTS

3.3.2.1 Functional complementation of growth defect of the S. cerevisiae mutant

The S. cerevisiae strain 22574d carries point-mutated nonfunctional three permeases involved in proline transport [general amino acid (GAP1), proline (PUT4), and γ-aminobutyric acid (UGA4) permease genes (JAUNIAUX et al., 1987a)]. Therefore, unlike the wild type strain 21278b, the mutant (22574d) is incapable of growing on medium containing proline as the sole N-source due to lack of both low- and high-affinity proline uptake systems (GRENSON et al., 1970; JAUNIAUX et al., 1987b; JAUNIAUX & GRENSON, 1990; ANDRE et al., 1993). To identify genes from C. albicans, which could complement the defect of 22574d, we functionally complemented it by genomic libraries of C. albicans
procured from different sources (Table 10). Several thousand Ura+ transformants resulted from complementation, were replica-plated on minimal medium containing L-proline (200μg/ml) as the only N-source. Out of these, 57 clones could show growth on L-proline as sole nitrogen source after 4-6 days.

### Table 10. List of Genomic Libraries of C. Albicans Used in This Study

<table>
<thead>
<tr>
<th>Strains of C. albicans</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1001</td>
<td>pYEURA3</td>
<td>C. Nombela, Madrid</td>
</tr>
<tr>
<td>1001</td>
<td>Yep352</td>
<td>C. Nombela, Madrid</td>
</tr>
<tr>
<td>655</td>
<td>p1041</td>
<td>S. Scherer, Berkeley</td>
</tr>
</tbody>
</table>

### 3.3.2.2 Growth characteristic of the transformants

We subsequently checked the growth prototypes of all the transformants on other amino acids; where the L-proline growing transformants were replica plated on different concentrations of amino acids to pick up genes involved in amino acid transport with different specificity or spectra. As discussed earlier, at lower concentrations γ-amino butyric acid (GABA) specifically enters only through inducible GABA permease (Uga4p), whereas D-proline and L-citrulline are the two amino acids, which specifically enters yeast cell through general amino acid permease (Gap1p).

Consequently, these transformants were also checked for their growth on D-proline (1 mM) and L-citrulline (1 mM) and GABA (50 μM & 500 μM) plates as the sole nitrogen source, to see if they complement Gap1p / Put4p / or Uga4p (Table 11). Most of the transformants showed different growth prototypes and were divided into three groups; (1) that can grow between 25-50 μM L-proline, (2) at 100-500 μM L-proline and (3) at 500 μM and above concentrations (Table 12). As indicated a few from each category were selected for further analysis. Two closely related transformants were selected from each of the three above-mentioned categories (i.e., 1C & 3C, 15C & 23C, 3S3 & 8S3), to determine if they could mediate L-proline uptake. Interestingly, it was observed that not only they grew on different concentrations of L-proline, they could also transport L-proline (of note, the mutant was unable to do both).
TABLE 11. GROWTH PATTERN OF TRANSFORMANTS ON DIFFERENT AMINO ACIDS.

<table>
<thead>
<tr>
<th>TRANSFORMANT</th>
<th>L-PRO 25 µM</th>
<th>L-PRO 50 µM</th>
<th>L-CIT 1 mM</th>
<th>D-PRO 1 mM</th>
<th>GABA 50 µM</th>
<th>GABA 500 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOMBELLA CENTROMERIC (NC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>-</td>
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<td>++++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3C</td>
<td>-</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>4C</td>
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</tr>
<tr>
<td>5C</td>
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<tr>
<td>7C</td>
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<td>++++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8C</td>
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<td>-</td>
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</tr>
<tr>
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<td>-</td>
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</tr>
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<td>13C</td>
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<tr>
<td>15C</td>
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<tr>
<td>16C</td>
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<td>24C</td>
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</tr>
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<td>36C</td>
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<td>37C</td>
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</tbody>
</table>

NOMBELLA EPISOMAL (NE)

<table>
<thead>
<tr>
<th>TRANSFORMANT</th>
<th>L-PRO 25 µM</th>
<th>L-PRO 50 µM</th>
<th>L-CIT 1 mM</th>
<th>D-PRO 1 mM</th>
<th>GABA 50 µM</th>
<th>GABA 500 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1E</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4E</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5E</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>6E</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
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<td>-</td>
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</tbody>
</table>

SCHERER 655-3 (S-3), SCHERER 655-4 (S-4)

<table>
<thead>
<tr>
<th>TRANSFORMANT</th>
<th>L-PRO 25 µM</th>
<th>L-PRO 50 µM</th>
<th>L-CIT 1 mM</th>
<th>D-PRO 1 mM</th>
<th>GABA 50 µM</th>
<th>GABA 500 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>353</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>853</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The different amino acids used as sole nitrogen source, and are added to the YNB minimal plates (without ammonium sulphate, and amino acids) medium supplemented with 2% glucose and the respective concentrations of amino acids as mentioned above (from the sterile stocks). Relative growth of the transformants after 4-5 days is indicated +/- signs, starting from + indicating very slow growth to ++++ a very good growth and - sign indicates no visible growth.
Table 12. Categorization of Transformants depending upon growth on different concentrations of L-proline as sole N-source.

<table>
<thead>
<tr>
<th>L-Proline (Conc.)</th>
<th>Transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-50 µM</td>
<td>1E, 3E, 3S3, 8S3</td>
</tr>
<tr>
<td>50-500 µM</td>
<td>1C, 3C, 23C, 38C, 4E</td>
</tr>
<tr>
<td>500 µM and above</td>
<td>2C-22C, 24C-37C, 5E, 6E</td>
</tr>
</tbody>
</table>

The growth curve of transformants on L-proline as sole nitrogen source and L-proline transport have clearly shown that the reacquired growth of the mutant on proline as sole nitrogen source was in fact due to their acquired ability to transport proline (Figure 34).

