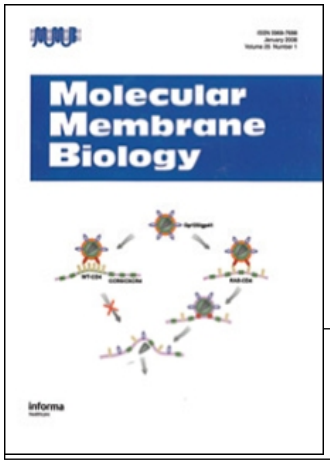


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Molecular Membrane Biology, 2000, 17, 47–57



# Amino acid residues $N^{450}$ and $Q^{449}$ are critical for the uptake capacity and specificity of UapA, a prototype of a nucleobase-ascorbate transporter family

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## Summary

**Specific carrier-mediated transport of purine and pyrimidine nucleobases across cell membranes is a basic biological process in both prokaryotes and eukaryotes. Recent *in silico* analysis has shown that the *Aspergillus nidulans* (UapA, UapC) and bacterial (PbuX, UraA, PyrP) nucleobase transporters, and a group of mammalian L-ascorbic acid transporters (SVCT1 and SVCT2), constitute a unique protein family which includes putative homologues from archea, bacteria, plants and metazoans. The construction and functional analysis of chimeric purine transporters (UapA-UapC) and UapA-specific missense mutations in *A. nidulans* has previously shown that the region including amino acid residues 378-446 in UapA is critical for purine recognition and transport. Here, we extend our studies on UapA structure–function relationships by studying missense mutations constructed within a 'signature' sequence motif [(F/Y/S)X(Q/E/P)NXGXXXXT(K/R/G)] which is conserved in the putative functional region of all members of the nucleobase/ascorbate transporter family. Residues $Q^{449}$ and $N^{450}$ were found to be critical for purine recognition and transport. The results suggest that these residues might directly or indirectly be involved in specific interactions with the purine ring. In particular, interaction of residue 449 with C-2 groups of purines might act as a critical molecular filter involved in the selection of transported substrates. The present and previous mutagenic analyses in UapA suggest that specific polar or charged amino acid residues on either side of an amphipathic α-helical transmembrane segment are critical for purine binding and transport.**

**Keywords:** Purine, mutagenesis, structure-function analysis, transmembrane domain, transport.

**Abbreviations:** *A. nidulans*, *Aspergillus nidulans*; TM, transmembrane domain; X, aliphatic hydrophobic amino acid residue; p, polar amino acid residue; ACZ, *A. nidulans* strain lacking all major purine transport activities; hc, high-copy; *K*m, Michaelis constant; *V*, velocity of transport; MM and CM, minimal and complete media respectively.

## Introduction

The filamentous ascomycete *A. nidulans* possesses three purine transporters encoded by the genes *uapA*, *azgA* and *uapC* (Scazzocchio 1994, Diallinas *et al.* 1995). The UapA protein is a high-affinity, high-capacity transporter, specific for uric acid and xanthine, the thioanalogues 2-thiouric acid and 2-thioxanthine, and allopurinol and oxypurinol (Darlington and Scazzocchio 1967, Diallinas and Scazzocchio 1989, Gorfinkiel *et al.* 1993). The AzgA protein is a high-affinity, high-capacity transporter, specific for adenine, guanine, hypoxanthine and the analogues 8-azaguanine and purine (Darlington and Scazzocchio 1967, Scazzocchio 1994, Diallinas *et al.* 1995). The UapC protein is a wide-specificity, low/moderate-capacity, carrier, transporting all natural purines as well as purine analogues (Scazzocchio and Gorton 1977, Diallinas *et al.* 1995). The *uapA* and *uapC* genes have previously been cloned, sequenced and studied in detail (Diallinas and Scazzocchio 1989, Gorfinkiel *et al.* 1993, Diallinas *et al.* 1995, Ravagnani *et al.* 1997). The UapA and UapC transporters share 62% identical amino acids throughout their amino acid sequence and belong to a distinct family of purine-pyrimidine-ascorbate carriers conserved in both prokaryotes and eukaryotes (Diallinas *et al.* 1995, Guimaraes *et al.* 1995, Schultes *et al.* 1996, Faaland *et al.* 1998, Rajan *et al.* 1999, Tsukaguchi *et al.* 1999). Members of this transporter family share 23–65% identical amino acid residues, a common hypothetical structure of 12–14 α-helical, transmembrane domains (TM) and a 'signature' motif (see figures 8 and 1A, respectively).

Recently, structure-function relationships in the UapA and UapC proteins have been analysed by constructing and studying chimeras and missense mutations. These studies have led to the identification of a short region, corresponding to residues 378–446 in UapA and 336–404 in UapC, and a number of specific amino acid residues within this region ($A^{404}$, $E^{412}$ and $R^{414}$ in UapA) critical for the function and specificity of the purine transporters. $A^{404}$ is important for UapA function, but probably not for its specificity *vis-a-vis* purines. It can be functionally replaced by $G^{404}$ but not by $D^{404}$. This agreed well with the fact that in all homologues of the nucleobase/ascorbate transporter family identified in the data bases, the residue at this position is either an alanine or a glycine. $E^{412}$ and $R^{414}$ are critical for both the function and specificity of UapA. Strains carrying either of the single amino acid replacements E412Q or R414E are indistinguishable from a total loss-of-function *uapA*$^{-}$ mutant. However, a strain carrying the double amino acid replacement has a novel purine transport specificity. While it fails to transport the physiological substrates of UapA (uric acid or xanthine), it gains low but detectable ability to transport hypoxanthine, similar to that of the general purine transporter, UapC, in which residues corresponding to positions 412 and 414 are already Q and E respectively. These residues are good candidates to form part of a purine binding site. However, the fact that the double replacement E412Q and R414E in UapA does not lead to a specificity identical to that of UapC suggests that other amino acid residues should also be involved in determining substrate specificity in these proteins. Such amino acid residues should, in principle, be present within the region

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defined, by chimeric and mutational analysis, as essential for function and specificity in UapA and UapC (Diallinas *et al.* 1998).

