Replacement of Lys by Glu in a transmembrane segment strongly impairs the function of the uracil permease from *Saccharomyces cerevisiae*

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The co-transport of uracil and protons through the plasma membrane of the yeast *Saccharomyces cerevisiae* is mediated by a specific permease encoded by the *FUR4* gene. The uracil permease is a multi-spanning membrane protein that follows the secretory pathway to the plasma membrane. Recent experimental data led to the proposal of a two-dimensional model of its topology. A spontaneous mutant corresponding to the substitution of Lys-272 by glutamic acid was obtained. The influence of this mutation was studied by comparing the wild-type and mutant permeases produced in a strain carrying a chromosomal deletion of the *FUR4* gene. The mutant permease is correctly

targeted to the plasma membrane and its stability is similar to that of the wild-type permease. The uptake parameters for the mutant permease were impaired and showed an approximately 65-fold increase of apparent K_m and a decrease in apparent V_{max} . Equilibrium binding measurements with enriched plasma membrane preparations showed an approximately 70-fold increase in apparent K_d in the mutant, whereas its B_{max} was similar to that of the wild type. Lys-272 is fully conserved in the uracil permease family and is predicted to lie in the fourth transmembrane segment of the protein. It seems to be essential for both efficient uracil binding and translocation.

INTRODUCTION

Cells contain carrier-type transport proteins to import actively exogenous nutrients which may be present only at low concentration in the medium. These transporters (or permeases) utilize electrochemical gradients to move solutes across membranes. The amino acid sequences of many of these integral membrane proteins have been deduced: they are extremely hydrophobic with a core of 10–12 putative membrane-spanning regions. These transmembrane segments contain some charged amino acids, the function of which has been investigated for some co-transporters, including the lactose carrier, the melibiose permease, the proline carrier, the tetracycline antiporter of *Escherichia coli* (see [1] for review), and the γ -amino butyric acid and glutamate neurotransmitter transporters from the mammalian brain [2,3]. Studies of mutations implicate three charged residues in the lactose carrier in an H^+ charge-relay mechanism (see [4] for review). However whether this motif is a model of a common proton pathway in carriers is unclear. It has been suggested that positively and negatively charged amino acids in the membrane domain may neutralize each other and so be stabilized in a hydrophobic environment by creating salt bridges. Experiments with second-site suppressor mutants of the lactose carrier are consistent with such pairing and give some insight into the spatial arrangement of transmembrane domains (see [4] for references).

The yeast *Saccharomyces cerevisiae* carries numerous plasma membrane transporters which have been the subject of extensive physiological and genetic investigations (reviewed in [5,6]) and more recently biochemical investigations [7–11]. The genes encoding more than 35 of these yeast permeases have been cloned and sequenced. To our knowledge, the possible involvement of charged residues in the activity of a yeast transporter has not been investigated.

Uracil transport-defective yeast strains (*fur4* mutants) were isolated by Grenson [12] and Jund and Lacroute [13]. The corresponding *FUR4* gene was cloned [14] and its nucleotide sequence determined [15]. It encodes a membrane protein of 633 amino acid residues. This permease is a symporter which actively translocates uracil with the co-transport of protons [16]. Uracil permease production in the yeast cell is weak, as is the production of other known yeast secondary transporters. Uracil permease is immunologically undetectable in cells expressing the chromosomal copy of the *FUR4* gene. To study this permease, we routinely overexpress the *FUR4* gene on multicopy plasmids without impairing the routing of the protein to the plasma membrane [8,11]. Newly synthesized uracil permease is delivered to the cell surface via the secretory pathway [15] and is phosphorylated on multiple serine residues on its arrival at the plasma membrane [8]. No other post-translational modification seems to take place along the secretory pathway. The orientation of both N- and C-termini of the protein, and the orientation of most hydrophilic connecting loops with respect to the membrane, have been determined, such that a two-dimensional model of its structure has been developed (C. Garnier, M. O. Blondel and R. Haguenauer-Tsapis, unpublished work). This model proposes that uracil permease contains ten transmembrane segments, with its N- and C-termini located in the cytoplasm. According to this model only three charged residues are localized in the transmembrane domains: two glutamic residues at positions 243 and 539 (in the third and ninth putative transmembrane segments) and a lysine residue (272) in the fourth segment (Figure 1). In this paper, we describe the properties of a mutant uracil permease carrying a glutamic residue instead of a lysine at position 272. The location of the mutated protein at the plasma membrane was investigated together with its activity *in vivo* and *in vitro*. The data emphasize the critical role of this lysine residue in the function of uracil permease.