In order to confirm that the regained function of mutant strain was contributed by transformant DNA fragment of C. albicans, transformants were grown in non-selective media. After several generations these transformants led to the loss of the plasmid and the resulting cells were found to be unable to grow in proline or transport proline. Thus, the regained function of the mutant was due to the functional complementation.

To further confirm that no reversions had occurred in the transformants, the recombinant plasmids were isolated and reintroduced into the mutant to show that the growth in YNB-Pro was dependent on the presence of pSS1 (PTH), pSS4 (PTH1), or pSS3 (P1112) (Figure 39). Reintroduction of PTH1 and PTH2 plasmids back to the mutant strain could independently complement the growth defect of the mutant strain. The mutant transformed with vector only (mock) could not show any growth on YNB-Pro plate (Figure 39, inset).

Although all of the transformants could grow on L-proline, but on the basis of growth patterns only 1C could grow on L-citrulline. D-proline and GABA as well (Table 11). Unlike two earlier cloned genes encoding specific permeases (Can1p and Lyp1p), 1C appears to be complementing an amino acid transporter with much broader substrate specificity.

3.3.2.3 Analysis of cloned DNA fragment of pSS1

The plasmid from the 1C transformant was isolated and amplified in E. coli strain (D115α). The XbaI- SalI endonuclease restriction enzymes digestion showed a distinct ~ 4.4 kb insert (pSS1), which was designated as PTH (Proline Transport Helper).
Several *PTH* sub-clones were constructed in pYeJra3 expression vector and transformed back into mutant strain (22574d), to ascertain the minimal functionality of the insert (Figure 35). The subclones carrying either *Xbal-PstI* fragment (pSS4) or *PstI-Smal* fragment (pSS5) of *PTH*, could complement the mutant for the growth on proline as sole N-source (Figure 36). While, *Xbal-Xhol* and *Xhol-PstI* fragments do not complement the growth mutation. Clones harboring pSS4 and pSS5 were named PTH1 and PTH2, respectively.

### 3.3.2.4 Sequence Analysis of PTH fragment.

For sequencing of complete PTH, several subclones were constructed in pBluescript II SK (+) vector (Stratagene) (Figure 36). pPTH-1, pPTH-2, pPTH-3, pPTH-4, and pPTH-5 were obtained by inserting *Xbal-PstI*, *PstI*, *Xhol-PstI*, *EcoRI* and *Xhol* fragments of pPTH into pBluescript II SK (+) (Stratagene vector). Sequencing was performed on both the DNA stands of *PTH* fragment by the dedoxy-chain termination method (SANGER et al., 1977), using strategy shown in Figure 36. Additionally, to accomplish complete sequencing of PTH insert, five oligonucleotide primers were also used; (1) 5'-GGGTGAGTCAGAATGTAGAC-3'. (2) 5'-CACTCAACACAGTAAATTAC-3'. (3) 5'-GAAAAGTAAAAAGTGCTTAG-3'. (4) 5'-TGTGGTCGCCCTTGCTGCG-3'. and (5) 5'-GTTGGGAGGAGAATCTGCTC-3' (Figure 36).

Sequencing has shown PTH fragment with nucleotide sequence of 4487 bp, carrying two putative open reading frames (ORFs). Upon complete sequencing it was found that minimal inserts of PTH, i.e., PTH1 and PTH2, which could complement the mutant for the growth on proline as sole N-source (Figure 37). Analysis of the PTH insert have shown that the both open reading frames PTH1 and PTH2 with length of 1134 bp & 369 bp, which encode for 377 & 122 amino acid proteins, respectively.

Nucleotide sequence analysis of PTH1 and PTH2 revealed the presence of TATA-like motifs at position -244 and -394 having A+T rich 5' region of the initiation codon ATG with correlated CCAAT-box, respectively, as assessed by using the EUKPROM algorithm (PC Gene, IntelliGenetics) (Figure 37). More putative promoter elements were found to be localized in the 5' untranslated region of PTH1 and PTH2 genes. During promoter element search in PTH1 gene, most striking was the presence of putative nit-2 binding element in
**PTH1** with a perfectly matching consensus sequence (-444 TATCTA); two heat shock factor binding sequences (HSF) (-223 AGAAA and -227 AGAAA); a putative AP-1 binding element (AP-1) (-313 GTGAGTCAG) with respect to ATG. NIT-2 is the positive-acting nitrogen regulatory gene encoding a sequence specific DNA binding protein involved in activation of nitrogen-regulated genes. While, AP-1 is a member of *jun* family of bZip transcriptional activators which binds sequence specific site (TGACTCA) including GCN4p (the 5'TGACTCA-3' sequence element is common in GCN4/AP-1 sites, also the amino-terminal domain of AP-1 is homologous to C-terminal DNA-binding domain of GCN4p).

The presence of two more putative response element (GCR1) (-453 GTCTTCCCT) and (ADR1) (-310 TGGGTG) were found to be localized at position mentioned with respect to ATG. Where, GCR1/SIT3 is high-affinity DNA binding protein regulate the expression of glycolytic genes. ADR1 is a zinc finger transcription factor, which binds to DNA and regulates peroxisomal genes. Two highly conserved putative promoter elements C/EBP (-534 AATTTTTCAAATA) and (HSF) (-471 TTTCT) were found in *PTH2* gene. The putative promoter elements present in *PTH1* and *PTH2* are listed in Table 13.