In this work, a series of strains, carrying *uapA* mutations within the 'signature' motif of the nucleobase/ascorbate transporter family, is constructed and studied. The results show that specific amino acid residues within this motif are indeed critical for purine recognition and transport and, together with results from a previous mutagenic analysis of UapA, strongly suggest that polar and charged amino acid residues on either side of a specific amphipathic transmembrane domain determine the function and specificity of this transporter.

## Results

### *Rationale of mutagenesis and mutant construction*

It was decided to mutagenize the three highly conserved polar amino acid residues, as well as the first hydrophobic residue overlapping with the upstream transmembrane segment, present into the consensus motif in UapA (figure 1B). The rationale of mutation design was the following. Close inspection of the motif sequence (figure 1A) revealed that position 3 (449 in UapA) is occupied by a Q in all known purine transporters (UapA, UapC and PbuX, a xanthine transporter in *Bacillus subtilis*), an E in pyrimidine transporters from *E.coli*, *B. subtilis* and *B. caldolyticus*, and a P in the recently

**A**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 447 | 448 | 449 | 450 | 451 | 452 | 453 | 454 | 455 | 456 | 457 | 458 |
| PURINE TRANSPORTERS - *A.NIDULANS* | F | X | Q | N | X | G | X | X | X | X | T | R |
| PURINE TRANSPORTERS - BACTERIA | F | X | Q | N | X | G | X | X | p | X | T | G |
| PYRIMIDINE TRANSPORTERS - BACTERIA | Y | X | E | N | X | G | X | X | X | X | T | R |
| L-ASCORBATE TRANSPORTERS- MAMMALS | S | X | P | N | X | G | X | X | X | X | T | K |
| TRANSPORTERS (?) - BACTERIA | F/Y | X | Q/E | N | X | G | X | X | p | X | T | R/K/G |
| TRANSPORTERS (?) - PLANT | S | X | E | N | X | G | X | X | X | X | T | R |
| TRANSPORTERS (?) - NEMATODE | F | X | E | N | X | G | X | X | X/p | X | T | R/K |
| TRANSPORTERS (?) - DROSOPHILA | F | X | E | N | X | G | X | X | X | X | T | K |
| TRANSPORTERS (?) - MOUSE | S | X | P | N | X | G | X | X | p | X | F | Q |
| TRANSPORTERS (?) - HUMAN | S | X | Q | S | X | G | X | X | X | X | T | R |
| CONSENSUS MOTIF | F Y S | X | Q E P | N | X | G | X | X | X/p | X | T | R K G |

**B**

| | 447 | 448 | 449 | 450 | 451 | 452 | 453 | 454 | 455 | 456 | 457 | 458 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| wild-type | F | A | Q | N | N | G | V | I | A | L | T | R |
| F447Y | Y | A | Q | N | N | G | V | I | A | L | T | R |
| Q449N | F | A | N | N | N | G | V | I | A | L | T | R |
| N450D | F | A | Q | D | N | G | V | I | A | L | T | R |
| N450Q | F | A | Q | Q | N | G | V | I | A | L | T | R |
| T457S | F | A | Q | N | N | G | V | I | A | L | S | R |

Figure 1. (*a*) A consensus sequence motif present in all known nucleobase transporters, two recently characterized mammalian ascorbate transporters, and a number of putative homologous proteins. The consensus sequence is based on a multiple amino acid sequence alignment of more than 70 proteins sharing significant similarity (> 22% overall identity) with UapA (Diallinas *et al.* 1998, Diallinas, unpublished observations). Lower numbers correspond to position in the UapA sequence. X and p indicate aliphatic hydrophobic and polar amino acid residues, respectively. Highly conserved amino acid residues are shown boxed. ? indicates unknown function. (*b*) Mutations introduced into the UapA sequence motif.

characterized ascorbate transporters (Rajan *et al.* 1999, Tsukaguchi *et al.* 1999). The same residue is either a Q or an E in other bacterial homologues, an E in all plant, insect and *Coenorhabditis elegans* homologues and a P in most mammalian homologues. To investigate its role in the function and/or specificity of nucleobase transporters *vis-a-vis* purines and pyrimidines, this residue was mutagenized from Q to E (mutation Q449E). Similarly, residue 1 (447 in UapA), which is always a F when residue 3 is a Q (purine transporters and other homologues from bacteria), but a Y or S when residue 3 is an E or P (bacterial pyrimidine transporters, mammalian ascorbate transporters, and other homologues from bacteria, plants and animals), was changed from F to Y (mutation F447Y). Positions 4 (450 in UapA) and 11 (457 in UapA) in the motif are the best conserved in all nucleobase transporter sequences and correspond to polar amino acids N and T respectively. To examine whether this exceptional conservation is significant for function, these residues were mutagenized to isofunctional (N to Q and T to S, respectively) or to isostructural (N to D) amino acids (mutations N450Q, T457S and N450D, respectively).

Mutations were constructed in plasmid pAN510 by oligonucleotide-directed mutagenesis as described in Experimental procedures. pAN510 is a pBLUESCRIPT derivative carrying a full-length *uapA* gene, in addition to the *A. nidulans argB* wild-type gene which allows selection of transformants in a genetic background of a total loss-of-function *argB*$^-$ mutant (Diallinas *et al.* 1998). Plasmids carrying mutations in *uapA* (pAN510-F447Y, pAN510-Q449E, pAN510-N450Q, pAN510-N450D, pAN510-T457S), and control vectors pAN510 and pIJL16 (a pUC8 derivative carrying only the *argB* gene; Diallinas *et al.* 1998), were introduced, by transformation, into a *uapA*$^-$ *uapC*$^-$ *azgA*$^-$ *argB*$^-$ *yA*$^-$ (ACZ; Diallinas *et al.* 1998) strain (for full genotypes and other experimental details see Experimental procedures). Several transformants (50–200), able to grow in the absence of arginine from minimal media, were obtained with 1 $\mu$g of plasmid DNA. A number of transformants obtained with each plasmid were selected and purified on selective media. Genomic DNA was isolated from all purified transformants and analysed by Southern blotting, as described in Experimental procedures (only selected results are shown, see below). Southern blot analysis was performed with both *uapA* and *argB*-specific radiolabelled probes, as described in Experimental procedures. Integration of plasmid sequences on the genome was detected in all cases, confirming that isolated strains were true transformants. Integration events took place, in most cases, by homologous recombination in either the *uapA* or the *argB* genomic loci. In most cases, transformants arose as the result of single integration events but in some cases integration of multiple copies of the transforming sequences also took place. Transformants resulting from identical integration events occurring at the *argB* locus (see figure 2) were selected. Both, single- and multi-copy transformants were selected for further analysis.