Abbreviations used: ORF, open reading frame; V_{max} , maximal rate of uptake; K_m (app.), apparent Michaelis constant of transport; B_{max} , maximal amount of specifically bound ligand; K_d (app.), apparent equilibrium constant of solute binding.

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Figure 1 Topological model of the uracil permease in the membrane according to C. Garnier (unpublished work)

Transmembrane segments are depicted as rectangles. Numbers at the top and bottom of each rectangle correspond to the first and the last residues of the helix. Charged residues in the membrane-spanning segments are indicated by the one-letter code followed by their position in the primary sequence. In the hydrophilic parts of the protein, positively and negatively charged residues are represented as closed and open circles, respectively.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The yeast strain used in this work was NC122-sp6, MATa, *fur4* Δ (*Xba*I-*Xba*II), *leu2* [17]. It was transformed as described [18]. The *E. coli* strain used for plasmid maintenance was DH5 α , from GIBCO-BRL. Plasmid pJDB207 is a multicopy shuttle plasmid with the *LEU2* gene as the selectable marker in yeast [19]. Plasmid pF is pJDB207 containing the *FUR4* gene [8]. Plasmid pF_{K272E} is a pF derivative containing the mutant *fur4* gene. The liquid minimal medium used for yeast strains was YNB (Difco), at a concentration of 6.7 g/l containing 20 g/l glucose. For plasma membrane preparation, the medium was buffered with 25 mM sodium phthalate, pH 5. Yeast cells were grown at 30 °C. *E. coli* was grown at 37 °C on Luria-Bertani (Difco) liquid or solid medium.

DNA sequencing

DNA sequences were determined as described in [20], using double-strand DNA and the Sequenase 2.0 kit (USB). DNA was labelled with deoxyadenosine [³⁵S]triphosphate (Amersham). An insert covering the complete open reading frame (ORF) of the *fur4* allele was sequenced using primer oligonucleotides derived from the sequence of the wild-type gene [15]. When the mutation was found, the sequencing of the mutant allele was repeated in parallel with that of the wild-type gene.

Membrane preparation

Plasma membrane-enriched fractions were isolated as described [21] from cells harvested during the exponential growth phase [(1–2) $\times 10^7$ cells/ml]. The cell suspension was ground with glass beads and submitted to differential centrifugation. Mitochondrial material was eliminated by acid precipitation.

Measurement of uracil uptake

The initial rate of uracil uptake was determined by a filtration technique after incubation of the cells, harvested in the exponential growth phase [(1–2) $\times 10^7$ cells/ml], in 50 mM sodium citrate, pH 4.5, containing 20 g/l glucose at 30 °C [7]. The cell concentration was (5–7.5) $\times 10^6$ cells/ml for cells transformed with pF and (15–21) $\times 10^6$ cells/ml for cells transformed with

pF_{K272E} or pJDB207. Uptake was started by addition of either [¹⁴C]uracil (Amersham) (218 MBq/mmol) for cells transformed with pF or [5,6-³H]uracil (1.6 GBq/mmol) for cells transformed with pF_{K272E} or pJDB207. Uptake rates were constant for up to 10 s under our experimental conditions. Maximal rate of uptake (V_{max}) and apparent Michaelis constant of transport [K_m (app.)] were determined as described [21].

Equilibrium binding procedure

Uracil binding to plasma membrane-enriched fractions was measured by a centrifugation technique at 4 °C in 50 mM sodium citrate, pH 4.5, containing 100 mM NaCl, as described in [21]. [¹⁴C]uracil (218 MBq/mmol) was included at various concentrations (0.1–150 μ M). Specifically bound ligand was defined as the difference between the amounts of ligand bound in the absence (total binding) and the presence (non-specific binding) of uracil (3 mM). The binding parameters B_{max} (maximal amount of specifically bound ligand) and K_d (app.) (apparent equilibrium constant of solute binding) were determined as described [21].