**Table 13. Putative Promoter Elements in PTH1 and PTH2.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Consensus Sequence</th>
<th>Sequence Present</th>
</tr>
</thead>
<tbody>
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<td>TATA</td>
<td>TATATTA</td>
<td>GTTTATATGCTAGTA</td>
</tr>
<tr>
<td></td>
<td>NIT-2</td>
<td>TATCTA</td>
<td>TATCTA</td>
</tr>
<tr>
<td></td>
<td>HSF</td>
<td>AGAAA</td>
<td>AGAAA</td>
</tr>
<tr>
<td></td>
<td>AP-1</td>
<td>GTGACTCAG</td>
<td>GTGACTCAG</td>
</tr>
<tr>
<td></td>
<td>GCR1</td>
<td>GTCTTCCCT</td>
<td>GTCTTCCCT</td>
</tr>
<tr>
<td></td>
<td>ADR1</td>
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<td>GGGGTG</td>
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<td>C/EBP</td>
<td>AATTTTTCAAATA</td>
<td>AATTTTTCAAATA</td>
</tr>
<tr>
<td></td>
<td>HSF</td>
<td>AGAAA</td>
<td>AGAAA</td>
</tr>
</tbody>
</table>
3.3.2.5 Chromosomal localization of PTH1 / PTH2

The chromosomal localization of the PTH showed that it mapped to Chromosome 5 SfiI fragment M of C. albicans, near PDE11, THR4, and GCD11 genes. The XbaI- SalI 4.487 kb complete PTH insert hybridized to fosmids 1B5, 7B8, 8D6, 9D9, 12C9, 17D7, 18E2 (courtesy Magee, B.B., USA). The nucleotide sequence of the PTH fragment was submitted to the EMBL/GenBank/DDBJ databases under accession number Y18210, appeared on 14-MAY-1999, and last updated on 05-OCT-1999.

3.3.2.6 Pth1p/ Pth2p are not homologous to permease proteins

Both Pth1p and Pth2p proteins showed no homology to any membrane transporter and in addition, no significant homology to any existing proteins in the database (discussed below). Hydropathy analysis of these proteins suggested that they are more of hydrophilic in nature, which also lacked hydrophobic transmembrane domains typical of membrane proteins (Figure 38).

The PTH1 ORF encodes a protein of 377 amino acids with a calculated molecular mass of 43.01 kDa and pl of 7.93 and as suggested by the hydropathy plot, Pth1p is a hydrophilic protein. PTH2 ORF on the other hand encodes a protein of 122 amino acids with a molecular mass of 14.26 kDa, a basic (pl = 9.62) and hydrophilic protein (Figure 38).

The protein sequence analysis of Pth1p and Pth2p proteins was made using ‘Protein software’ of (DNASTAR Scientific Software Co.) and motif searches were made using MotifFinder software available at Internet site (http://www.motif.genome.ad.jp/motif-bin/).

A cAMP-dependent protein kinase phosphorylation site at position 2 (KRWT) in Pth2p and numerous casein kinase II phosphorylation sites and other protein kinase C phosphorylation sites present in both Pth1p and Pth2p may suggest their functional importance. Important features of PTH1 and PTH2 encoded proteins are summarized in Table 14.
Figure 34. (A) Growth Curves of Transformant Strains; 1C, 3C, 15C, 23C, 3S3 & 8S3 and (B) Their L-Proline Transport Kinetics.

For both growth curve and transport assays, transformants were grown on YNB-Pro media, at 30 °C and L-[C\textsuperscript{14}] proline (100 μM) uptake measurements were done as described under Materials and Methods, at 30 °C and pH 4.5.
Figure 35. Restriction map of the PTH nucleotide fragment of chromosome V cloned in pSS1 plasmid and various subclones. Only the restriction sites pertinent to the cloning experiments are indicated with their nucleotide positions within the PTH sequence. The horizontal lines indicate the DNA segments present in the various plasmids and the segments that were sequenced. The arrows indicate the extent of the PTH1 and PTH2 genes. The ability of each plasmid to complement the L-proline uptake mutation and restore normal uptake of L-[14C]-proline is indicated on the right.
<table>
<thead>
<tr>
<th>X</th>
<th>X</th>
<th>H</th>
<th>E</th>
<th>X</th>
<th>M</th>
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<tr>
<td>M</td>
<td>E</td>
<td>Sw</td>
<td>S</td>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| pPTH | pPTH-1 | pPTH-2 | pPTH-3 | pPTH-4 | pPTH-5 |

**Figure 25. Restriction map and sequencing strategy of the 4487 bp PTH (pSS1) insert.**

pPTH-1, pPTH-2, pPTH-3, and pPTH-5 were obtained by inserting XbaI-PstI, PstI, XhoI-PstI, XhoI and XhoI fragments of pPTH into pBluescript SK-1 vector. Restriction sites are represented as follows: X, XhoI; Xb, XbaI; H, HpaI; E, EcoRI; M, MspI; P, PstI; V, VspI; Sw, Smal; S, SmaI. Sequencing was performed by the dideoxy-chain termination method (Sanger et al., 1977). Arrows denote sequencing directions and lengths of the sequence data obtained from the fragments. The primer 3'-end primers (●) and five 8-mer oligonucleotide primers (○) synthesized according to the determined pPTH sequences. The open rectangles indicated the PTH1 and PTH2 open reading frames (ORFs) and the box in pPTH indicates the vector backbone.
Figure 37. Nucleotide sequences of PTH1 and PTH2 and the ORFs position with their deduced primary structures. Nucleotides are numbered (right), with +1 at the initiator ATG codon; amino acids are numbered (left) and displayed under the codons.
FIGURE 38. HYDROPATHY PLOTS OF (A) PTH1 PROTEIN, AND (B) PTH2 PROTEINS. Hydrophobicity (below the line) and hydrophilicity (above the line) values were determined using Kyle and Doolittle algorithm (ref.). They were obtained using ‘SOAP’ Software of ‘PC Gene Sequence Analysis Package’, by averaging hydropathy values for a window of 15 amino acid residues acids (Gravy = -4.59).
### Table 14. Features of PTH1p and PTH2p.