### *Phenotypic analysis of uapA mutants*

Selected transformants carrying different *uapA* mutations in single (ACZ:pAN510-F447Y ACZ:pAN510-Q449E, ACZ:pAN510-N450Q ACZ:pAN510-N450D, ACZ:pAN510-T457S) or multiple (ACZ:pAN510-Q449E-hc, ACZ:pAN510-N450Q-hc ACZ:pAN510-N450D-hc) copies were tested for their ability to grow on minimal media supplemented with various purines as nitrogen sources at 37°C. Representative growth tests are shown in figure 3. Mutation N450D had the most dramatic effect on UapA function. Transformants carrying this allele, in single (figure 3(*a*)) or multiple copies (not shown), are indistinguishable from total loss-of-function mutations in the *uapA* gene. Transformants carrying mutation F447Y were indistinguishable from transformants expressing a wild-type *uapA* gene. Mutations T457S and N450Q had a mild negative effect for growth on both xanthine (figure 3(*a*)) or uric acid (not shown) at 37°C. However, strains expressing N450Q exhibited a dramatic loss of their ability to grow on these media at lower temperatures (22–30°C).

Mutants expressing the Q449E allele had the most complex phenotype. Growth of single copy transformants on xanthine (figure 3(*a*)) or uric acid (not shown) at 37°C was reduced compared to wild-type strains, but it was significantly restored in high-copy transformants. Q449E transformants also grew marginally better than other transformants or control strains on hypoxanthine at 37°C (marginally visible in figure 3(*a*)). This indicated that Q449E mutants might have gained a very low capacity for hypoxanthine transport. The effect of the Q449E mutation for growth on purines was also found to be highly conditional, depending on both the temperature and the pH of the medium. At temperatures lower than the physiological (22–30°C), the Q449E mutation resulted in significant reduction of UapA function, even in multiple-copy transformants (figure 3(*a*)). On the other hand, at an elevated pH (7.5), compared to the standard physiological pH (6.5–6.8) for *A. nidulans*, Q449E mutants showed an enhanced ability to grow on both uric acid or xanthine (see figure 3(*b*)).

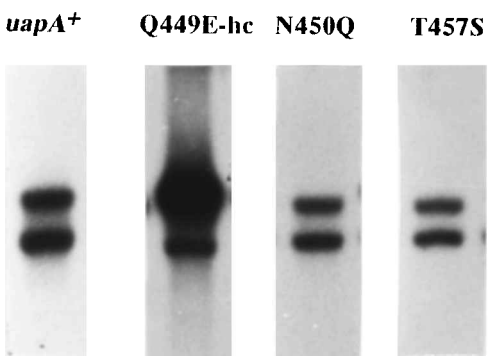


Figure 2. Southern blot analysis of selected mutants. Genomic DNA (5 $\mu$g) from transformants carrying plasmids pAN510 (*uapA*$^+$), pAN510-Q449E-hc, pAN510-N450Q, pAN510-T457S was digested with *Eco*RI, fractionated in 0.8% agarose gel, transferred to Hybond nylon filters and hybridized to radiolabelled *uapA*-specific sequences. The lower band corresponds to an intact single copy of genomic *uapA* sequences (carrying a total loss-of-function mutation). The upper band corresponds to single (pAN510, pAN510-N450Q, pAN510-T457S) or multiple (pAN510-Q449E-hc) copies of introduced plasmidic *uapA* sequences integrated into the *argB* locus.

