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Research Article

Amino acid residues important for substrate specificity of the amino acid permeases Can1p and Gnp1p in *Saccharomyces cerevisiae*

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Abstract

Deletion of the general amino acid permease gene *GAP1* abolishes uptake of L-citrulline in *Saccharomyces cerevisiae*, resulting in the inability to grow on L-citrulline as sole nitrogen source. Selection for suppressor mutants that restored growth on L-citrulline led to isolation of 21 mutations in the arginine permease gene *CAN1*. One similar mutation was found in the glutamine-asparagine permease gene *GNP1*. L-[^{14}C]citrulline uptake measurements confirmed that suppressor mutations in *CAN1* conferred uptake of this amino acid, while none of the mutant permeases had lost the ability to transport L-[^{14}C]arginine. Substrate specificity seemed to remain narrow in most cases, and broad substrate specificity was only observed in the cases where mutations affect two proline residues (P148 and P313) that are both conserved in the amino acid–polyamine–choline (APC) transporter superfamily. We found mutations affecting six predicted domains (helices III and X, and loops 1, 2, 6 and 7) of the permeases. Helix III and loop 7 are candidates for domains in direct contact with the transported amino acid. Helix III was affected in both *CAN1* (Y173H, Y173D) and *GNP1* (W239C) mutants and has previously been found to be important for substrate preference in other members of the family. Furthermore, the mutations affecting loop 7 (residue T354, S355, Y356) are close to a glutamate side chain (E367) potentially interacting with the positively charged substrate, a notion supported by conservation of the side chain in permeases for cationic substrates.

Keywords: **arginine transport; *CAN1*; *GNP1*; citrulline; glutamine transport; *YEL063c*; *YDR508c***

Introduction

Uptake of amino acids into living cells has importance in widely different contexts, from nutrition to re-uptake of neurotransmitters. Uptake can be driven by a cation gradient, as is the case for glutamate re-uptake in cells of the central nervous system by the EAAT3 transporter (Zerangue and Kavanaugh, 1996) and aromatic amino acid uptake in *Escherichia coli* (Cosgriff and Pittard, 1997), or it can be driven directly by ATP hydrolysis, which is, for example, the case for histidine uptake in *Salmonella typhimurium* (Liu *et al*., 1997). In the yeast *Saccharomyces cerevisiae*, amino acid uptake is mediated by a family (André, 1995; Nelissen *et al*., 1997; Paulsen *et al*., 1998; Regenberg *et al*., 1999) of almost two dozen proton-driven permeases predicted to consist of one subunit with 8–12 transmembrane domains (TMD). This family is part of a larger family of cation-driven permeases, the amino acid–polyamine–choline (APC) transporter superfamily, including mammalian, plant and prokaryotic members (Fischer *et al*., 1998; Reizer *et al*., 1993). Amino acids are important nutrients, but excessive uptake of some of them can inhibit growth (Ljungdahl *et al*., 1992). It is therefore not surprising that amino acid uptake is controlled in several ways. The relatively large number of amino acid permease genes in yeast opens the possibility of fine tuning of the uptake of each amino acid from a

complex mixture. Preference of each permease towards one or a limited set of amino acids is thus likely to be an important physiological factor in amino acid permease function.

Experimental approaches to an understanding of the basis for substrate specificity must obviously involve mutant forms of permeases with altered substrate preference. Among side chains that are important for determining substrate preference, some are likely to form part of the amino acid binding site. Especially in the case of membrane proteins, mutagenesis is essential in elucidation of molecular functions, since crystallization can seldom be carried out as to determine the helix packing and substrate binding. The lactose permease from *E. coli* is a prime example of application of such technology. Site-directed mutagenesis and Cys-scanning mutagenesis have revealed residues that are essential for the coupling between H^+ translocation and sugar translocation; together with extensive analysis of the helix packing, this has led to a proposed mechanism for this coupling (Kaback, 1997). Site-directed *N*-ethylmaleimide labelling (Venkatesan and Kaback, 1998) and cysteine replacements were used to predict residues involved in sugar binding.

We have investigated the relationship between the primary structure of an APC transporter and its substrate specificity by selecting for mutations that alter the substrate range of the L-arginine permease Can1p in *S. cerevisiae*. The mutants were selected by requiring that the mutant permeases transport sufficient amounts of L-citrulline to allow the yeast to grow on this amino acid as nitrogen source. Sequencing of the mutant genes revealed the mutations to be located in six small clusters, which, according to current topological models for the permease, correspond to locations in transmembrane domains, cytoplasmic regions and external regions.