<table>
<thead>
<tr>
<th></th>
<th>PTH1</th>
<th>PTH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotides (ORF)</td>
<td>1134 bp</td>
<td>369 bp</td>
</tr>
<tr>
<td>Amino Acids</td>
<td>377</td>
<td>122</td>
</tr>
<tr>
<td>Molecular Weight</td>
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<td>19.06 kDa</td>
</tr>
<tr>
<td>Isoelectric Point</td>
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</tr>
<tr>
<td>Transmembrane Domains</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Putative N-glycosylation site</td>
<td>Five sites (Positions)</td>
<td>One site (Position)</td>
</tr>
<tr>
<td>PKC phosphorylation site</td>
<td>Six sites (Positions)</td>
<td>One site (Position)</td>
</tr>
<tr>
<td>CKII Phosphorylation site</td>
<td>Five sites (Positions)</td>
<td>Two sites (Positions)</td>
</tr>
<tr>
<td>Myristyl N-myristoylation site</td>
<td>Two sites (Positions)</td>
<td>Two sites (Positions)</td>
</tr>
</tbody>
</table>

Charged, basic, acidic, hydrophobic and polar amino acids are: RSHEYDDE, KRDE, NCQSTY, ALFWV, respectively. Where, PKC and CKII stands for Protein Kinase C and Casein Kinase II, respectively.

3.3.2.7 L-proline transport in yeast transformants.

Analysis of transport characteristics of PTHd, PTH1d and PTH2d strains showed that these transformants are able to transport 10- to 20-fold L-proline, as compared to the mutant and mock (vector only) transformed strains (Figure 40). The PTHd clone which carries both *PTH1* and the *PTH2* genes have shown maximal uptake (20-fold) as compared to 11- and 17-fold uptake shown by PTH1d and PTH2d clones, respectively.

The kinetics studies of restored proline uptake showed that PTH1d and PTH2d had half saturation constants (*K*<sub>T</sub>) of 150 μM and 800 μM, respectively (Figure 41). It appeared that *PTH1d* complements a high-affinity transport system as compared to that by PTH2d.

To further ascertain that the retained growth on L-proline and uptake of L-proline of mutant strain (22574d) was neither due to the diffusion nor due to the altered membrane
permeability towards L-proline, uptake was performed at different temperatures (between 4 °C and 50 °C). Results have shown that at temperature of 30 °C uptake rate in PTH1d and PTH2d clones were 0.036 and 0.021 (nmoles/mg dry wt. of cell/s), whereas at 4 °C, the proline uptake rate was only 0.003 and 0.002 (nmoles/mg dry wt. of cell/s), respectively (Figure 39A).

The proline uptake in transformant strains was sharply reduced with decrease in temperature and reached almost to the activity of non-transformed yeast, where both mutant and mock-transformed mutant strains have not shown any significant change in basal level of uptake rate determined between 4°C and 30°C.

The increase in the L-proline uptake at 30 °C compared with that at 4 °C, was 5 to 6-fold greater than that expected from a change due to diffusion, which has a change in rate with 10 °C increase (Q10) of ≈1.2 and would result in a change in uptake rate of ≈2.1-fold between 4 °C & 30 °C. The temperature dependence of the uptake confirmed that the increased uptake of proline in the transformant strains are protein mediated, which had an optimal pH 4.5 (Figure 42B).

3.3.2.8 Substrate specificity of transport system complemented by PTH1 and PTH2

Although it was apparent from the above results that, both PTH1d and PTH2d clones could complement the proline transport, kinetically they appear very different from each other. Competition studies revealed that PTH1 mediates a specific L-proline uptake, since only its structural analogue azetidine-2-carboxylic acid (Azt) could substantially inhibit L-proline transport (Figure 43). The restored uptake was stereo-specific, as there is no inhibition of L-proline uptake by D-proline.

The amino acids, which could also compete with L-proline uptake mediated by PTH1d were GABA, tyrosine and glutamic acid, but the extent of inhibition was less than 40% (Figure 43A).

On the other hand, mediated proline uptake in PTH2d strain showed broader specificity for amino acids (Figure 43B). It appeared that the PTH2 complements a low-affinity amino acid transporter with broad substrate specificity. In S. cerevisiae only Gap1p mediates the uptake of both, L- and D-amino acids.
FIGURE 39. GROWTH CURVES OF WILD TYPE Σ1278b, MUTANT 22574d, PTH1d, PTH1d, AND PTH2d STRAINS.

Wild type and mutant strains were grown on YPD media, and PTH1d, PTH1d, PTH2d strains were grown on YNB-proline media, at 30 °C. In inset shown the growth of wild type Σ1278b, mutant 22574d, PTH1d, PTH1d, and PTH2d strains on YNB-Pro minimal plate. Table below shows the generation time for each of the transformant clones calculated as described in section “Material and Methods.”
FIGURE 40. UPTAKE OF L-[\textsuperscript{14}C]PROLINE IN WILD TYPE Σ1278b, MUTANT 22574b, PTHd, PTH1d, AND PTH2d STRAINS.