Western-blot analysis

Proteins from (2–4) $\times 10^7$ cells or 200 μ g of protein from enriched plasma membrane fractions were treated as described [11]. Aliquots corresponding to (0.5–1) $\times 10^7$ cells or 2 μ g of membrane proteins were subjected to electrophoresis, then transferred to a nitrocellulose membrane and probed with antibodies as described [8]. Uracil permease was detected with an antiserum against a C-terminal peptide (provided by M. R. Chevallier, CNRS Institut de Botanique, Strasbourg, France) that had been affinity purified by incubation with a nitrocellulose strip containing the same peptide. Antibodies specific for the plasma membrane H⁺-ATPase (provided by R. Serrano, Universidad Politécnica, Valencia, Spain) were used without further purification. Primary antibodies were detected with a horseradish peroxidase-conjugated anti-(rabbit IgG) second antibody and enhanced chemiluminescence (ECL; Amersham).

Miscellaneous

Cell concentration was determined by measuring absorbance (A) at 600 nm (one A_{600} unit corresponding to 2 $\times 10^7$ cells/ml). Protein concentration was determined by the method of Lowry et al. [22] with BSA as a standard. Radioactivity was counted with a Beckman LS 3801 scintillation counter.

RESULTS

Characterization of the mutant uracil permease

The *fur4* mutant studied was obtained fortuitously during site-directed mutagenesis experiments for another purpose as follows: a yeast strain in which the chromosomal copy of *FUR4* had been disrupted was transformed with plasmids carrying the mutagenized *fur4* genes. Transformants with mutant uracil permeases were isolated and the *fur4* genes characterized by DNA sequencing. An unexpected type of transformant exhibiting an almost total defect in uracil transport was also recovered. DNA sequence analysis of the corresponding gene showed that an additional spontaneous mutation had occurred: a single base-pair transversion (AAA $\rightarrow$ GAA) leading to the substitution of Lys-272 by Glu (K272E). The mis-sense mutation was isolated by subcloning for subsequent studies. Lys-272 would map in the fourth transmembrane segment of the protein (Figure 1). All

Figure 2 Immunological detection of uracil permease in cells transformed with pF or pF_{K272E}

Total protein extracts (lanes 1–6) or enriched plasma membrane fractions (lanes 7 and 8) were prepared from cells transformed with pF (lanes 1–3 and 7) or pF_{K272E} (lanes 4–6 and 8) and harvested after various times of growth. Extracts from equal amounts of growing cells or enriched plasma membrane fractions were separated by SDS/PAGE and analysed by Western blotting with uracil permease C-terminal antipeptide antibodies and H⁺-ATPase antibodies. It may be noted that the pattern of the different permease species was less well resolved when using membrane fractions. WT, wild type.

algorithms to predict the boundaries of hydrophobic segments give the same results for this region (M. R. Chevallier, personal communication). Thus, Lys-272 is believed to be the fifth residue from the external face of the membrane.

The mutant permease is located at the plasma membrane

The *fur4* null mutant strain was transformed with the *FUR4* gene or its mutant allele carried on multicopy plasmids. Total extracts from equal amounts of growing cells and enriched plasma membrane fractions (see Materials and methods section) were analysed for their content of uracil permease and plasma membrane H⁺-ATPase by immunoblotting. Exponentially growing wild-type cells contain several permease species with electrophoretic mobilities corresponding to different phosphorylation levels [8]. The mutant gene expressed under the same cell growth conditions gave an amount of uracil permease similar to the wild type and an indistinguishable banding pattern (compare lanes 4 and 5 with lanes 1 and 2 in Figure 2). This indicates that the mutant protein did reach the plasma membrane where it could be phosphorylated on multiple residues. Uracil permease has a moderate turnover in growing cells. However, adverse conditions, such as the approach of the stationary growth phase, induce rapid degradation of this protein [11]. The decrease in the amount of immunodetectable uracil permease when yeast cells reached the stationary growth phase was similar for the wild-type and the mutant proteins, indicating that the mis-sense mutation did not modify the turnover of the protein (Figure 2, lanes 3 and 6). In addition, the banding patterns of the mutant and the wild-type permease species in the stationary phase differed similarly from that observed in exponentially growing cells, with preferential disappearance of bands corresponding to species with higher levels of phosphorylation. The presence of the wild-type and mutant uracil permeases was also checked in enriched plasma membrane fractions (Figure 2, lanes 7 and 8). The uracil permease:H⁺-ATPase ratios in the plasma membrane fractions were roughly the same for the two preparations and were in agreement with the pattern and content of total extracts from exponentially growing cells. Thus, the mutant uracil