Materials and methods

Strains and media

The *Escherichia coli* strain DH5α (Grant *et al.*, 1990) served as plasmid host. A full deletion of the general amino acid permease gene (*GAP1*) was made in *Saccharomyces cerevisiae* strain M3750 (*MAT***a** *ura3* with S288C background, i.e. also *gal2*; Grauslund *et al.*, 1995). *GAP1* was deleted from 500 bp upstream the start codon to 200 bp downstream the stop codon by a one-step gene disruption applying the *kan*MX marker (Wach *et al.*, 1994). The deletion was verified by PCR, and the resultant strain was denoted M4269 (*MAT***a** *ura3 gap1::kanMX*). A similar deletion of *GAP1* was available in a strain of opposite mating type, M4577 (*MATα lys2 gap1::kanMX* with S288C background; Düring-Olsen *et al.*, 1999). The L-arginine permease gene (*CAN1*) was further deleted in a Δ*gap1* strain background, to avoid activity of the wild-type L-arginine permease when mutant forms were studied. This was carried out by inserting the *kan*MX marker (Wach *et al.*, 1994) between a point 100 bp upstream the start codon and a point 100 bp downstream the stop codon in strain M4054 (*MAT***a** *ura3* Δ*gap1* with S288C background; Grauslund *et al.*, 1995). This resulted in a complete deletion of *CAN1*, which was verified by PCR, and the resultant strain was denoted M4542 (*MAT***a** *ura3* Δ*gap1 can1::kanMX*). Yeast standard media, such as synthetic complete medium (SC), were made according to Sherman (1991), while minimal proline medium (MP) was made as described previously (Regenberg *et al.*, 1998). Minimal citrulline medium (MC) was made according to Regenberg and Hansen (2000).

DNA manipulation and sequence analysis

Unless otherwise noted, DNA work was carried out according to standard procedures (Guthrie and Fink, 1991; Sambrook *et al.*, 1989). All plasmids containing alleles of *GNP1* or *CAN1* suppressing *gap1* were sequenced from upstream of the predicted start codon to downstream of the predicted stop codon. Sequencing of each of the two open reading frames (ORFs) was effected with a set of 10 17-base primers that anneal to the coding strand with an interval of approximately 200 bases. The sequencing reaction was performed with the Perkin-Elmer BigDye Terminator Mix on Promega Wizard Plus Midiprep-purified plasmid, and the reactions were run, read and analysed on the ABI PRISM™ 377 DNA sequencer, all according to manufacturer's instructions.

Selection procedures

The L-citrulline uptake-deficient Δ*gap1 S. cerevisiae* strain M4269 was used to select for suppressors that restored growth on MC medium. Cells were

exposed to ethyl methanesulphonate (EMS) for mutagenesis according to Lawrence (1991). Mutagenesis was stopped after 10 min, at which point 90% of the cells were still alive. Approximately 2×10^8 cells were spread on selective medium (MC + uracil) and grown for 5 days at 30°C, after which 13 colonies appeared. These suppressors arose as expected by independent mutations, as a similar number of cells from the same culture did not give rise to any colonies on MC + uracil when not treated with EMS.

A strain containing a plasmid-linked wild-type copy of *CAN1* (obtained as described below) was used for selection of more alleles of *CAN1* suppressing *gap1*. Mutants were obtained by exposing 2×10^8 cells to 254 nm UV light to 50% survival. The mutants were selected directly on 10 MC plates, and 450 colonies appeared after 5 days. The 450 mutants had as expected almost exclusively arisen by independent mutation events, since 10 control plates that had not been exposed to UV light gave only two colonies. Another 44 mutants were obtained as spontaneous mutants. To ensure that these mutants were independent, single-cell colonies of the parental strain were isolated before selection, and only one mutant was selected from each. The selection yielded 61 mutants, of which 44 carried the mutation on the plasmid, according to the 5-fluoro-orotic acid (FOA)-test. Spontaneous mutation rates were not determined. Plasmids containing the *CAN1* mutant alleles were then prepared from yeast and introduced into *Escherichia coli*. To test that the plasmid indeed carried a suppressor allele, plasmids were reintroduced into the Δ*gap1* strain M4269, and the resultant transformants were tested for growth on MC. Plasmids from eight of the UV mutants were introduced into M4269, and three of these, *CAN1-201*, *CAN1-204* and *CAN1-209*, were able to support growth on MC. In the case of the spontaneous mutants, plasmid DNA from 35 clones was tested by retransformation; 17 of the plasmids were confirmed to carry suppressor alleles (*CAN1-306*, *CAN1-307*, *CAN1-315*, *CAN1-316*, *CAN1-318*, *CAN1-327*, *CAN1-328*, *CAN1-329*, *CAN1-331*, *CAN1-332*, *CAN1-335*, *CAN1-337*, *CAN1-339*, *CAN1-340*, *CAN1-341*, *CAN1-342* and *CAN1-343*).

Construction of a *GNP1-1* and a *CAN1-1* library

In order to clone some of the genes containing the primarily selected dominant mutations, we constructed genomic libraries in a centromere-based vector from two of the 13 EMS-induced suppressor mutants. A 30 ml overnight culture from each mutant was harvested and genomic DNA was isolated according to Hoffman and Winston (1987). Each DNA preparation was divided into two parts of 50 μg for partial digestion with 75 units *Bgl*II and *Bam*HI, respectively, for 45 min. The DNA was separated by agarose gel electrophoresis, and fragments in the range 3–11 kb were purified from the gel by QIAEX II (QIAGEN), according to the supplier's instructions. A total of 3 μg genomic DNA was then ligated with 2 μg *Bam*HI-digested, dephosphorylated pRS316 (Sikorski and Hieter, 1989). Strain M4269 was transformed (Becker and Guarente, 1991) with the ligation mixture and plated directly on MC medium for selection of positive clones. Parallel plating on SC without uracil showed that each of the two libraries gave approximately 120 000 transformants, of which 30% contained vector without insert. The library derived from the first mutant gave six colonies, of which one carried the suppressor gene on the plasmid, as evidenced by concomitant loss when the transformant was cured for the plasmid by selection for Ura3$^-$ cells on FOA-containing medium (Sikorski and Boeke, 1991). The second mutant library gave 14 colonies, of which six carried the suppressor gene on the plasmid, according to the same test. Plasmid DNA from one clone of each of the libraries was prepared by the Amersham yeast DNA extraction kit (RPN8518) and reintroduced into *E. coli* for amplification and purification. Partial sequencing of the two plasmids showed that the first, pRB152, contained a 10 452 bp *Bgl*II fragment of chromosome V from 28 025 to 38 477, including the *CAN1* gene, while the second, pRB156, contained a 5410 bp *Bgl*II fragment of chromosome IV, including the *GNP1* gene.

