Only one of the charged amino acids located in membrane-spanning regions is important for the function of the *Saccharomyces cerevisiae* uracil permease

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The transport of uracil into the yeast *Saccharomyces cerevisiae* is mediated by uracil permease, a specific co-transporter encoded by the *FUR4* gene. Uracil permease is a multispan membrane protein that is delivered to the plasma membrane via the secretory pathway. Experimental results led to the proposal of a two-dimensional model of the protein's topology. According to this model, the membrane domain of Fur4p contains three charged amino acid residues (Glu-243, Lys-272 and Glu-539) that are conserved in the members of the FUR family of yeast transporters. We have previously shown that a mis-sense mutation leading to the replacement of Lys-272 by Glu severely impairs the function of uracil permease. In the present paper, the role of the three charged residues present in the membrane-spanning regions of Fur4p was further investigated by using site-directed mutagenesis. The variant permeases were correctly targeted to

the plasma membrane and their stabilities were similar to that of the wild-type permease. The effect of the mutations was studied by measuring the uptake constants for uracil on whole cells and equilibrium binding parameters on plasma membrane-enriched fractions. We found no evidence for ionic interaction between either of the glutamic residues in transmembrane segments 3 and 9 and the lysine residue in transmembrane segment 4. Of the three charged residues, only Lys-272 was important for the transport activity of the transporter. Its replacement by Ala, Glu or even Arg strongly impaired both the binding and the translocation of uracil.

Key words: binding, plasma membrane, translocation, transporter, yeast.

INTRODUCTION

Transporters specifically catalyse the translocation of solutes across the membrane by undergoing reversible conformational changes that expose their solute-binding site alternately on each side of the membrane. Secondary active co-transporters use electrochemical gradients to move solutes across membranes. A recent computer-based analysis of the genome sequence of the yeast *Saccharomyces cerevisiae* identified 186 potential transporters [1]. Transporters in bacteria, fungi, plants and animals generally have a common structural topology, which is readily identified by hydropathy analysis: a central core with 10–12 membrane-spanning regions flanked by hydrophilic extremities that presumably face the cytoplasm. The membrane-spanning regions of these proteins contain a few charged amino acids, the function of which has been investigated for some co-transporters, including the lactose permease, the melibiose permease, the proline carrier, the tetracycline antiporter of *Escherichia coli* (reviewed in [2]) and members of the neurotransmitter transporter family in mammalian cells [3,4]. It has been suggested that such positively and negatively charged amino acids in the membrane domain might form charge-neutralizing pairs, thereby stabilizing the folding of the protein in a hydrophobic environment. For the lactose permease (lacY) of *E. coli*, one of the most extensively studied members of this class of proteins, it has been shown that charged residues are involved in the carrier function (reviewed in [5]). By using site-directed mutagenesis it was shown that pairs of interacting charged residues (Asp-231/Lys-358 and Asp-240/Lys-319) were involved in the stability and activity of the carrier. The importance of such interactions has been demonstrated in

other integral membrane systems such as the bacteriorhodopsin of *Halobacterium halobium* [6] and the melibiose permease of *E. coli* [7]. Therefore in studies of transporter mechanisms it is important to investigate whether charged residues in the hydrophobic environment have structural, as well as functional, significance.

The uracil permease from *S. cerevisiae* is a good model for investigating the role of charged residues in the activity of a transporter. Uracil permease is a symporter that actively translocates uracil with the co-transport of protons [8]. This transporter is a multispan membrane protein encoded by the *FUR4* gene [9]. Newly synthesized uracil permease is delivered to the plasma membrane via the secretory pathway, and several of its serine residues are phosphorylated in a post-Golgi compartment on the way to, or at, the plasma membrane [10]. This post-translational modification affects mostly a PEST-like sequence at the extreme of the N-terminus of Fur4p (PEST is a peptide motif that targets proteins for degradation), which has been shown to be essential for uracil permease turnover [11]. The *FUR4* gene belongs to a family of homologous yeast genes [1,12]. The deduced amino acid sequences of the proteins encoded by these genes are all very similar to that of uracil permease, particularly in the region of the hydrophobic core, which is involved in transport.

A two-dimensional model of the structure of uracil permease has been developed in which the cytoplasmic orientation of both termini and the orientation of most connecting loops with respect to the membrane have been determined [13]. According to this model, uracil permease has 10 transmembrane spans (TMs). There are only three charged residues in the trans-

Abbreviations used: ORF, open reading frame; TM, transmembrane span.