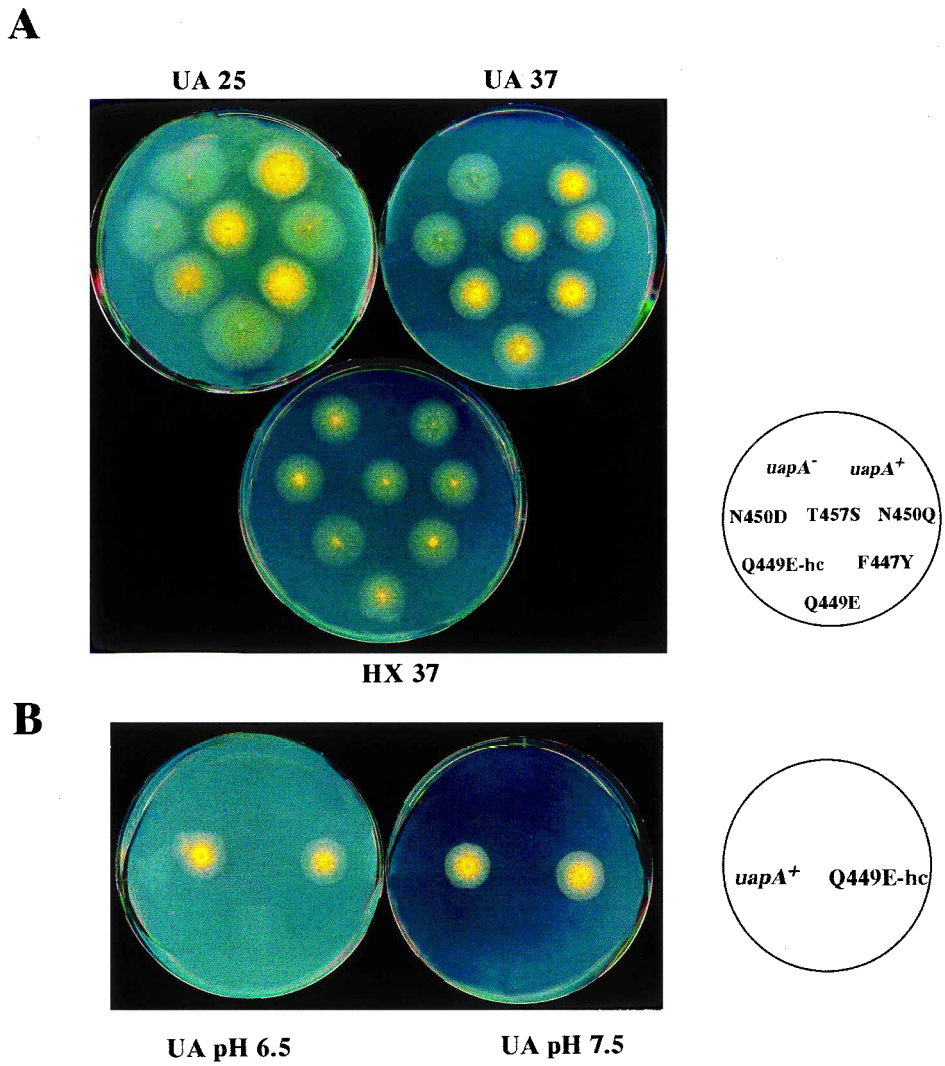


Figure 3. Growth of mutants on purines as sole nitrogen sources. Strain terminology is described in the text. The media are *Aspergillus* minimal media (MM) supplemented with 0.1 mg/ml uric acid (UA) or hypoxanthine (HX) as sole nitrogen source. All strains grow equally well on control media supplemented with urea as sole nitrogen source (not shown). Growth tests were carried out for 48 h at 37°C or for 80 h at 25°C. When indicated, the pH of the medium is 7.5. In all other cases, the pH is 6.5 which is the physiological pH for *A. nidulans*. (*a*) Growth of control strains and mutants on UA or HX and effect of the temperature for growth on UA. (*b*) Effect of the pH on growth of a control strain (*uapA*$^+$) and the mutant Q449E on UA. Strains are as described in the text. This figure is reproduced in colour in the on-line version of the paper.

*Kinetic analysis of purine transport in uapA mutants*

Transformants carrying mutations T457S, N450Q, N450D, F447Y and Q449E were further analysed by direct measurements of purine uptake rates. Mutation Q449E was studied in transformants expressing the mutant *uapA* allele from either single or multiple copies. Radiolabelled $C^{14}$-xanthine or $C^{14}$-hypoxanthine initial uptake rates were determined in germinating conidiospores of strains ACZ:plJL16, ACZ:pAN510, ACZ:pAN510-F447Y, ACZ:pAN510-Q449E, ACZ:pAN510-Q449E-hc, ACZ:pAN510-N450Q, ACZ:pAN510-N450D and ACZ:pAN510-T457S (Diallinas *et al.* 1995, 1998; for details see Experimental procedures). Uptake assays were performed at 10 $\mu$m xanthine or 50 $\mu$m hypoxanthine. Results are summarized in figure 4 and are in agreement with growth tests presented above. Strains expressing mutations N450Q and T457S showed approximately 18 and 31% of wild-type xanthine initial uptake rates, respectively (figure 4(*a*)). These values were estimated after subtraction of a 'background' xanthine accumulation rate measured in a strain lacking all major purine transporter activities (ACZ:plJL16; Diallinas *et al.* 1998). The strain expressing mutation F447Y showed similar to wild-type (86%) xanthine accumulation rate, while the strain expressing mutation N450D showed no xanthine uptake (figure 4(*a*)). Strains carrying mutation Q449E in single (pAN510-Q449E) or multiple (pAN510-Q449E-hc) copies showed 7 and 21% of wild-type xanthine accumulation rates, respectively (figure 4(*a*)). Similar results were obtained for $C^{14}$-uric acid accumulation rates (results not shown). Only the Q449E mutant showed the ability to

transport radiolabelled $C^{14}$-hypoxanthine (see figure 4(*b*)). This activity was very low, comparable to that of a previously constructed double mutant (E412Q/R414E) which had also gained the ability to transport hypoxanthine (Diallinas *et al.* 1998).

The kinetic parameters of purine transport were also determined in selected mutants. Transformants ACZ:pIJL16, ACZ:pAN510, ACZ:pAN510-T457S, ACZ:pAN510-N450Q and ACZ:pAN510-Q449E-hc were selected for direct measurements of radiolabelled xanthine in the range 1–100 $\mu$M. Transformants carrying mutations F447Y, N450D and Q449E in single copies were not analysed any further since they either do not affect UapA function significantly (F447Y) or result in no or very low transport rates (N450D, Q449E). *K*ms and *V*s were estimated after subtracting background xanthine accumulation measured in strain ACZ:pIJL16 and are shown in table 1. None of the mutations significantly affect the affinity of UapA for xanthine. In T457S, the *K*m rises from $9 \pm 2$ to $15 \pm 10$ $\mu$M, in Q449E to $25 \pm 4$ $\mu$M and in N450Q to $36 \pm 2$ $\mu$M. All mutations lower the maximum xanthine accumulation capacity (*V*) of the UapA transporter to 28–37% of the wild-type control strain ACZ:pAN510. It should be stressed that these are apparent values since *V*s also depend on the absolute amount of protein inserted in the membrane, which could not be estimated for reasons discussed both in this work (see Discussion) and previously (Diallinas *et al.* 1998).