Figure 3 Uracil uptake by cells transformed with pF or pF_{K272E}

Cells harvested in the exponential growth phase were assayed for uracil uptake as described in the Materials and methods section. Experiments were done with uracil concentrations between 0.1 and 25 μM [¹⁴C]uracil (218 MBq/mmol) for cells transformed with pF (a), and 10 and 300 μM [³H]uracil (1.6 GBq/mmol) for cells transformed with pF_{K272E} (b). Permease-independent uracil sequestration for cells transformed with pJDB207 was deducted in (b). Data reported are those from one experiment. V: nmol uracil · min⁻¹ · 10⁷ cells⁻¹. Data are displayed according to Eadie-Hofstee. Insert: saturation curves.

permease appears to fold correctly and is found in the normal location (the plasma membrane).

The mutation affects both the kinetic and the equilibrium binding parameters of uracil permease

Transport activity parameters were determined using a *fur4* null mutant strain containing the wild-type *FUR4* gene or its mutant allele carried on multicopy plasmids. $K_m(\text{app.})$ and $V_{\text{max.}}$ could be measured more accurately due to the overproduction of the corresponding proteins. For the overproduced wild-type protein, $V_{\text{max.}}$ was about 40-fold higher than that for the chromosomally encoded protein (Figure 3 and Table 1) [17]. We determined the apparent Michaelis constant of transport [$K_m(\text{app.})$] to be 2.5 μM (Figure 3 and Table 1). This is lower than previously described (5 and 19 μM) [12,23]. The disagreement may be due to the difference in the experimental conditions used for uptake measurements.

The rate of uptake by cells containing the K272E mutant protein was much lower and therefore the determination of the apparent uptake parameters was difficult. In addition, an apparent sequestration of uracil was observed in control experiments with the permease null strain transformed with the

Table 1 Uracil uptake and binding parameters of wild-type and mutant uracil permease

K_m (app.) and V_{max} , K_d (app.) and B_{max} values were determined from data presented in Figures 3 and 4 respectively. Non-linear regression of the saturation curves was calculated with a computer program written in the laboratory. Values were determined twice for cells transformed with pF and three times for cells transformed with pF_{K272E}.

	Uracil uptake		Uracil-specific binding	
	K_m (app.) (μ M)	V_m (nmol $\cdot$ min ⁻¹ $\cdot$ 10 ⁷ cells ⁻¹)	K_d (app.) (μ M)	B_{max} (pmol/mg of protein)
Wild type	2.5 $\pm$ 0.3	8.8 $\pm$ 0.6	0.74 $\pm$ 0.04	889 $\pm$ 42
K272E	162 $\pm$ 72	1.1 $\pm$ 0.3	50.3 $\pm$ 10.3	> 700

Figure 4 Uracil binding to plasma membrane preparations isolated from cells transformed with pF or pF_{K272E}

Plasma membrane fractions were isolated as described in the Membrane preparation section. Binding was measured at the following concentrations of radioactive uracil: for pF from 0.1 to 35 μ M and for pF_{K272E} from 0.5 to 145 μ M. The amount of radioactivity bound in the presence of 3 mM uracil in the incubation medium was considered as non-specific binding and was 20 ± 0.5 pmol/mg of protein for 1 μ M free ligand at equilibrium. Data reported are from one experiment. B: pmol/mg of protein. Specific binding is displayed as a Scatchard plot. The insert shows saturation curves for: $\circ$, pF; and $\bullet$, pF_{K272E}.

multicopy plasmid which did not harbour the *FUR4* gene. This permease-independent uracil sequestration was not large enough to perturb significantly the determination of the uptake parameters for the wild-type protein, but led to inaccuracies for the determination of uptake when only the mutant permease was present. Therefore this sequestration had to be considered. Nevertheless, the K272E mutant cells did import uracil. The transport was saturable and its K_m (app.) was much higher than that of wild-type cells (Figure 3 and Table 1).

The equilibrium binding parameters, K_d (app.) and B_{max} , were determined using enriched plasma membrane preparations. Only one class of specific binding site was present in cells containing either wild-type or mutant permeases (Figure 4). B_{max} for the mutant protein was difficult to determine precisely, but was similar to that for cells producing the wild-type protein (Figure 4 and Table 1). This is consistent with the immunological assays of the proteins (Figure 2). K_d (app.) values for the two types of cells were very different. The apparent affinity of the mutant for its ligand was approximately 70-fold lower than that of the wild-type permease (Figure 4 and Table 1).