Cloning of wild-type *CAN1* and *GNP1*

The *CAN1* gene and the *GNP1* gene were cloned by gap repair of the two isolated alleles, *CAN1-1* and *GNP1-1*. The *CAN1-1* mutation in pRB152 was removed by digestion with *Aat*II and *Nru*I, while the *GNP1-1* mutation was removed from pRB156 with *Aat*II and *Sph*I, respectively, to remove the *CAN1-1* and the *GNP1-1* alleles from the two genomic clones. Strain M4269 was then transformed with the linearized plasmids for gap repair

by homologous recombination. Transformants that grew on SC without uracil were tested for growth on MC. Of 200 tested transformants, 45% of the *GNP1* clones showed no growth on MC, while the same was the case for 83% of the *CAN1* clones. One *CAN1* clone that did not complement on MC was chosen for further mutagenesis, and to confirm that this was indeed a wild-type copy of the L-arginine permease gene, the complete ORF was sequenced and found to be identical to that described in the Saccharomyces Genome Database (SGD) and reported by Hoffmann (1985) and Ahmad and Bussey (1986).

Amino acid uptake

Overnight cultures were diluted and grown to a cell density corresponding to OD_{600} 0.5–0.7 in MP medium at 30°C. Cells were harvested from 50 ml culture and resuspended in 1 ml minimal medium without nitrogen source. The assay was started by adding 100 µl resuspended cells to 150 µl minimal medium with radiolabelled amino acid without nitrogen source. In experiments where cells were exposed to only a single, high concentration of L-arginine (250 µM), 0.1 µCi L-[^{14}C]arginine was added per sample. When saturation kinetics was determined, L-arginine concentrations varied from 5 to 600 µM, whereas L-citrulline concentrations varied from 50 to 1600 µM. The specific radioactivity of the radiolabelled amino acid was constant, and the tube with the highest concentration contained 0.3 µCi for L-arginine and 1.0 µCi for L-citrulline. After 1 or 2 min, uptake of L-arginine was stopped by the addition of 1.0 ml ice-cold 100 mM non-radioactive L-arginine. In the case of L-citrulline uptake, mutant cells were exposed to the radiolabelled amino acid for 30 min before the reaction was stopped, while wild-type (*GAP1*) cells were exposed to L-citrulline for only 3 min. The cells were collected on a glass-fibre filter (GC50, Advante Toyo), washed twice with 2 ml ice-cold SC, dried and counted in a Beckman L8S 60000IC scintillation counter. For background activity, cells were added to the assay medium after addition of ice-cold amino acid. All uptake measurements were made in two or three independent experiments, and average values are given. ^{14}C-labelled amino acids were obtained from New England Nuclear.

Results

Mutations in *CAN1* and *GNP1* that restore citrulline uptake in a Δ*gap1* strain

Deletion of the general amino acid permease gene *GAP1* (Grenson *et al.*, 1970; Jauniaux and Grenson, 1990) in *Saccharomyces cerevisiae* abolishes uptake of L-citrulline, resulting in the inability to grow on moderate concentrations of L-citrulline as sole nitrogen source. In a search for mutations affecting substrate specificity in other permease genes, ethyl methanesulphonate (EMS)-induced mutants that could suppress the citrulline-negative phenotype were selected in the Δ*gap1* strain M4269. Mutagenesis to 90% survival of 2×10^8 cells gave rise to 13 mutants that could grow on L-citrulline, i.e. a frequency of 7×10^{-8}. They were all found to be dominant, which is expected if the suppressor phenotype arises from a change in the substrate specificity of an existing transport protein. Plasmid libraries were constructed from two mutants, and the mutations were subsequently cloned by complementation in the parental strain, M4269. From the first mutant, a plasmid with a 5 kb insert was cloned, and partial sequencing showed that it contained the complete L-glutamine transporter gene *GNP1*, and a short ORF, *YDR509W*. Cloning from the library from the second mutant resulted in a 10 kb fragment from chromosome V. This comprised the L-arginine transporter gene, *CAN1* plus *NPR2* and ORF *YEL064C*. Excision of an *Aat*II–*Nru*I fragment in *CAN1* and an *Aat*II–*Sph*I fragment in *GNP1*, followed by gap repair in strain M4269 with the genetic information of the wild-type genes, resulted in plasmids that could not complement growth on L-citrulline. Hence, the mutations were located in the two transporter genes, and the alleles were therefore denoted *GNP1-1* and *CAN1-1*.