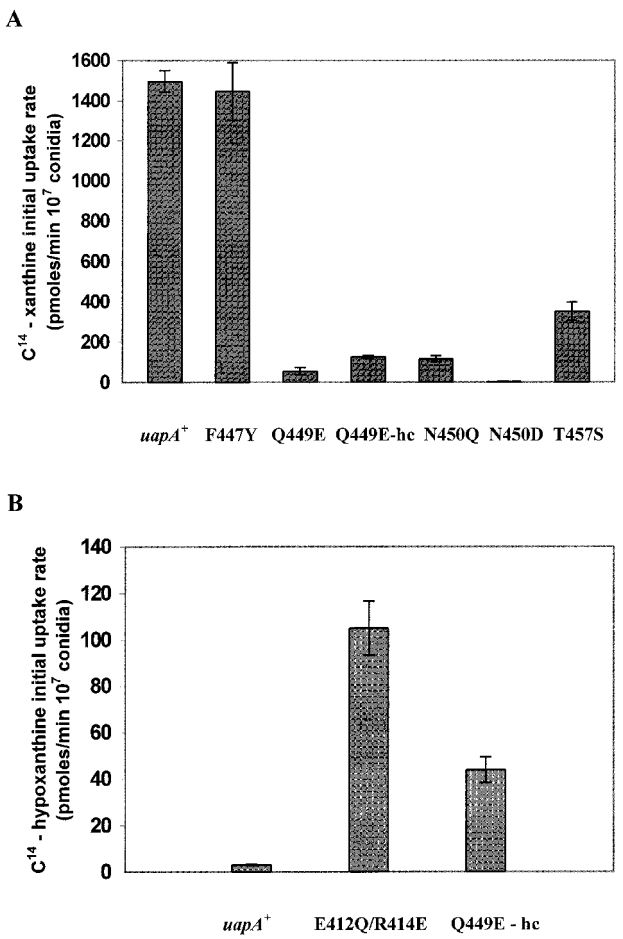


Figure 4. $C^{14}$-xanthine (*a*) and $C^{14}$-hypoxanthine (*b*) initial uptake rates in control strains (*uapA*$^+$ and *uapA*$^-$) and *uapA* mutants (F447Y, Q449E, Q449E-hc, N450D, N450Q, T457S). Uptake assays were performed as described in Experimental procedures. Purine initial uptake rates are expressed in pmoles/min per $10^7$ viable conidiospores. Results shown represent averages of at least six independent assays. Vertical lines represent mean standard deviation values.

### *Competition of xanthine transport by other nucleobases in uapA mutants*

The role of the amino acid residues changed by mutations Q449E, N450Q and T457S on the substrate specificity of UapA was tested. $C^{14}$-xanthine (10 $\mu$M) initial uptake rates were measured, in UapA wild-type and mutant strains, in the presence and absence of excess (30-fold) non-radiolabelled purines (adenine, guanine, hypoxanthine, uric acid and xanthine), purine analogues (8-azaguanine, 2-thioxanthine, 6-thioguanine), or pyrimidines (thymine, cytosine, uracil). Results are summarized in table 2. As expected, wild-type, UapA-mediated, xanthine uptake was efficiently competed (72–94% competition) by purines which are established substrates for this transporter (uric acid, xanthine, 2-thioxanthine). A more or less similar profile of competition was observed for mutant strains expressing mutations T457S (84–91% competition) and N450Q (96–98% competition). The only important difference observed with these mutants was the absence of significant competition by 2-thioxanthine (13% competition) in the N450Q mutant strain. In contrast to the above observations, mutants expressing the Q449E substitution showed a clearly novel $C^{14}$-xanthine uptake competition profile. Among physiological substrates, xanthine and uric acid were efficient competitors (81–90% competition) but 2-thioxanthine was not (22% competition). More significantly, guanine (71%), 8-azaguanine (68%), 6-thioguanine (98%), and hypoxanthine (94%), were all efficient competitors of $C^{14}$-xanthine uptake.

The competitive effect of uric acid, hypoxanthine and guanine on xanthine accumulation in the Q449E mutant was also analysed kinetically. Results (table 3) show the concen-

Table 1. $K_m$ and *V* values for $C^{14}$-xanthine uptake in strains carrying UapA mutations.

| Strain | $K_m$ | *V* |
|---|---|---|
| ACZ: pAN510 (wildtype UapA) | $9 \pm 2$ | $4305 \pm 540$ |
| ACZ:pAN510-T457S | $15 \pm 10$ | $1375 \pm 125$ |
| ACZ:pAN510-N450Q | $36 \pm 2$ | $1650 \pm 550$ |
| ACZ:pAN510-Q449E hc | $25 \pm 4$ | $1214 \pm 205$ |

Uptake assays were performed as described in Experimental procedures with conidia germinating for 4 h 30 min under inducing conditions. $K_m$ and *V* values were calculated by double reciprocal Lineweaver–Burk plots and represent average values from three independent experiments. The substrate range chosen was optimized for the determination of $K_m$. $K_m$ is in $\mu$M, *V* is in pmoles/min $10^7$ conidia. For other details see Experimental procedures.

Table 2. Competition of $C^{14}$-xanthine (10 μM) uptake by excess (300 μM) unlabelled nucleobases.

| Unlabelled nucleobase (competitor) | Strain ACZ: pAN510 (wild type) | ACZ:pAN510-Q449E-hc | ACZ:pAN510-N450Q | ACZ:pAN510-T457S |
|---|---|---|---|---|
| (–) | 100±3 | 100±11 | 100±9 | 100±4 |
| XAN | 6±0.4 | 10±1 | 7±0.5 | 9±1.4 |
| UA | 20±1.5 | 19±2 | 4±0.5 | 9±0.4 |
| HX | 96±10 | 6±0.3 | 73±7 | 77±7 |
| ADE | 100±5 | 135±13 | 78±2 | 88±5 |
| GUA | 99±5 | 29±6 | 136±3 | 107±11 |
| 2-THIOX | 26±5 | 78±6 | 87±10 | 16±2 |
| 6-THIOG | 100±14 | 11±0.6 | 115±22 | 125±17 |
| 8-AZAG | 101±13 | 32±2 | 136±10 | 142±9 |
| THY | 126±8 | 118±10 | 113±12 | 105±5 |
| CYT | 91±3 | 71±10 | 105±17 | 110±9 |
| URA | 127±9 | 94±14 | 108±13 | 108±8 |

Uptake assays were performed as described in Experimental procedures with conidia germinating for 4 h 30 min under inducing conditions. Values shown express per cent of $C^{14}$-xanthine (10 μM) uptake in the presence of excess (300 μM) unlabelled nucleobases (competitor). For all strains uptake values obtained in the absence of competitors were taken as 100%. Results represent averages of at least six independent experiments. XAN is xanthine, UA is uric acid, HX is hypoxanthine, ADE is adenine, GUA is guanine, 2-THIOX is 2-thioxanthine, 6-THIOG is 6-thioguanine, 8-AZAG is 8-azaguanine, THY is thymine, CYT is cytosine, URA is uracil. For other details see Experimental procedures.

trations of competitors, resulting in 50% inhibition of xanthine accumulation. For uric acid, this was found to be very close to the physiological *K*m of UapA for this purine (15±5 μM). The mutant was also found to have acquired the ability to bind guanine and hypoxanthine with affinities 10–15-fold lower than the physiological *K*m of UapA for its natural substrates (100±50 and 98±15 μM, respectively). These results also established that relevant competition assays described above were all performed in concentrations of competitors (300 μM) well above saturation values for maximum inhibition by uric acid, guanine or hypoxanthine.