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membrane domain: two glutamic residues at positions 243 and 539 in TM3 and TM9 respectively, and a lysine residue at position 272 in TM4. The locations of these charged residues in the hydrophobic environment of the membrane suggests that they could be involved in transporter structure and function. We have shown previously that the replacement of Lys-272 by Glu (K272E) greatly impairs uracil permease function [14]. In the present study we used site-directed mutagenesis to investigate the role of the three charged amino acid residues. The location of the mutated proteins at the plasma membrane was investigated, together with their activity *in vivo* and *in vitro*.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The *S. cerevisiae* strain used in this work was NC122-sp6, MATa, *fur4Δ*, *leu2* [15]. The chromosome-encoded uracil permease is produced in very small amounts; cells producing the permease from multicopy plasmids were used for the accurate measurement of permease activity and for the immunodetection of the protein [10]. The multicopy plasmid, pF (2 μ *LEU2 FUR4*) is pJDB207 containing the *FUR4* gene [9]. Yeast cells were transformed as described by Gietz et al. [16]. Cells were grown at 30 °C in minimal medium (YNB) containing 0.67% yeast nitrogen base without amino acids (Difco). The carbon source was 2% (w/v) glucose. Cells used for plasma membrane preparation were grown in a medium buffered with 25 mM sodium phthalate, pH 5.5.

Mutagenesis

Point mutations were introduced into the *FUR4* gene by site-directed mutagenesis with the Stratagene chameleon double-stranded site-directed mutagenesis kit, in accordance with the manufacturer's instructions. Mutations were detected by testing for restriction site polymorphism introduced by the mutagenic primers. Mutations were confirmed by sequencing with double-stranded DNA and the Sequenase 2.0 kit (USB) [17]. For each mutagenesis, two independent mutant plasmids were used to transform yeast; two yeast transformants were analysed for each mutant plasmid.

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 [18]. The cell concentration was (5–7.5) $\times 10^6$ cells/ml for cells transformed with pF or plasmid derivatives harbouring substitutions E243K or E539K in the *FUR4* gene. The cell concentration was 2 $\times 10^7$ cells/ml for cells transformed with pJDB207 or plasmid derivatives harbouring substitutions at K272 in the *FUR4* gene. Uptake was started by adding [¹⁴C]uracil (Amersham) (218 MBq/mmol). Uptake rates were steady for up to 10 s under our experimental conditions. The apparent accumulation of radioactivity measured with the pJDB207-null strain, resulting from passive uracil diffusion across the plasma membrane, has been subtracted from all the kinetic analyses presented here. The maximal rate of uptake, $V_{\max}$, and the apparent Michaelis constant of transport, K_m (app), were calculated by non-linear regression analysis of the initial rates of uptake against the solute concentration by using the method of Cleland, as described previously [19].

Membrane preparation and equilibrium binding

Binding of uracil to plasma-membrane-enriched fractions was determined by a centrifugation technique, as described elsewhere [19]. Plasma-membrane-enriched fractions were isolated from cells harvested during the exponential growth phase (3 $\times 10^7$ cells/ml). Uracil binding was measured at 4 °C in 50 mM sodium citrate, pH 4.5, containing 100 mM NaCl. [¹⁴C]Uracil (218 MBq/mmol) was included at various concentrations (0.1–250 μ M). Specifically bound ligand was calculated as the difference between the amounts of ligand bound in the absence (total binding) and the presence (non-specific binding) of uracil (2–3 mM). The binding parameters $B_{\max}$ (maximum amount of specifically bound ligand) and K_d (app) (equilibrium constant of solute binding) were determined by non-linear analysis of the saturation curves, as described previously [19].

Western blot analysis

Whole-cell extracts were prepared from cells in the exponential growth phase; proteins from approx. (1–2) $\times 10^6$ cells were resolved by SDS/PAGE and analysed by immunoblotting as described previously [20] by using antibodies directed against Fur4p and Pma1p (the H⁺-ATPase, an integral membrane-bound protein of the plasma membrane) [21]. Primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody and by enhanced chemiluminescence (ECL[®]; Amersham).