Uptake was assayed in wild type and mutant strains grown on YPD media, and PTHd, PTH1d, PTH2d strains were grown on YNB-proline media. Uptake measurements were done as described under Materials and Methods, at 30 °C and pH 4.5.
FIGURE 41. LINEWEAVER-BURK PLOT OF L-[\textsuperscript{14}C]-PROLINE UPTAKE BY PTHd, PTH1d, AND PTH2d AT DIFFERENT CONCENTRATIONS OF PROLINE (5 µM-3 mM). 490 µl aliquots from a 5% wet weight suspension of mid-exponential grown cells were added to the reaction mixture (100 mM Tris-citrate pH 4.5, 200 µg/ml cycloheximide, and indicated concentrations of L-[\textsuperscript{14}C]-proline (Sp. Activity = 3 µCi/µmole), in a final reaction volume of 700 µl). The reaction was started by the addition of L-[\textsuperscript{14}C]-proline (Sp. Activity = 3 µCi/µmole), and 100 µl aliquots withdrawn at intervals as described under section "Material and Methods." Data represent the average of 3-4 independent sets of experiments and the standard deviation was less than 5% in each case.
**Figure 42.** L-[^14]C PROLINE UPTAKE IN PTH1D AND PTH2D CLONES AS A FUNCTION OF (A) TEMPERATURE, AND (B) pH.
Figure 43. Effect of different amino acids on L-[^14]C|proline uptake in (A) PTH1D; (B) PTH1D; (C) PTH2D strains. Percentage inhibition for each amino acid was calculated by comparing rate of uptake in presence and absence of the 100-fold amino acid or structural analogue, at pH 4.5. Uptake measurements were done as described under section “Materials and Methods”. Data represent the average of 3-4 independent sets of experiments and the standard deviation was less than 5% in each case.
In order to ensure if the complemented function by \textit{PTH2} was due to the restored Gap1p activity, the competitive effect of citrulline on L-[\textsuperscript{14}C]proline uptake was determined. Citrulline is exclusively transported by the general amino acid permease, thus can compete with proline uptake only when the GAP1 permease function is restored. As shown in the figure 43B, \textit{PTH2} mediated L-[\textsuperscript{14}C]proline uptake was inhibited by citrulline, while in case of \textit{PTH1d} strain, citrulline did not compete (Figure 43B). Thus, \textit{PTH1d} and \textit{PTH2d}, both strains could restore transport of L-proline but apparently through two very different transport systems. Interestingly, the competitive studies with \textit{PTH1d} strain (carrying full \textit{PTH} insert) showed more of mixed type of results (Figure 43A).

Out of amino acid permeases involved in L-proline transport only PUT4 and GAP1 permeases could show ammonium repressibility (NCR) of transport activity (Lasko & Brandriss, 1981). In order to establish if the restored growth and transport function elicit ammonium repressibility, it was followed in \textit{NH}_4\textsuperscript{+} grown cells. It was found that the L-proline transport was severely effected when \textit{PTH1d} and \textit{PTH2d} strains were grown on YNB-NH\textsubscript{4}\textsuperscript{+} (Figure 44). This ammonium repressibility of \textit{PTH1} and \textit{PTH2} mediated restored transport is found consistent with the growth pattern of the transformants on YNB-Pro and YNB-NH\textsubscript{4}\textsuperscript{+}. Where, only \textit{PTH} transformants could grow on L-proline as sole nitrogen source (YNB-Pro), while their growth became sensitive upon supplementing a toxic proline analog, azetidine-2-carboxylic acid (Azt) to the media (which, enters through proline transporter proteins) (Figure 45C). But, Azt failed to inhibit the growth, when transformants were grown on YNB-NH\textsubscript{4}\textsuperscript{+} (Figure 45D). This restored Azt-resistance was due to \textit{NH}_4\textsuperscript{+}-repression of regained L-proline transport activity complemented by the transforming genes, which in turn stops the entry of toxic Azt into the transformants.

Only mutant and mock transformants strain could grow in presence of Azt (a toxic proline analogue), while \textit{PTH1d}, \textit{PTH1d} & \textit{PTH2d} strains could not grow on Azt (Figure 45C). This growth loss of \textit{PTH1} and \textit{PTH2} transformants was because toxic Azt could enter via regained transport activity of Put4p & Gap1p. Thus, results obtained comprising of affinities, competition and ammonium repression studies of L-proline transport, together have supported that the transport systems restored in \textit{PTH1d} and \textit{PTH2d} strains are high-affinity Put4p and Gap1p, respectively.
RESULTS AND DISCUSSION

Figure 44. Effect of ammonium repression on 1 mM L-[14C]-proline uptake in (a) PTH1d; (b) PTH1d; and (c) PTH2d strains.

The strains were grown on YNB minimal medium (w/o amino acids and ammonium sulphate) plus 1 mM L-proline in presence or absence of 10 mM ammonium sulphate. Uptake is measured at pH 4.5. L-[14C]proline used was at Sp. Activity - 3 μCi/μmole), and done as described under section "Materials and Methods". Data represent the average of 3-4 independent sets of experiments.
Figure 45. Growth of the 22574d mutant, mutant transformed with pYeUra3 plasmid containing PTH, PTH1 & PTH2, and 22574d mutant transformed with pYeUra3 plasmid only (Mock) on YNB-Pro (w/o amino acids and ammonium sulfate) supplemented with specified additives. (A) Streaking pattern of strains grown on (B) YNB-Pro, (C) YNB-Pro plus Azetidine-2-carboxylic acid, and (D) YNB-NH$_4$ plus Azetidine-2-carboxylic acid.
3.3.2.9 Effect of Energy Inhibitors and Cytoskeletal Disruptant Cytochalasin E on proline uptake.

The transformed yeast strains appeared to take up L-proline from the external medium by two independent transport systems. We further checked the effect of energy inhibitors on proline transport in these cells (Figure 46). Most of the energy inhibitors including proton ionophore CCCP could lead to a remarkable (more than 80 percent) drop in restored L-proline uptake in PTH1d strain (Figure 46B).

Almost the same extent of inhibition could be seen for PTH2 mediated transport, but sodium vanadate and cytochalasin E had shown relatively lesser extent of inhibition (approximately 50 percent only) of L-proline uptake (Figure 46C). Inhibition by proton ionophore CCCP clearly supported that the L-proline transport complemented by PTH clones, is mediated through a amino acid membrane transporter (as proton coupled transport of amino acid is typical feature of yeast AAP family).

Further, cytochalasin E (a cytoskeleton disruptant; MACLEAN-FLETCHER & POLLARD, 1980; COOPER, 1987) was selected as one of the inhibitors, since PTH showed homology over short stretch of actin binding protein domain. In order to check if cytoskeleton could play any role in L-proline transport in transformant cells, we have used cytochalasin E (80µg/ml) to disrupt cytoskeletal organization. Addition of cytochalasin E resulted in more than 90% inhibition of proline uptake in PTH1d strain (Figure 46). Whereas, all the inhibitors have shown no effect on the background level of proline transport in mock transformed mutant 22574d, demonstrating that the residual uptake shown by mutant cells have little or no proton coupled transport of proline and is energy independent too (Figure 46D).