### *pH–dependence of UapA function in Q449E mutants*

It was shown in figure 3(*b*) that Q449E mutants exhibited an enhanced ability to grow on xanthine (or uric acid) at an elevated pH (7.5), compared to the standard physiological pH (6.5–6.8) for *A. nidulans*. It was examined whether the pH-dependence of the Q449E mutant phenotype was due to direct pH-dependence of the process of transport catalysis. Figure 5 compares $C^{14}$-xanthine uptake measurements in strains ACZ:pAN510 and ACZ:pAN510-Q449E-hc performed at various pHs. These results demonstrated that, while wild-type UapA transport of xanthine is constant in the pH range 5–7 and drops significantly at pH 8 (65% of the physiological capacity), the same activity in the Q449E mutant is lowest at pH 5 (17% of wild-type), increases as the pH rises (30–35% at pH 6 and 7) and becomes highest at pH 8 (75% of wild-type).

### *Cryosensitivity of N450Q and Q449E mutants is basically due to defective transport*

Cryosensitive growth of strains carrying mutations N450Q or Q449E might be due to either a *de novo* translated unstable protein, or a protein that is not properly sorted into the plasma membrane, or a protein which is properly sorted in the membrane but in which limited misfolding at the non-

Table 3. Kinetic analysis of competition $C^{14}$-xanthine (10 μM) accumulation by uric acid, hypoxanthine or guanine in the Q449E-hc mutant.

| Purine competitor | Concentration of competitor resulting in 50% of maximum inhibition of $C^{14}$-xanthine (10 μM) uptake |
|---|---|
| UA | 15±5 |
| HX | 98±15 |
| G | 100±14 |

UA is uric acid, HX is hypoxanthine, G is guanine. Uptake assays were performed as described in Experimental procedures. Values represent averages of six different experiments. Competitor purines and $C^{14}$-xanthine were added simultaneously.

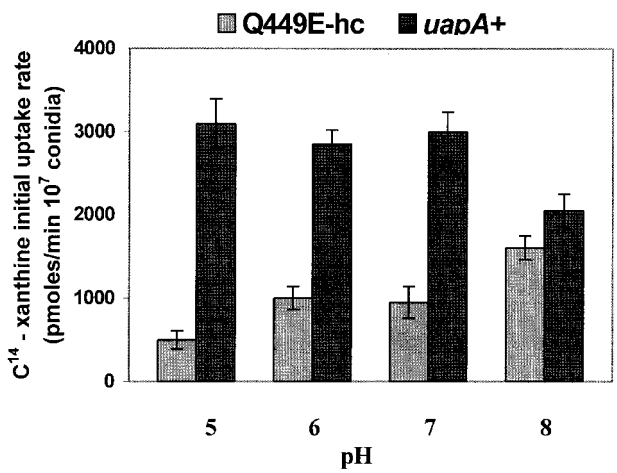


Figure 5. Effect of pH on xanthine initial uptake rate in a wild-type (*uapA*$^+$) and the Q449E-hc mutant. Uptake assays were performed basically as described in Experimental procedures, but the MM medium was adapted to different pH. Xanthine initial uptake rates are expressed in pmoles/min per $10^7$ viable conidiospores. Results shown represent averages of at least three independent assays. Vertical lines represent mean standard deviation values.

permissive temperature affects purine binding and/or transport. To distinguish among these possibilities in cryosensitive mutants, conidia were allowed to germinate at the permissive (37°C) or non-permissive (25°C) temperature, and transport assays were performed, in both cases, at either 37 or 25°C. If cryosensitivity is due to protein instability or/and defective topogenesis to the plasma membrane, mutant UapA will not be expected to function when germination takes place at 25°C, but might function when germination takes place at 37°C. On the other hand, if cryosensitivity is due to a protein properly sorted at the plasma membrane but unable to perform transport catalysis at 25°C, this would be expected to function in conditions in which conidia germinate at 37 or 25°C and transport measurements are carried out at 37°C. In case that UapA does not function in these mutants under any of the above conditions, it would suggest that cryosensitivity is due to a more complex defect, probably as the result of improper folding, affecting both stability or sorting at the plasma membrane and the ability of UapA to bind and transport purines.

Conidia from strains ACZ:pAN510-N450Q2 and ACZ:-pAN510-Q449E2, as well as a control strain ACZ:pAN510 carrying the wild-type *uapA* gene, were allowed to germinate either at 37°C for 4.5 h, or at 25°C for 9 h (it had previously been established that in *A. nidulans* germination at 25°C proceeds with a rate half that of germination at 37°C; unpublished observations). Initial uptake rate assays were carried out essentially as described above. The results are summarized in figure 6 and show that (i) in the control strain expressing the wild-type UapA protein, xanthine accumulation is not affected significantly by the germination temperature (compare bars 1 and 3, 2 and 4), and is approximately 30% higher in assays performed at 37°C rather than at 25°C (compare bars 1 and 2, 3 and 4), and (ii) in both mutants, xanthine accumulation is little affected by the germination temperature, but is significantly increased when transport assays are performed at 37°C. For reasons which are not clear, this increase is higher when germination took place at 25°C. These results strongly suggest that in both mutants cryosensitivity is basically due to defective transport catalysis, rather than to defective transporter topogenesis, at the non-permissive temperature.