Cell fractionation and equilibrium density centrifugation

Cell organelles were fractionated on equilibrium density gradients, as described previously [22]. For fractionation of membranes, half of the clear cell extracts from (0.5–1) $\times 10^9$ cells were layered on a 12 ml linear sucrose gradient [20–60% (w/v)] and centrifuged for 17 h at 30000 rev./min (150000 g) in an SW40 rotor at 4 °C. Fractions (375 μ l) were collected from the top of the gradient, the volume was made up to 1 ml with water and proteins were precipitated by adding 100 μ l of 100% (w/v) trichloroacetic acid. The proteins in each fraction were resolved by SDS/PAGE and analysed by Western blotting [20] with antibodies directed against Fur4p, Pma1p [21] and Sss1p (an integral membrane-bound protein of the endoplasmic reticulum) [23].

Miscellaneous

Protein concentrations were determined as described by Lowry et al. [24], with BSA as a standard.

RESULTS

Conservation of the charged residues in the membrane domain of the FUR transport proteins

The *FUR4* gene belongs to the FUR transport family comprising eight homologous yeast genes with representatives in *S. cerevisiae* and *Schizosaccharomyces pombe*. The amino acid sequences deduced from these genes are very similar, especially in the region of the hydrophobic core, which is involved in the transport function. The functions of five of the deduced sequences are known: uracil permease (Fur4p), allantoin permease (Dal4p) [25], uridine permease (Fui1p) [26] and thiamin permease (Thi10p/Thi17p) [27,28] from *S. cerevisiae*, and the uracil permease from *Schizosaccharomyces pombe* [29]. Dal4p has 67% amino acid sequence identity with Fur4p, Fui1p has 41%, Thi10p 30% and the uracil permease of *Schizosaccharomyces*

Figure 1 Location of the charged amino acid residues within the transmembrane domain of the proteins of the FUR transporter family

(A) Each column corresponds to a membrane-spanning segment (TM1 to TM10). Charged residues predicted to reside within the membrane-spanning segments are indicated by the one-letter amino acid code. The locations of the charged amino acid residues within the transmembrane domain of the deduced sequences from ORF YOR071c and YOR192c are not shown so as not to bias the comparison. Abbreviations: *S.c.*, *S. cerevisiae*; *S.p.*, *Schizosaccharomyces pombe*; Ura. P.: uracil permease. (B) Alignment of the fourth transmembrane segment of yeast FUR transport proteins. Sites completely conserved are indicated by capital letters in the consensus line. Sites in which a single change has occurred are indicated by lower-case letters in the consensus. The deduced sequences from ORF YOR071c and YOR192c are not shown so as not to bias the comparison.

pombe 37%. Two other deduced protein sequences, those encoded by the open reading frames (ORFs) YOR192c and YOR071c from *S. cerevisiae*, are extremely similar to that of Thi10p over their entire length (respectively 87% and 84% identical with Thi10p). They both transport thiamin, although less efficiently than Thi10p [27]. The remaining ORF, from *Schizosaccharomyces pombe* (Swissprot accession number O14035), encodes a protein that is most similar to the uracil permease of *Schizosaccharomyces pombe* (69% identity) and least similar to Fur4p (44% identity). The hydropathy profiles of these eight proteins are very similar. All are predicted to have a central core with 12–13 TMs according to the prediction program of Sipos and von Heijne [30], based on the GES hydrophobicity scale [31]. Ten of these segments, with equivalent locations in each of the proteins, have a mean hydropathy score above 1.1 (except for the proposed TM2 from Thi10p and derivatives with less average hydropathy) and could be considered to be true transmembrane segments ([13], and results not shown). A two-dimensional model of the Fur4p structure has been developed on the basis of experimental data and it is consistent with the topological model with 10 transmembrane segments [13]. We extended this model to all members of the yeast FUR family. There are three charged amino acid residues in the 10 membrane-spanning regions of Fur4p and they are conserved throughout the FUR transport protein family (Figure 1A). Glu-243 is located in TM3 and is conserved throughout the FUR family except in YOR071c and the *Schizosaccharomyces pombe* FUR members, in which it is replaced by a Gln residue. A second negatively charged residue, Glu-539, is located in TM9. It is conserved in Thi10p and derivatives and is replaced by Asp, a conservative substitution, in Dal4p. There is a single positively charged residue, Lys-272, in TM4. It is predicted to be the fifth residue of TM4 (Figure 1B) and to lie on the external face of the membrane (different algorithms used to predict the boundaries of hydro-