Sequencing of *GNP1-1* revealed a single base pair substitution (Table 1) changing residue 239 from tryptophan to cysteine in a putative TMD of Gnp1p. The tryptophan in position 239 is conserved as an aromatic residue in the amino acid permeases, being tryptophan, tyrosine or phenylalanine (Figure 1). Substitution of the corresponding residue in the amino acid permeases AroP and PheP from *E. coli* also leads to a change in substrate preference (Cosgriff *et al.*, 2000). The mutation in *CAN1-1* results in a one-residue substitution of a conserved side chain. In this case a proline residue in position 148 is changed to leucine as the result

Table 1. *CAN1* and *GNP1* mutations that restore growth on L-citrulline in a Δ*gap1* mutant

Suppressor allele	Nucleotide exchange	Amino acid exchange	Predicted location
CAN1-343	C338T	P113L	Loop 1
CAN1-1, -201, -204, -209, -337	C443T	P148L	Loop2
CAN1-315	G445T	V149F	Loop2
CAN1-342	C455T	S152F	Loop2
CAN1-306, -332	T517G	Y173D	Helix III
CAN1-327	T517C	Y173H	Helix III
CAN1-341	G923C	G308A	Loop6
CAN1-329	C938T	P313S	Loop6
CAN1-318	Δ1060–1065	Δ354–355	Loop7
CAN1-339	T1066A	Y356N	Loop7
CAN1-340	T1066C	Y356H	Loop7
CAN1-316, -331	G1352T	W451L	Helix X
CAN1-335	G1352C	W451S	Helix X
CAN1-328	G1353C	W451C	Helix X
CAN1-307	T1382C	F461S	Helix X
GNP1-1	A717C	W239C	Helix III

of a T-to-C transition in position 443 of the open reading frame of *CAN1* (Table 1). This proline residue is conserved among most members of the amino acid permeases (Figure 1). The fact that an L-arginine transporter and an L-glutamine transporter can be altered to transport L-citrulline is not surprising from a structural point of view. All three compounds are L-α-amino acids, and they only differ slightly in their side chains. L-citrulline is a precursor in the biosynthesis of L-arginine, and the two amino acids have side chains that are sterically almost identical. L-glutamine has a shorter side chain than the two other amino acids, but resembles L-citrulline through the amide group.

Several substitutions in Can1p confer L-citrulline transport

In order to reveal more residues in the amino acid permeases important for substrate recognition, more mutants were selected in the Δ*gap1* strain (M4269) harbouring *CAN1* on a centromere-based plasmid. UV mutagenesis to 50% survival yielded citrulline-utilizing mutants at a frequency of 2.3×10^{-6}. The plasmid-borne copy of *CAN1* was sequenced, and it was confirmed that the sequence of the coding region corresponded to that previously reported (Ahmad and Bussey, 1986; Hoffmann, 1985). Selection was performed on L-citrulline as sole nitrogen source, with or without preceding UV mutagenesis. Linkage of the mutation to the plasmid was investigated by curing the strain for the plasmid. Eight plasmid-linked mutations expected to be independent were isolated from UV-mutagenized cells, and given allele numbers of three digits beginning with 2 (*CAN1-2xx*). Upon propagation in *E. coli* and reintroduction into the Δ*gap1* strain, each of the mutant plasmids restored growth of the cells on medium with 2 mM L-citrulline as nitrogen source. Complete sequencing of the *CAN1* gene in three such plasmids revealed that *CAN1-201, CAN1-204* and *CAN1-209* contained the same mutation, namely transition of cytosine 443 to thymine, also found in *CAN1-1* (Table 1). Another 35 plasmids were isolated from independent spontaneous mutants, and denoted *CAN1-3xx*. Of these, 17 plasmids suppressed the *gap1* mutation on L-citrulline as sole nitrogen source when reintroduced into yeast.

A similar experiment was carried out with *GNP1* on a centromere-based plasmid. However, in this case none of 100 independent mutants carried the mutation on the plasmid, suggesting that few *GNP1* mutations allow import of L-citrulline as compared with the situation in *CAN1*. We find it probable that mutations in the (chromosomally located) *CAN1* gene or other amino acid permease genes were responsible for citrulline utilization in these mutants; however, this was not investigated.