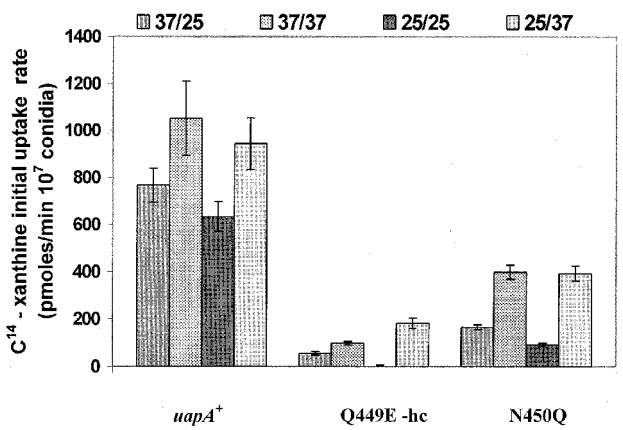


Figure 6. Effect of germination and/or uptake temperature on xanthine uptake rates in cryosensitive mutants. Histograms show $C^{14}$-xanthine initial uptake rates in a control strain (*uapA*$^+$) and mutants Q449E-hc and N450Q. Uptake assays were performed as described in Experimental procedures. Xanthine accumulation is expressed in pmoles/min per $10^7$ viable conidiospores. 25/25 is germination and transport carried out at 25°C, 37/37 is germination and transport at 37°C, 25/37 is germination at 25°C and transport at 37°C, 37/25 is germination at 37°C and transport at 25°C. Results shown represent averages of at least six independent assays. Vertical lines represent mean standard deviation values.

## Discussion

This work examines the qualitative role of specific amino acid residues, which are located within a sequence motif conserved in all members of a specific nucleobase/ascorbate transporter family. The model system was UapA, a prototype of this protein family. Although antibodies have been obtained which recognize the cognate UapA epitopes at very high titres, the authors have been unable to detect UapA whole proteins in Western blots of mycelial extracts or membrane fractions (J. Valdez, A. Rosa, C. Scazzocchio and G. Diallinas, unpublished observations), and, thus, could not study quantitatively UapA expression in the mutants. This is a common problem in studies concerning transporters expressed from their physiological promoters. However, with the exception of N450D, none of the mutations studied (F447Y, Q449E, N450Q and T457S) abolishes completely the function of UapA. Thus, even in the absence of immunological detection, UapA was at least partially expressed and correctly inserted in the membrane in all these mutants. This allows one to perform uptake rate assays and examine the effect of these mutations on UapA affinity and, specificity, towards different purines.

Amino acid residues $N^{450}$ and $Q^{449}$ were found to be critical for the function and/or specificity of UapA. Substitution of $N^{450}$ by an isostructural charged residue (D) results in total loss-of-function, while substitution by an isofunctional residue (Q) generates a transporter with significantly reduced transport capacity, especially at 20–30°C. These observations are in agreement with the absolute conservation of $N^{450}$ in all nucleobase/ascorbate transporter homologues present in databases. Mutation Q449E causes a similar effect on UapA transport capacity as mutation N450Q, but more interestingly, it also has a significant qualitative effect on UapA specificity. While the presence of a Q residue at position 449 allows transport of purines which have a carbonyl (uric acid and xanthine) or a thiol (2-thioxanthine) group at position C-2 of the purine ring, the presence of an E residue allows binding and transport, albeit with reduced efficiency, of uric acid and xanthine, reduces binding of 2-thioxanthine significantly, and allows the binding and transport of other purines which have either an amino group (guanine and its analogues) or a hydrogen atom (hypoxanthine) at C-2. These results suggest that residue Q449, and possibly the neighbouring $N^{450}$, might be involved in interactions with groups at or close to the C-2 position of purines. In fact, mutagenesis, crystallographic and functional analyses in the *S. cerevisiae* purine-cytosine transporter (Bloch *et al*, 1992 Ferreira *et al*. 1997, 1999), the $Na^+$-dependent mammalian nucleoside transporters (Wang and Giacomini