To further counter check whether cytochalasin E inhibitory effect seen is specific to the PTH transformants or is a more generalized effect, we have also checked the effect of cytochalasin E on the uptake of different amino acids in wild type strain 10261 of C. albicans. Expectedly, we could see remarkable decrease in different amino acids uptake in 10261 strain, showing that the organization of the cytoskeleton affect amino acid transport (Figure 47). The result supports that apart from its role in helping their right insertion in
the membrane, cytoskeleton has a dynamic role in stabilizing the membrane transporters (Liu et al., 1996; Liorca et al., 1999).

3.3.2.10 Functional Expression of PTH1 and PTH2 in other amino acid permease mutants

Since, PTH1 and PTH2 encoded proteins are not membrane transporter proteins, but could restore transport of proline, we wanted to explore if they only restore Gap1 and Ptt4p function, or could also help in restoring other amino acid transport systems. Two mutant strains, HS100-3C (gap1-1, lyp1, can1-1, ura3Δ) and HS50-7 (lyp1, can1-1, lys, ura3Δ) were transformed with PTH1, PTH2 and PTH (which carries both PTH1 and PTH2). [Where, HS100-3C has non-functional general amino acid, lysine-specific and arginine-specific permeases, whereas, HS50-7 has non-functional mutations in lysine- and arginine-specific permeases (Sychrova & Chevallier, 1993a).]

HS50-7 was unable to grow on YNB-NH₄⁺ medium supplemented with uracil (10 μg/ml) and low concentration of lysine (3 μg/ml), (under these conditions GAP1 is repressed by NH₄⁺ and no transport system for lysine is present). For transformation, the cells were grown on YNB-Pro medium (derepression of GAP1) supplemented with uracil and lysine. Plasmids carrying PTH, PTH1 or PTH2 were used to transform both HS100-3C and HS50-7 stains and Ura+ transformants were selected on YNB plates and then replica plated on YNB-Lysine (3 μg/ml) plates. Out of several Ura+ transformants obtained after transforming HS50-7 with PTH, PTH1 and PTH2, one transformant was selected from each and were subsequently designated as PTH-7, PTH1-7, PTH2-7, respectively.

Similarly, out of several Ura+ transformant obtained after transforming HS100-3C with PTH, PTH1 and PTH2, and one transformant resulting from each plasmid was selected that could grow on YNB-Lysine, and were designated PTH-3C, PTH1-3C, PTH2-3C, respectively. Unlike HS50-7 transformants, HS100-3C transformants PTH1-3C and PTH2-3C could grow on L-lysine or L-arginine as sole nitrogen source and were sensitive to L-canavanine (Figure 48B & C).

Consistent with the growth of PTH-3C and PTH2-3C strains could show significant uptake of L-proline, L-lysine and L-arginine as compared to that in the mutant (Figure 49).
Figure 7: Effect of energy inhibitors, and cytochalasin E on L-[14C]-proline uptake in PTHd, PTHd, PTH2d, and vector transduced (mock) strains.

Uptake was compared in presence and in the absence of the inhibitor, at pH 4.5. L-proline concentration used was 1 mM. Uptake measurements were done at 60 seconds, rest remain the same as described under section "Materials and Methods". Cells were pretreated for 10 min with DNP (5 mM), CCCP (25 μM), sodium azide (10 mM), sodium vanadate (10 mM), cytochalasin E (30 μg/mL) before assaying for transport. Uptake was started by addition of L-[14C]-proline. cells were washed on nitrocellulose filters, and the radioactivity retained on dry filters was calculated by liquid scintillation counting.
FIGURE 47. EFFECT OF CYTOCHALASIN E ON UPTAKE OF DIFFERENT $[^{14}C]$-RADIO-LABELED AMINO ACIDS. L-proline, L-arginine, L-glutamate, L-leucine, L-aspartate, and L-lysine at concentration of 100 μM in *C. albicans* ATCC10261 strain. Uptake for each amino acid was checked at 90 seconds time interval from the start of transport experiment, and compared with the rate in presence and in the absence of the inhibitor, at pH 4.5. Uptake measurements were done as described under section "Materials and Methods". Cells were pre-incubated for 10 min with cytochalasin E (80 μg/ml) before assaying for transport. Uptake was started by addition of $[^{14}C]$-radio-labeled amino acids, cells were washed on nitrocellulose filters, and the radioactivity retained on dry filters was calculated by liquid scintillation counting.
When the uptake of L-arginine, L-lysine and L-proline in PTH-3C, PTH1-3C and PTH2-3C strains were performed under ammonium repressing condition (NCR), marked repression of uptake activity of these amino acids was observed (Figure 49). Also, further reduction in background L-proline transport in HST00-3C was observed under NCR condition, which was due to the repression of proline transport mediated by wild type Put4p (Figure 43A). Since, only Gap1p transport activity is NCR regulated (Grenson, 1983a; Jauniaux et al., 1987a; Sychrova & Chevallier, 1993b; Sychrova & Souciet, 1994; Opekaro et al., 1998), the ammonium repressible transport results suggests that, the complemented growth and transport phenotype in PTH-3C and PTH2-3C strains were solely due to PTH2 restored transport activity of GAP1 mutant permease. Thus, Pth1p and Pth2p specifically restore the transport activity of the mutated Put4p and Gap1p and do not complement other mutated permease proteins.
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**Figure 48. Growth of the mutant strains transformed with pYeUra3 plasmid containing PTH, PTH1 & PTH2, and mutants transformed with pYeUra3 plasmid only (Mock), on YNB-Lysine (w/o amino acids and ammonium sulfate) supplemented with specified additives.**