Figure 2 Variant permeases are present in the plasma membrane

(A) Immunological detection of uracil permease in cells transformed with variant uracil permeases. Total protein extracts were prepared from cells transformed with pF or derivatives harbouring mutations in the *FUR4* gene. Half of each extract was subjected to SDS/PAGE and analysed by Western blotting for Fur4p and Pma1p. Lane a, fur4Δ; lanes b and j, wild-type; lane c, K272E; lane d, K272A; lane e, K272R; lane f, E243K; lane g, E539K; lane h, K272E-E243K; lane i, K272E-E539K. (B) Extracts were prepared from 10⁹ cells transformed with pF or derivatives with substitutions of Lys-272 in the *FUR4* gene. They were fractionated on 20–60% (w/v) sucrose density gradients containing 10 mM EDTA. Aliquots of the various fractions were analysed by Western blotting with antibodies directed against Fur4p, Pma1p (marker for plasma membrane) and Sss1p (marker for endoplasmic reticulum). Gradients for the wild-type permease and a single variant (K272A) are shown. Similar results were obtained for the other mutant permeases (results not shown).

phobic segments all give the same results for this region). Lys-272 is conserved at the same position in TM4, in all members of the FUR family except for Thi10p and its two related proteins.

Variant permeases are located at the plasma membrane

We used site-directed mutagenesis to mutate the three charged amino acid residues located in the transmembrane domain of FUR4p. A *fur4*-null mutant strain was transformed with the *FUR4* gene or one of its mutant alleles carried on multicopy plasmids. The variant uracil permeases were detected in total extracts from equivalent amounts of growing cells by immunoblotting (Figure 2A). Blots were reprobed with plasma membrane H⁺-ATPase (Pma1p) antibodies to provide loading controls. Similar amounts of uracil permease were detected in all assays, demonstrating that the mutant permeases were stable species. Exponentially growing cells expressing the wild-type *FUR4* gene contain several permease species with different electrophoretic mobilities, corresponding to different phosphorylation levels [10]. The mutant genes, expressed under the same conditions, gave an identical banding pattern to that of the wild type. The phosphorylation of Fur4p is a plasma membrane event [10], so the mutant proteins must indeed have reached the plasma membrane. The location of the variant permeases was also checked by density-gradient fractionation. Extracts from exponentially growing cells producing the variant permeases were

Table 1 Uracil uptake and binding parameters for wild-type and mutant uracil permeases

Uracil uptake parameters were determined as described in the Materials and methods section over a concentration range of 0.1–310 μM [^{14}C]uracil (218 MBq/mmol). The rate of passive uracil diffusion measured with the *fur4*-null strain was 0.02 nmol/min per 10^7 cells at an external uracil concentration of 10 μM . This value was subtracted from all measurements. For the determination of equilibrium binding parameters, the amount of radioactivity bound in the presence of an excess of unlabelled uracil (4 mM) in the incubation medium was considered to represent non-specific binding and was 30 ± 1 pmol/mg of protein for 1 μM uracil. Uptake and binding parameters were determined six times and three times respectively for each variant permease. Parameters were calculated as described previously [19]. The values for the wild-type and K272E variant proteins are taken from [14]. Abbreviations: n.d., not determined; n.m., not measurable under the experimental conditions [K_d (app) values were too high to be measured (see the text)]. In the first column the designations refer to the native and mutant overproduced permeases.

Fur4p	Uracil uptake		Uracil-specific binding	
	K_m (app) (μM)	$V_{\max}$ (nmol/min per 10^7 cells)	K_d (μM)	$B_{\max}$ (pmol/mg of protein)
Wild-type	2.5 ± 0.3	8.8 ± 0.6	0.74 ± 0.04	889 ± 42
K272E	162 ± 72	1.1 ± 0.3	50.3 ± 10.3	> 700
K272A	1000 ± 700	5.0 ± 3.7	64 ± 41	1300 ± 300
K272R	> 1000	> 5	40 ± 22	1400 ± 300
E243K	3.5 ± 0.5	11.5 ± 0.9	n.d.	n.d.
E539K	4.8 ± 0.5	8.8 ± 0.3	n.d.	n.d.
K272E-E243K	600 ± 350	2.5 ± 1.1	n.m.	n.m.
K272E-E539K	300 ± 150	1.5 ± 0.5	n.m.	n.m.