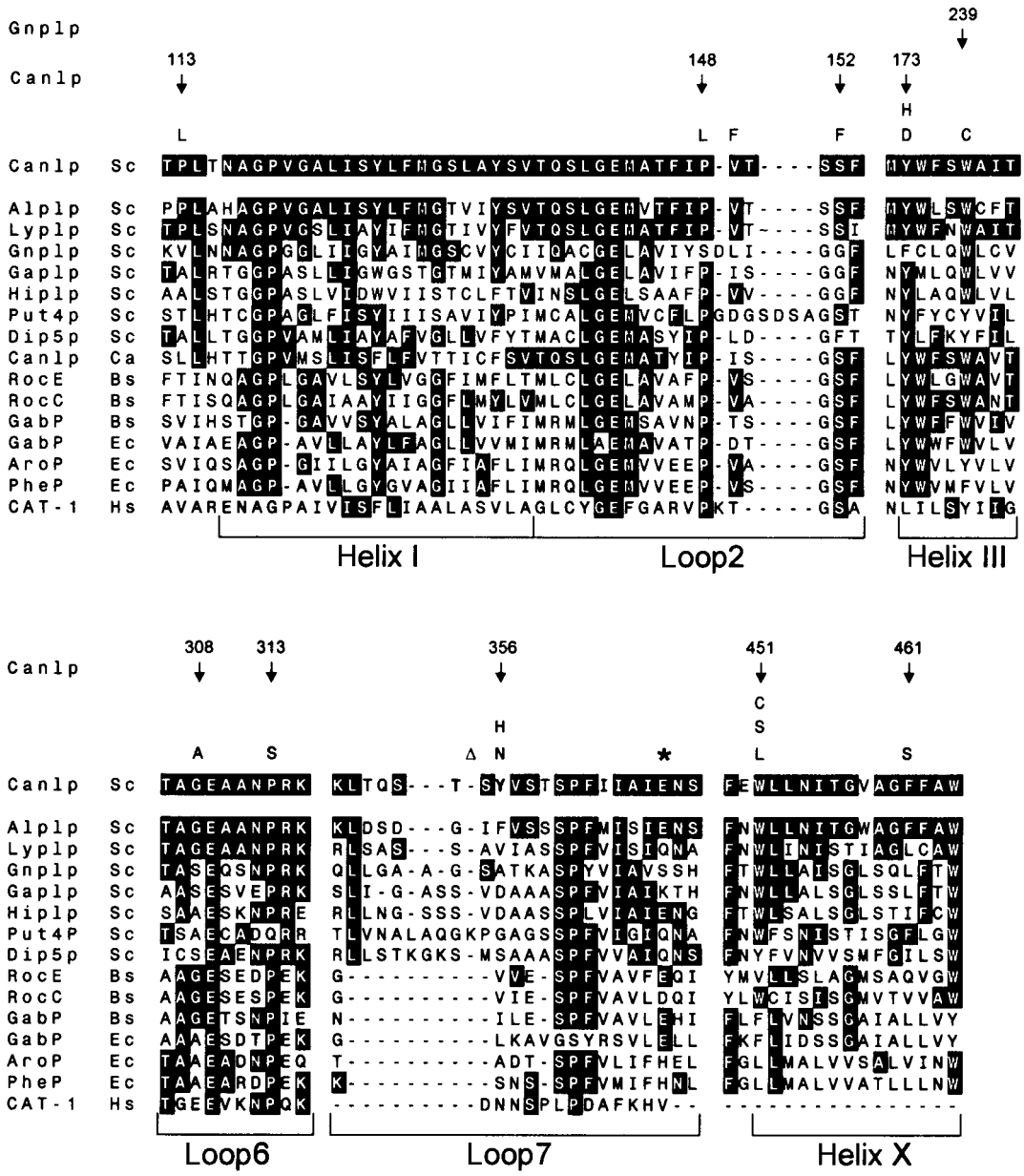

Figure 1. Alignment of the affected domains in Gnp1p and Can1p mutant permeases with 13 similar amino acid permeases. Species name is given after gene name: Bs, *Bacillus subtilis*; Ca, *Candida albicans*; Ec, *Escherichia coli*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*. Upper lines indicate the amino acid substitutions described in Table 1. Identical residues are shown in black boxes. Asterisk indicates the glutamate residue only found in substrates with a positive charge in the side chain. The alignment is part of that given by ProDom (**http://www.toulouse.inra.fr/prodom/doc/prodom.html**)

The mutations affect transmembrane domains as well as loops of Can1p

DNA sequence analysis of the 17 new *CAN1* alleles showed that each had one mutation in the open reading frame (Table 1 and Figure 1). In total, 15 distinct alleles were found, affecting 12 different codons, situated in six clusters (Table 1 and Figure 1). Can1p is located in the plasma membrane (Opekarová *et al.*, 1993) and is expected to contain

close to a dozen TMDs (Ahmad and Bussey, 1988; Hoffmann, 1985, 1987). Indeed, fusion of alkaline phosphatase in the putative loop regions of the homologous permeases AroP and PheP indicated the presence of 12 TMDs (Cosgriff and Pittard, 1997; Pi and Pittard, 1996). This topology was confirmed for the *S. cerevisiae* permease Gap1p by insertion of signals for *N*-linked glycosylation and a protease factor Xa cleavage site in putative loop regions (Gilstring and Ljungdahl, 2000). It can be assumed that Can1p does not deviate in its overall structure from the other permeases (for review, see Grenson, 1992; Reizer *et al*., 1993; Sophianopoulou and Diallinas, 1995), and we find that topological work described above gives strong evidence for the model shown in Figure 2. The mutations found here are located in TMDs, loops on the cytosolic side of the membrane, and loops on the extracellular side (Figure 2).