DISCUSSION

This paper describes the properties of a mutant permease carrying a glutamic in place of a lysine residue in a transmembrane segment of the uracil permease of *S. cerevisiae*. Cells containing the mutant protein accumulate uracil poorly and the mutant permease is impaired in its ability to bind uracil.

The *FUR4* gene encoding uracil permease belongs to a family of homologous yeast genes comprising three other *S. cerevisiae* genes, the *DAL4* gene, encoding the allantoin permease [24], the ORF YLB0407 and a more recently discovered sequence, identified by systematic sequencing of chromosomes II and XV respectively (A. Goffeau, personal communication; B. Daignan-Fornier, personal communication) and the gene encoding the uracil permease of *S. pombe* (M. R. Chevallier, personal communication). Three of the corresponding deduced amino acid sequences are very similar to the uracil permease from *S. cerevisiae*. Allantoin permease is 68 % identical with and 82 % similar to uracil permease. These two proteins do not share any overlap of specificity. However a *dal4* mutation has been complemented by the *FUR4* gene overexpressed on a multicopy plasmid [24]. The ORF YLB0407 of unknown function and the uracil permease from *S. pombe* are respectively 48 and 39 % identical and 69 and 63 % similar to the uracil permease of *S. cerevisiae*. The similarity is greatest for the hydrophobic core of the four proteins, i.e. excluding the dissimilar hydrophilic N- and C-termini. All these deduced sequences exhibit the same hydrophathy profile. A lysine residue seems to be the only positively charged residue present in the hydrophobic segments of these proteins. This lysine residue is predicted in each case to lie at the beginning of the fourth putative transmembrane segment by analogy with the topological model proposed for the uracil permease from *S. cerevisiae* (C. Garnier, M. O. Blondel and R. Haguenaer-Tsapis, unpublished work). The conservation of this residue and its position suggest that it might be involved in the carrier function.

If the K272E mutation caused a major conformational change, it might be expected to have a large effect on the stability of uracil permease 'en route' to the plasma membrane. Misfolded proteins are retained early in the secretion pathway for subsequent degradation [25]. This was the case for various uracil permease mutants constructed in our laboratory (results not shown; C. Garnier, M. O. Blondel and R. Haguenaer-Tsapis, unpublished work). In contrast, the K272E mutant permease appears to be correctly integrated into the plasma membrane, indistinguishably from the wild-type permease. It thus seems unlikely that the severe impairment in transport activity and ligand binding observed for the mutant was due to major changes in the spatial arrangement of the molecule.

Experiments *in vitro* showed that the K272E mis-sense mutation greatly reduced the affinity for uracil. Thus, the lysine residue may be part of the substrate-binding site. However a conformational change affecting the binding site could lead to the same result. For example, Lys-272 might be part of a salt bridge with one of the two glutamic residues located in transmembrane segments three and nine, resulting in a juxtaposition of two defined transmembrane domains. The charge inversion due to the mutation would disrupt this bridge.

In addition to the modification in the affinity, the mutation also affects uracil translocation since the V_{max} of uptake for the mutant protein is very low. Lys-272 might be involved in a conformational change of the protein needed for the correct translocation of the cosubstrates and/or their release. For example, it has been postulated that specific charged residues might be involved in a gating mechanism that may contribute to the obligatory coupling of cosubstrate flows by symporters [4]. Lys-272 found in the membrane domain of uracil permease might be involved in such a mechanism.

This work does not indicate whether it is the presence of the negative charge, the absence of a positive one or the side chain of glutamic acid being shorter than that of lysine, which impairs the transporter activity. Systematic mutagenesis of Lys-272 *in vitro* should give a better understanding of the role of this residue in the function of uracil permease. A genetic approach could also be developed to isolate intragenic suppressors from the original mis-sense K272E mutation. Looking for revertants *in vivo* should lead to the identification of the amino acids whose interaction with Lys-272 are essential for transport. It would also be of interest to investigate the role of the corresponding lysine residue in the two homologous proteins of known function, the allantoin permease and the uracil permease from *S. pombe*.

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