fractionated on sucrose density gradients under experimental conditions giving a good separation of internal membranes from the more dense plasma membrane [22]. The fractions collected from the sucrose gradients were tested for Fur4p, plasma-membrane Pma1p and the integral membrane-bound Sss1p used as a marker of the endoplasmic reticulum [23] (Figure 2B). The wild-type uracil permease was detected, as expected, in the same fractions as the plasma-membrane marker, Pma1p. The mutant permeases had the same distributions on sucrose density gradients as that of the wild-type permease. Moreover, the ratios of Fur4p to Pma1p in the plasma-membrane fractions were similar for all variant extracts and were consistent with the amounts in total extracts. Therefore all the variant uracil permeases were in the correct location (the plasma membrane) and were presumably correctly folded.

Only Lys-272 is important for uracil binding and translocation

The only basic residue predicted to reside within the membrane domain is Lys-272. A mis-sense mutation in the *FUR4* gene leading to a replacement of Lys-272 by Glu (K272E) gave rise to a mutant permease impaired in both uracil binding and transport [14]. We used site-directed mutagenesis to analyse the function of Lys-272 further by replacing this residue with a neutral residue, Ala (K272A), or a positive residue, Arg (K272R). Transport activity was studied with the use of a *fur4*-null mutant strain expressing the variant permease genes from multicopy plasmids so that K_m (app) and $V_{\max}$ could be measured more accurately owing to the overproduction of the corresponding proteins. Passive, permease-independent, uracil diffusion was subtracted from the total amount of uracil accumulated by cells transformed with the variant permeases. Therefore the results presented in Table 1 correspond to active uracil uptake.

The replacement of Lys-272 by Ala or Arg led to significant changes in uptake parameters, involving a much higher K_m (app) (400-fold higher) and a slightly lower $V_{\max}$ (40% lower) than those of cells containing the wild-type Fur4p. Furthermore, these mutant cells had a slightly higher K_m (app) (6-fold higher) and a significantly higher $V_{\max}$ (4.5-fold higher) than cells containing the K272E mutant protein. The equilibrium binding parameters, K_d (app) and the maximum number of binding sites, $B_{\max}$, were determined with plasma-membrane-enriched preparations. Satu-

ration curves showed that only one class of binding site was present in cells containing wild-type FUR4p or the K272E [14], K272A or K272R variant permeases (results not shown). $B_{\max}$ was difficult to determine accurately for the mutant permeases, but the values obtained were similar to that from cells producing the wild-type permease (Table 1). This is consistent with there being equivalent amounts of variant permeases per cell, as shown by immunoblotting experiments (Figure 2). K_d (app) values for the K272A and K272R variants were much higher than that of the wild-type permease and were similar to those previously measured for the K272E variant. The apparent affinities of the K272A and K272R mutant permeases for the ligand were 1/50 to 1/80 of that of the wild-type permease.

We investigated whether Lys-272 was involved in an ionic interaction with the glutamic residues located in TM3 and TM9 respectively, by replacing the glutamic residues with positively charged ones. We determined the K_m (app) and $V_{\max}$ values for the variant permeases in which Glu-243 or Glu-539 was replaced by Lys (E243K and E539K). We found that $V_{\max}$ was almost the same, whereas K_m (app) was slightly higher (1.4–1.9-fold higher) than that of the wild-type permease (Table 1). The production of the variant permeases by expression from *FUR4* genes carried on multicopy plasmids might account for the small difference in $V_{\max}$ between cells containing the E243K variant protein and those containing the wild-type protein. Therefore the presence of the two acidic residues in TM3 and TM9 is not critical for optimal uptake. These results also suggest that neither of these two residues is involved in an ionic interaction with Lys-272. We checked this result and further investigated the uracil transport impairment observed with the variant permeases in which Lys-272 was replaced by another residue, by constructing double mutants in which Lys-272 and one of the Glu residues were simultaneously replaced with a residue of opposite charge. Cells transformed with K272E/E243K or K272E/E539K variant proteins had low transport activity, similar to that described for cells transformed with the K272E variant. They had much higher K_m (app) values (approx. 400-fold higher) and lower $V_{\max}$ values (72–83% lower) than cells transformed with the wild-type protein (Table 1). Equilibrium measurements were undertaken. Despite the presence of specific binding, the amount of ligand specifically bound was so low that it could not be measured accurately within the range of concentrations of ligand used (0–275 μM)

and no saturation could be observed (results not shown). This indicates that K_d (app) was very large (up to 275 μ M) and that the two amino acid substitutions caused severe impairment of uracil-binding affinity. However, these results confirmed that neither Glu-243 nor Glu-539 was involved in ionic interactions with Lys-272 to stabilize the structure of an active permease.