Substrate preferences of the mutant transporters

Prior to testing the mutant phenotypes, the different *CAN1* alleles were introduced into a Δ*can1*Δ*gap1* strain (M4542) in order to study the characteristics of the mutant transporters in the absence of the wild-type Can1p and Gap1p transporters. The resulting strains were cultured in the presence of different toxic amino acid analogues to test whether any of the mutant permeases had gained the ability to take up a broader range of substrates than just L-citrulline. Two of the 15 mutants, *CAN1-329* and *CAN1-337*, were more sensitive to several of the analogues. *CAN1-337* gave sensitivity to L-glutamic acid α-hydroxamate, α-aminoisobutyrate, 3-chloro-L-alanine, L-ethionine, L-allylglycine, and D-histidine, but not sensitivity to L-aspartic acid α-hydroxamate or *p*-fluoro-L-phenylalanine, while *CAN1-329* con-

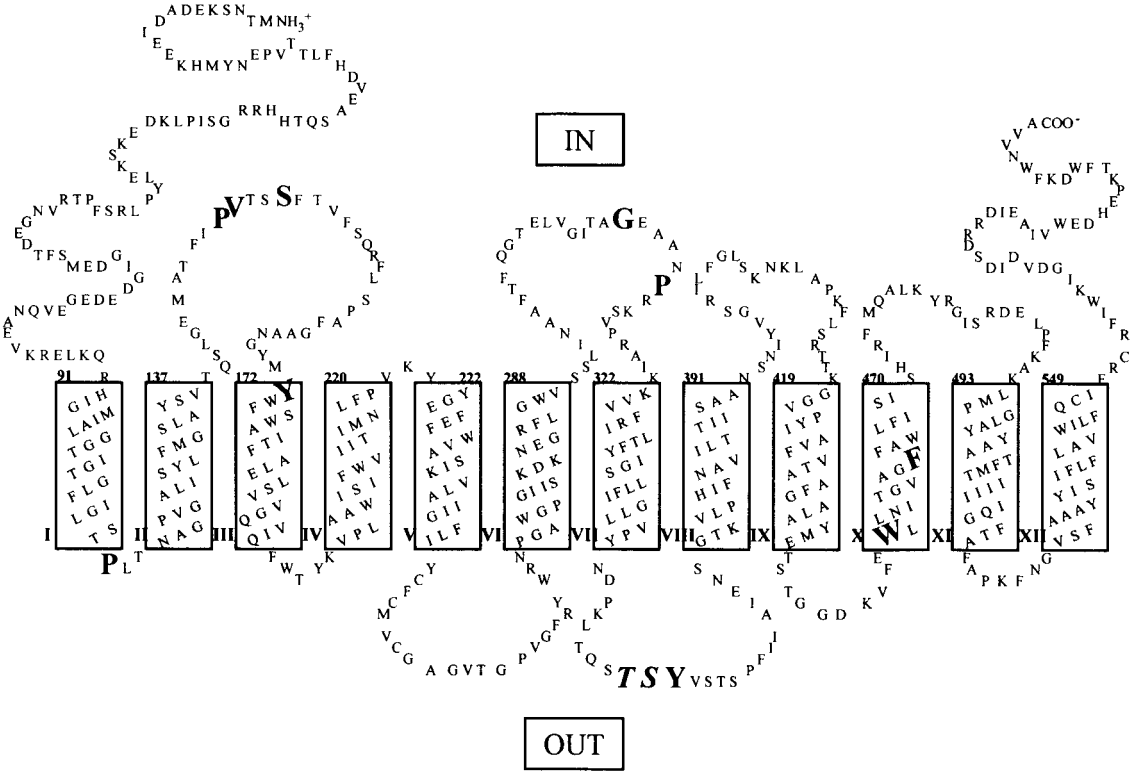

Figure 2. Secondary structure model with residues affecting substrate specificity of Can1p. The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. The residues altered by single substitutions are enlarged and marked in black bold, whereas the two that are absent as a result of a 6 bp deletion are shown in enlarged italics. The model is based on alignment of the primary sequence of Can1p to AroP and subsequent fitting of the topological model of AroP (Cosgriff and Pittard, 1997), as well as the models previously suggested for Can1p (Hoffmann 1985, 1987; Ahmad and Bussey, 1988) and Gap1p (Gilstring and Ljungdahl, 2000)

ferred sensitivity to all eight compounds. These drastic changes in substrate specificity are noteworthy, considering that *CAN1-329* and *CAN1-337* encode changes in two highly conserved residues (P313 and P148, respectively). These residues might be of more general importance for the function of the amino acid transporters than the other mutated residues, and therefore not directly involved in substrate binding.

Kinetics of L-citrulline transport

The ability to transport L-citrulline was further investigated by exposing the mutant strains to L-[^{14}C]citrulline. As with the amino acid analogue assay, mutant alleles were expressed, at low copy number, in the Δ*gap1*Δ*can1* strain M4542, so that background activity from the general amino acid permease Gap1p was avoided. Cells were grown to mid-exponential phase in medium with proline as nitrogen source, harvested and exposed to a range of L-citrulline concentrations from 50 μM to 1600 μM. Under these conditions, L-citrulline transport (Figure 3) follows Michaelis–Menten kinetics in the *GAP1* strain with an apparent $K_t \approx 50$ μM for L-citrulline and a $J_{max} = 19.5$ nmol/min/mg dry weight (Figure 3). As expected, all mutant strains had detectable transport of L-citrulline; however, none of them reached saturation over the range of substrate concentrations used here. Rather, the mutant transporters exhibit a linear relationship between the concentration of substrate and the rate of transport, as exemplified with the alleles *CAN1-337* (P148L), *CAN1-329* (P313L), *CAN1-331* (W451L), *CAN1-335* (W451S) and *CAN1-328* (W451C) in Figure 3. We conclude that no high-affinity L-citrulline-binding site was created in any of the mutants.