Growth of HS100-3C mutant transformants (A) Streaking pattern of strains grown on (B) YNB-Lysine, and (C) YNB-Pro plus L-canavanine. Growth of HS50-7 mutant transformants (D) Streaking pattern of strains grown on (E) YNB-Pro plus L-canavanine.
FIGURE 49. EFFECT OF AMMONIUM DEPRESSION ON 100 μM L-PROLINE, L-ARGININE, AND L-LYSINE UPTAKE IN PTH1d-3C, PTH1h-3C, PTH2d-3C, AND HS100-3C STRAINS. The strains were grown on YNB minimal medium (two amino acids and ammonium sulphate) plus 1 mM L-proline in presence or absence of 10 mM ammonium sulphate. Uptake is measured at pH 4.5. L-[14C]amino acids used were at Sp. Activity of 1 μCi/μmole), done as described under section “Materials and Methods”. Data represent the average of 3-4 independent sets of experiments.
3.3.3 Discussion

Functional complementation of 22574d mutant (defective in proline transport) gave several transformants that could grow on L-proline as the only N-source. One such transformant carrying DNA fragment of 4.487 kb (PTH; Proline Transport Helper) was finally selected. Upon complete sequencing PTH was found to harbor two putative open reading frames (ORF) named PTH1 and PTH2. The PTH1 and PTH2 genes were independently cloned and reintroduced in the mutant strains to establish that the complemented function. The 10- to 20-fold higher L-proline uptake in PTH1-d and PTH2-d strains clearly established that the growth of mutant on proline as sole N-source was indeed due to the retained L-proline uptake. Effect of energy inhibitors confirmed that the complemented proline transport in PTH1 and PTH2 was energy-dependent and proton-coupled.

Interestingly, though both PTH1 and PTH2 mediate L-proline uptake, the kinetics and competitive proline uptake studies have shown that both restored different L-proline transport systems. Where, PTH1 mediates a high-affinity proline-specific, while PTH2 mediates a low-affinity with low-specificity transport system. The PTH1 and PTH2 complemented proline transport activity was ammonium ion repressible (NCR). In S. cerevisiae only three amino acid transporters are reportedly NCR regulated, i.e., GAP1, PUT4 and DAL5 (a high-affinity allantoin permease). It would mean that PTH1 complemented and restored Put4p mediated transport while, PTH2 restored a general amino acid permease.

In recent years, the molecular cloning has proven to be an effective tool for isolation of genes encoding proteins involved in amino acid transport not only from yeasts but also from mammalian systems. Although, a number of cDNAs identified from animal systems that stimulate amino acid transport, most of these proteins did not resembles integral membrane proteins (WANG et al., 1991; PACHOLCZYK et al., 1991; BERTRAN et al., 1992a; BERTRAN et al., 1992b; TATE et al., 1992; WELLS & HEDIGER, 1992; PINES et al., 1992; KANAI & HEDIGER, 1992; SEGEL et al., 1992; LIN et al., 1996; TAKAGI et al., 2000). Similarly, in an attempt to clone a proline transporter gene of C. albicans, we have also
encountered *PTH1* and *PTH2* genes encoding hydrophilic proteins, which themselves are not transporter proteins but are still involved in transport of proline.

Since, the mutant strain has non-functional point mutations in all three proline permease proteins and *PTH1* and *PTH2* proteins are not transporter proteins, the question arises as to how *PTH1* and *PTH2* could help in retaining the transport activity of otherwise non-functional amino acid permeases. The Pthlp and Pth2p do not show any homology to proteins existing in the Protein Databases; they do however show some notable similarities in conserved domains of the proteins. For example, Pthlp contains regions with considerable sequence homology to chaperonin proteins conserved motifs (the regions displaying the highest degree of sequence conservation are shown in Figure 50). It also has significant homology to *S. cerevisiae* ORF YGR046w with 40% identity and 57% similarity, over 250 amino acids towards C-terminal region of Pthlp. This overlapping region of YGR046w and Pthlp are also found conserved in other proteins from *S. pombe*, *A. thaliana*, *D. melanogaster* (http://dot.imagen.bcm.tmc.edu:9331/; http://pedant.mips.biochem.mpg.de/ Complete; http://genome-www.stanford.edu/ Saccharomyces/; http://www.rcsb.org/pdb/; http://dove.embl-heidelberg.de/Blast2/) (Figure 51). The YGR046w ORF disruption have been found lethal in *S. cerevisiae*, and two-hybrid interaction studies have shown it to be physically interacting with *SIN4* gene in *S. cerevisiae*, where *Sin4p* is reported as a global regulator protein (http://portal.curagen.com).

The Pth2p has shown homology to Pac2p protein (a c-AMP independent regulatory protein) involved in mating of *S. pombe* and its homologue ORF YHI.007w of *S. cerevisiae* (up to 70% homology over 107 amino acids towards N-terminal of Pth2p). A stretch of 42 amino acid was found very conserved in Pth2p, Pac2p, YHI.007w and another protein Gti1p “involved in gluconate transport” from *S. pombe*, which probably constitute a conserved functionally important domain (Figure 52). The N-terminal sequence of Pth2p protein has also shown significant similarity to *S. cerevisiae* proteins, Hal5p and YHR177w over 65-70 amino acid stretch 30 to 43% identity and 48-69% similarity (Figure 52). It is interesting to note that the protein encoded by *HAL5* and *YHR177w* are serine/threonine protein kinases, in the absence of any precise function
known for the proteins with which the Pth1p and Pth2p have shown homology, no direct role can be assigned to these ORF's. However, on the basis of the limited homology shown with chaperone and some regulatory proteins (like, Hsp90, Pac2p and Hal5p), there could be two possible rationales that could explain the restored activity of point mutated non-functional GAP1 and PUT4 permeases. First possibility could be that, Pth1 and Pth2 proteins directly interact with the mutated Gap1p and Put4p present on the plasma membrane, and thus somehow restores their functions. It is also possible that Pth1 and Pth2 proteins assist in trafficking of mutated but still potentially functional Gap1p and Put4p permeases to plasma membrane, enabling them to escape endoplasmic reticulum/Golgi quality control machinery. The later probability appears to be more expedient, since, earlier using anti-Gap1p antibodies Jauniaux et al. have shown that in 22574d mutant strain, the mutated Gap1p permease was not assembled at plasma membrane (unpublished results). The result obtained by them suggested that the mutated Gap1p was defective in its targeting to plasma membrane and perhaps remained in ER/Golgi sorting pathway, or are accumulated in sub-plasma membrane structures.