DISCUSSION

This study is a first step towards identifying the amino acid residues that are important for the function of the uracil permease of *S. cerevisiae*. The uracil permease is responsible for the transport of uracil coupled to a proton electrochemical gradient across the cytoplasmic membrane [8]. Charged residues within the membrane domains are logical candidates for involvement in the transport mechanism and might also have a structural role involving, for example, ionic interactions between groups of opposite charge. Here we have described the kinetic and binding properties of variant permeases with mutations of the three charged residues located in the membrane-spanning regions of Fur4p. The two Glu residues in TM3 and TM9 are not essential, because mutant proteins with substitutions of Glu-243 and Glu-539 are active. In contrast, all mutations corresponding to substitutions of Lys-272 led to severe defects in binding and transport. Therefore only Lys-272 was important for uracil binding and translocation by the transporter.

The severe decrease in uracil uptake activity observed when Lys-272 was replaced by other amino acids might be due to a direct or indirect effect of the mutations on the active site of the permease, or to a failure in permease targeting to the plasma membrane. A major conformational change would be expected to have a large effect on the stability and hence on the distribution of the permease. Misfolded proteins are usually retained early in the secretion pathway for subsequent degradation [32]. It has been shown that a mutant uracil permease, with a three-residue insertion into a cytoplasmic connecting loop, does not progress beyond the endoplasmic reticulum, and is degraded by the 26 S proteasome [33]. In this study the variant mutant permeases were correctly targeted to the plasma membrane, where they were phosphorylated, giving the same banding pattern as that of the wild-type permease. It is therefore unlikely that the severely impaired ligand binding and transport activity of variants with substitutions of Lys-272 resulted from misfolding of the molecule.

The replacement of K272 by Glu, Ala or Arg affected uracil binding and translocation. The replacement of K272 by Ala or Arg only slightly decreased the V_{max} of uracil translocation, whereas a huge decrease in V_{max} was observed with the replacement of K272 by Glu. The amounts of these mutated proteins were similar to that of the wild-type protein. These changes therefore reflected changes in the catalytic constant, the largest being observed with K272E. In terms of uptake affinity, there was in each case a large increase in K_m (app) that was mainly attributable to a large increase in K_d (app). This suggested that Lys-272 is not an irreplaceable residue for active transport, but that it does have a crucial role in the correct organization of the binding site. As already done for other carriers [5], it would be interesting to perform accurate efflux and counterflow experiments to identify the steps of translocation in which Lys-272 is involved; however, such experiments are not yet possible.

There was no electrostatic interaction between Lys-272 and either Glu-243 or Glu-539. The replacement of either Glu-243 or Glu-539 by a residue of opposite charge (E243K and E539K) had no effect on turnover, but led to an increase in K_m (app). Therefore none of the negatively charged residues in the membrane domain were involved in any major change in the active

structure. Moreover the behaviour of the double-mutant proteins was very similar to that of the K272E variant protein in terms of V_{max} , but with a larger K_m (app) probably owing to the second mutation. Thus these results emphasize the crucial role of Lys-272, which is required for both the optimal structure of the uracil-binding site and the catalytic process in a very precise way, because its replacement by Glu, Ala or Arg led to a severe impairment of these activities. These results also demonstrate that the positive charge at position 272 is not in itself sufficient to sustain the activity of the carrier.

Only one of the three charged residues located in the membrane domain of Fur4p, Lys-272, is important for its activity. Lys-272 is predicted to occupy the fifth position in TM4, on the external face of the membrane, and is conserved at the same location in all members of the FUR family except Thi10p and its two related proteins. However, there is in the membrane-spanning regions of Thi10p and related proteins a single lysine residue in the third position from the external face of the membrane in TM3. As TM3 and TM4 are connected by a very small loop of four residues, the Thi10p lysine residue might be a functional equivalent of the lysine residue in TM4, conserved in the other FUR transport proteins. It would be of interest to investigate the role of the corresponding lysine residues of other members of the FUR family of transporters with known function, particularly the thiamin transporter.

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