Mutants maintain ability to transport L-arginine

To determine whether the Can1p mutant permeases had gained their ability to transport L-citrulline at the expense of L-arginine transport, accumulation of L-[^{14}C]arginine was monitored in the mutant strains. In the first series of experiments, accumulation was measured with a single concentration of L-arginine (250 μM). The plasmids with the *CAN1* mutant alleles conferred L-arginine transport to the Δ*gap1*Δ*can1* strain in all cases, although accumulation varied in the range of 30–100% of that monitored in a wild-type *CAN1* strain. The L-arginine transport was then further investigated

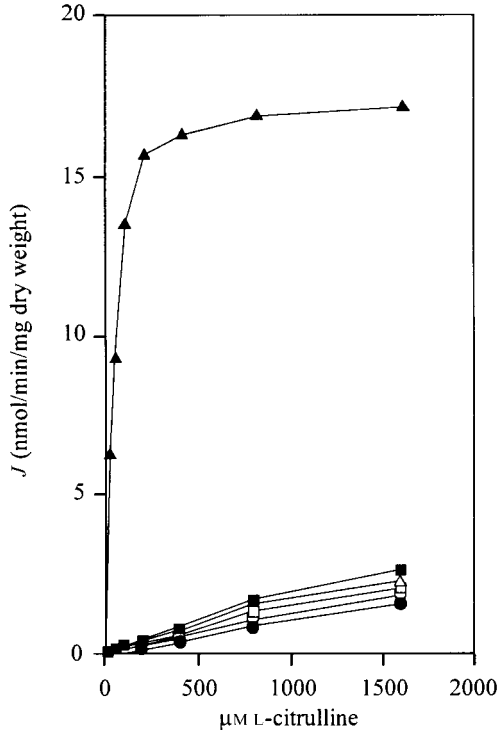

Figure 3. L-citrulline transport in the *CAN1* mutants. L-citrulline transport was monitored in strain M4542 (Δ*can1* Δ*gap1 ura3*) with plasmids carrying *CAN1-328* (closed circles), *CAN1-329* (closed squares), *CAN1-331* (open squares), *CAN1-335* (open circles), or *CAN1-337* (open triangles). Mutants were compared with the *CAN1* (wild-type) strain (closed triangles) M3750 (also *GAP1*, which in this strain is responsible for the L-citrulline transport). Cells were grown on proline as nitrogen source prior to the experiment, to ensure a high expression of *GAP1*

in five of the mutant strains, by exposing cells to varying concentrations of L-arginine. All five mutant permeases followed Michaelis–Menten kinetics with affinities in the range of that for the wild-type permease (3–10 μM L-arginine), and J_{max} somewhat lower (7–10 nmol/min/mg dry weight as compared to 22 nmol/min/mg dry weight).

Discussion

We have found that a number of mutations in the *S. cerevisiae* L-arginine permease gene (*CAN1*) confer an ability of the permease to transport L-citrulline, which is sufficient to support growth on this amino acid as sole nitrogen source. One

similar mutation was detected in *GNP1*, the glutamine-asparagine permease gene.

Functional changes were generally not dramatic. All of the Can1p mutant permeases retained some of their ability to transport L-arginine in addition to their new ability to transport L-citrulline. The affinity for L-arginine was changed by no more than a factor of two up or down in the analysed five mutants. Most of the suppressor mutants harbour a single base pair substitution, and this may be insufficient to turn Can1p into a high-affinity L-citrulline transporter, since none of the mutant permeases exhibited saturated uptake of L-[^{14}C]citrulline (Figure 3). These kinetics could be explained by a statically open channel-like mechanism for L-citrulline transport through the mutant permeases, but we find it unlikely that they transport L-arginine and L-citrulline by different mechanisms. Rather, we presume that the mutant permeases transport L-citrulline by a conventional four-state carrier mechanism (Stein, 1990), at which the affinity is so low that the transport appears linear and non-saturated within the range of substrate concentrations used in this study.

The mutations affect 13 different residues of Can1p and Gnp1p, and the concerned residues must somehow be involved in substrate recognition. The most N-terminal substitution, P113L, faces the extracellular space. The next three substitutions, P148L, V149F and S152F, are interesting in several respects; first, they are found in a cluster in the second loop, emphasizing the importance of this domain. Second, P148 and S152 have pronounced conservation patterns in the amino acid permease family (Figure 1). P148 is conserved among all yeast amino acid permeases except Gnp1p, Agp1p and Tat1p (**http://www.toulouse.inra.fr/prodom/doc/prodom.html**), with Gnp1p and Agp1p being able to transport L-glutamine and L-asparagine (Regenberg *et al*., 1999; Schreve *et al*., 1998). Hence, proline at this position might ensure a sterical constraint on the permease, preventing transport of certain amino acids such as the amido amino acids L-asparagine, L-glutamine and L-citrulline. S152 is interesting, since it is conserved among bacterial, fungal, and mammalian cationic amino acid transporters, including CAT1 from humans (Figure 1). Therefore the presence of serine in this site of Can1p could be important for the recognition of L-arginine as substrate. Two other loop regions seem to influence the substrate recognition: two substitutions in the sixth loop gave L-citrulline transport (G308A and P313S), while the deletion of residues 354–355 or substitution of the tyrosine side chain for asparagine or histidine in residue 356 suggests the importance of loop 7 (Table 1, Figure 2). The proline residue 313 in loop six is highly conserved within the APC superfamily (Reizer *et al*., 1993), and only in the sequence of the proline transporter Put4p has the proline been exchanged for a glutamine side chain (Figure 1). Loop 7, on the other hand, is highly variable in size and sequence within the APC superfamily (Figure 1). In addition to these mutations, another eight suppressor alleles were predicted to affect sites in the TMDs. Residue Y173 in helix III appears interesting, since two different substitutions of the tyrosine residue led to L-citrulline transport (Table 1). It is noteworthy that the W239C substitution of the *GNP1-1* allele is also predicted to locate in helix III of the L-glutamine transporter (Figure 2), supporting a role of helix III in substrate recognition of this family of transporters. Cosgriff *et al*. (2000) studied chimeras of AroP and PheP in *E. coli*, and identified a position (103 and 111, respectively) in TMD III of AroP and PheP as being important for substrate preference, supporting a rather general role of helix III in substrate recognition by the APC transporters. In addition to the mutations affecting helix III, the remaining suppressor mutations were found to affect two residues in helix X (Figure 2). In one case (residue 451), tryptophan could be altered to leucine, serine and cysteine, while in the second case (residue 461), a phenylalanine substitution to serine gave the L-arginine transporter the ability to transport L-citrulline.