Like most of the proteins, integral membrane proteins destined for the plasma membrane have to undergo strict quality control trafficking through sorting machinery comprised of ER/ Golgi complex (Nothwehr et al., 1995; Ellgaard et al., 1999). Where, ER/ Golgi sorting decides the fate of the protein and its localization to intended destinations (Broxterman et al., 1996; Loayza et al., 1998; Ellgaard et al., 1999; Wickner et al., 1999; Aridor & Banch, 2000; Hill & Cooper, 2000). ER mediated quality control of synthesized proteins mainly works by structural rather than by functional criteria. However, the purely architectural nature of selection can lead to problems and at times potentially functional mutant proteins are retained because of minor structural defects (Nothwehr et al., 1993; Nothwehr et al., 1995; Ellgaard et al., 1999).

Recently, it has been shown that there is a specialized transport step for delivery of Gap1p and Put4p to the plasma membrane (PM) that requires Sec13p function and is regulated by nitrogen source. It has been also shown that Gap1p take route to the vacuole in sec13-1 mutant cells (Roberg et al., 1997a; Roberg et al., 1997b; Bryant & Stevens, 1998; Beck et al., 1999; Beck & Hall, 1999)
FIGURE 50. SEQUENCE ALIGNMENT OF PTH1P WITH CHAPERONIN PROTEINS ANC2P AND TCP1-LIKE PROTEIN, FROM SACCHAROMYCES CEREVISIAE AND MUS MUSCULUS (MOUSE), RESPECTIVELY.

Amino acids with identity and similarity are shown in blue and yellow shaded boxes, respectively. The horizontal lines indicate the absence of the corresponding amino acid residues at this position. The chaperonin proteins of this class contains highly conserved amino acid signature motif: \( [\text{RKEL}]:[\text{ST}]-\text{X}[\text{LMFY}]:[\text{G}-\text{P}-\text{X}-\text{X}-\text{K}]:[\text{LIVMF}] \). The GeneBank accession number of Pth1p, Anc2p and Tcp1_mo are Y18210, P39078 and P80315, respectively.
Amino acids with identity and similarity are shown in blue and yellow shaded boxes, respectively. The horizontal lines indicate the absence of the corresponding amino acid residues at this position. The GeneBank accession number of hypothetical proteins from *S. cerevisiae*, *S. pombe*, *A. thaliana*, *D. melanogaster* and *C. elegans* are P53230, O74339, AL132955, AF003706 and AC024201, respectively.
**Figure 52. Sequence alignment of Pth2p with Pac2p, Gti1p, Hal5p, and hypothetical proteins from Saccaromyces cerevisiae, S. pombe, Plasmodium falciparum, Neisseria meningitidis and Ureaplasma urealyticum.**

Amino acids with identity and similarity are shown in blue and yellow shaded boxes, respectively. The horizontal lines indicate the absence of the corresponding amino acid residues at this position. The GeneBank accession numbers: Pac2p (Q10294), Gti1p (X92655), Hal5p (P38970), and hypothetical proteins from S. cerevisiae, YEL007w, YHR177w, Plasmodium falciparum, Neisseria meningitidis and Ureaplasma urealyticum, are U18530, U000027, AL034560, U73942, AE002142 and AE002113, respectively.
In sec13-1 pep12Δ double mutant (where, *pep12Δ* blocks transport of proteins from Golgi to the pre-vacuolar compartment at 24 °C), however lead to accumulation of Gap1p at the Golgi and result in non-specific packaging of Gap1p into cargo-vesicles by *SEC13*-independent process, which in turn effect the transport of Gap1p from Golgi to PM. It is thus possible that when the pathway for transport from the Golgi to the prevacuolar compartment is blocked, vacuolar membrane proteins can be transported to the cell surface instead (NOTHWEHR et al., 1995). During earlier studies it has been shown that some vacuolar protein sorting mutants could suppress certain missense alleles of *CIN1*, the gene encoding arginine permease (JONES, 1983) and particularly, mutations in *PEP12* are capable of suppressing *can1* mutations (BECHERER et al., 1996). In *pep12Δ*, the absence of a functional transport pathway from Golgi to the vacuole allows the concentration of membrane proteins that would otherwise be transported to vacuole (FISCHER VON MOLLARD et al., 1997).

The possible involvement of *PTH1* and *PTH2* during post-Golgi sorting of mutant Put4p and Gap1p can not be excluded, whether by blocking pre-vacuolar targeting or specifically assisting the packaging of Put4p and Gap1p (in particular, from the Golgi to cargo-vesicles destined to plasma membrane) (COHEN et al., 1999; HAMILTON et al., 1999; LAU et al., 2000). Although, the accumulation of these mutant proteins in post-ER compartments are yet to be ascertained, altogether the results obtained with 22574d, HS100-3c and HS50-7 mutants transformed with plasmids carrying *PTH1* and *PTH2* genes suggest that, *PTH1* specifically implicated in retaining transport activity of a high-affinity proline transport system (Put4p). While, *PTH2* restores a broad and lower affinity amino acid transporter (Gap1p).