Mutations that affect predicted TMDs and influence substrate preference have previously been found in the *Escherichia coli* lactose permease (Varela *et al*., 1997; Venkatesan and Kaback, 1998), the *Aspergillus nidulans* purine transporter (Diallinas *et al*., 1998) and the *Saccharomyces cerevisiae* hexose transporters Gal2 and Hxt2p (Kasahara *et al*., 1996). This is in accordance with our finding of several mutations affecting residues in TMD III and X of Can1p, as well as a substitution in the third predicted TMD of Gnp1p (Figure 1).

The mutations in helix X exchanged bulky residues (W451 and F461) to shorter polar side chains. These substitutions could potentially enlarge a substrate-binding site or move a charged group that would otherwise interact with the charged side

chain of L-arginine, allowing for L-citrulline to fit into Can1p.

The positive charge of the transported arginine could have a negatively charged counterpart in Can1p substrate recognition. A candidate for such a residue is found in the periplasmic loop 7, between TMD VII and VIII. This is a glutamate residue (E367) conserved among the yeast and bacterial arginine permeases [Figure 1; Can1p and Alp1p in *S. cerevisiae* (Ahmad and Bussey, 1986; Hoffmann, 1985; Regenberg *et al*., 1999), Can1p in *Candida albicans* (Sychrova and Souciet, 1994), RocE and RocC in *Bacillus subtilis* (Gardan *et al*., 1995)], the bacterial γ-aminobutyric acid permeases [GabP in *B. subtilis* (Borriss *et al*., 1996), GabP in *Escherichia coli* (Niegemann *et al*., 1993)], a yeast histidine permease [Hip1p in *S. cerevisiae* (Tanaka and Fink, 1985)] and a potential *S*-methyl methionine permease [YkfD from *E. coli* (Thanbichler *et al*., 1999)]. In short, substrates that contain a basic extension might be recognized by an acidic environment in the seventh loop. Three mutations were found to affect this loop in Can1p: Δ354–355, Y356N and Y356H. These changes could have a local effect on the loop, directly altering the interaction with the substrate, thereby allowing for the transport of L-citrulline through Can1p. The seventh loop is also a good candidate for recognition of the variable substrate side chains by other members of the APC family. It is predicted to face the periplasm, and it has variable size and sequence that could be adopted to fit different amino acid side chains, such as the carboxyl groups of glutamate and aspartate, which might be recognized by one of the two lysine residues in the aspartate and glutamate transporter Dip5p (Figure 1).

Besides a direct interaction with the transported amino acid, one can also imagine that a side-chain substitution causes a change of the conformation of the permease, giving more room for amino acids that might otherwise not be tolerated as substrates. The latter explanation may apply to the substitutions found in the second and the sixth loop, in particular P148L and P313L, which confer broad substrate specificity. As opposed to the seventh loop, several residues in these two loops are conserved (Figure 1), implying the general importance of these loops. Proline causes specific constraints on the backbone conformation, and the fact that the two proline substitutions result in broad substrate specificity suggests that these substitutions have overall effects on the conformational equilibrium rather than local effects directly on substrate binding. It may be noted that Pi *et al*. (1998) found relatively modest effects of substituting the corresponding proline residues in the phenylalanine-specific permease pheP on *E. coli* with alanine.

In view of the small size of the substrate, it is noteworthy that mutations are found to affect loops on both sides of the membrane. This is consistent with the idea that some of these mutations affect substrate recognition in an indirect way. However, it is likely that residues on both sides of the membrane make contact to the substrate in a transport process involving more than one step.

With this work we have added to an initial understanding of substrate recognition in a vast group of transporters with heterologous substrates. The domains defined by mutational analysis of *CAN1* as involved in side chain recognition will assist in pinpointing the residues attaching the substrate to the other permeases of bacterial, fungal, plant and mammalian origin, by construction of further point mutations and chimeras between Can1p and heterologous permeases. The availability of the present selection system and the similarity between Can1p and other permeases suggests this *S. cerevisiae* permease as an attractive model for studying the mechanism of H^+/amino acid transport in the APC transporters.

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