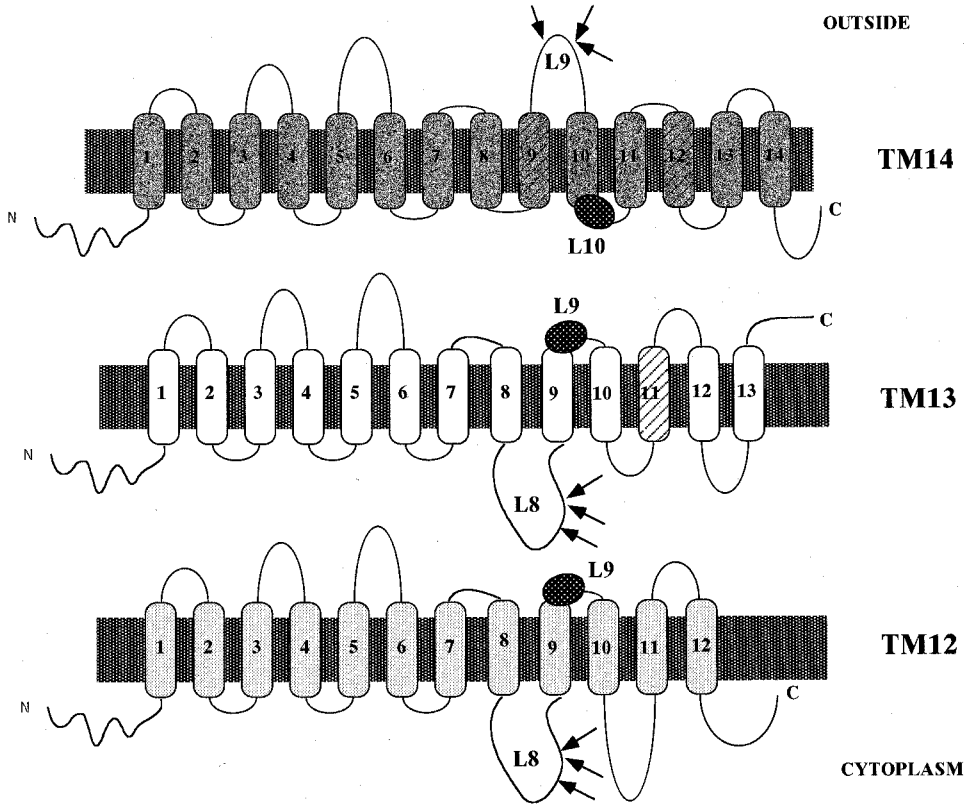


Figure 7. Alternative topological models of UapA showing the position of the consensus sequence motif. TM indicates putative transmembrane domains. L indicates hydrophilic loops linking two TM. TMs are shown as cylinders connected to each other by lines corresponding to Ls. The membrane environment is shown as a dotted black bar. The extracellular compartment is shown above, and the intracellular compartment below the protein. (*a*) topological model with 14 TM (grey), (*b*) topological model with 13 TM (white), and (*c*) topological model with 12 TM (dotted white). Different models are generated depending on the programs used, as described in Experimental procedures. Ambiguous TM (TM9 and TM12 in the 14 TM model or TM11 in the 13 TM model) are shown with stripes. The domain which has previously been shown to affect UapA function and specificity comprises L8-TM9-L9-TM10-L10 in the 14 TM model (Diallinas et al. 1998), or L8-TM9-L9 in 13 and 12 TM models. Arrows show approximate positions of specific missense mutations affecting UapA function and specificity (Diallinas et al. 1998). The consensus motif is shown as an oval domain (black with white dots) overlapping with TM10-L10 (14 TM model) or TM9-L9 (13 and 12 TM models).

1997, 1999, Loewen *et al.* 1999), the cyclic-nucleotide-gated ion channels (Varnum *et al.* 1995), and a number of soluble enzymes binding nucleobase moieties (Kang *et al.* 1994, Hoffenberg *et al.* 1995, Erion *et al.* 1997, Stoeckler *et al.* 1997, Sunahara *et al.* 1998, Tucker *et al.* 1998) have shown that interactions of polar or charged residues with both positions N-1 and C-2 of nucleobase rings are critical for specific substrate binding. If not fortuitous, similar mechanisms in active-site interactions of soluble and transmembrane purine-binding proteins with their substrates might well demonstrate an example of convergent evolution.

Both Q449E and N450Q mutants were shown to be cryosensitive for xanthine or uric acid transport, basically due to defective transport catalysis, rather than defective topogenesis, at the non-permissive temperature (see figure 6). This suggests that these substitutions might not cause global protein misfolding, but rather locally affect a domain involved in transport catalysis. The partially restored function of the Q449E mutant in a more basic environment (pH 7.5–8.0) suggests that the effects which this mutation produces on UapA function might, at least partly, be due to the degree of protonation of residue E449. Protonation of an amino acid residue, which probably interacts through hydrogen bonding with the purine ring, might alter purine binding and transport. In fact, Gordon *et al.* (1996) have shown that protonation of a D residue in the ligand binding domain of cyclic nucleotide-gated channels alters their specificity.

The predicted topology of nucleobase transporters and their homologues is consistent with 12, 13 or 14 $\alpha$-helical TM domains depending on the programme used (Diallinas *et al.* 1995, 1998). In UapA, the region proposed to be critical for its function and specificity (Diallinas *et al.* 1998) would include, in any model, two TMs (TM9 and TM10 in a 14 TM model, or TM8 and TM9 in 12 or 13 TM models) and the hydrophilic segments joining these hydrophobic domains (figure 7). In the 14 TM model, the amino acid residues $A^{404}$, $E^{412}$ and $R^{414}$, previously shown to affect UapA function and specificity (Diallinas *et al.* 1998), would be located in an extracellular hydrophilic loop downstream TM9, while the amino acid residues $Q^{449}$ and $N^{450}$, shown in this work to play a similar functional role, would be located in the following cytoplasmic loop, downstream TM10. In the alternative 12 or 13 TM models the positioning of these residues would be inverted, $A^{404}$, $E^{412}$ and $R^{414}$ becoming cytoplasmic and $Q^{449}$ and $N^{450}$ extracellular. In all

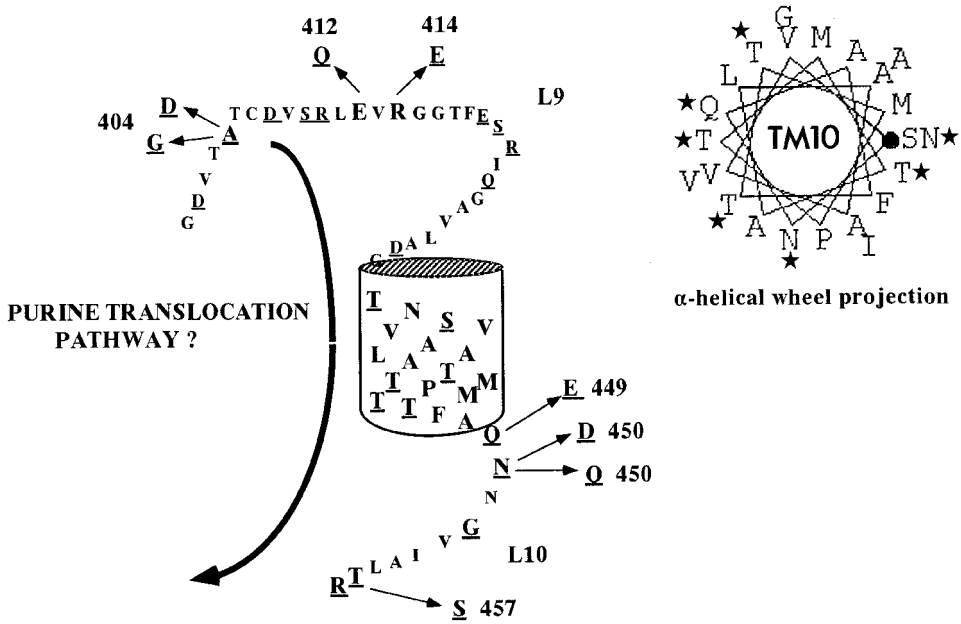


Figure 8. Topology of a polar pathway for purine transport via UapA. The model highlights L9-TM10-L10 (in a 14 TM UapA topology). Arrows indicate missense mutations affecting the function and specificity of transport (Diallinas et al. 1998; and work herein). Polar residues in TM10 are underlined. Mutated residues are numbered. α-helical wheel projection of TM10 shows that polar residues (marked with an asterisk) occupy one side of the TM surface.

models however, these functional amino acid residues are located on either side of a specific transmembrane segment (figure 8). This α-helical hydrophobic segment (TM9 or TM10), and the equivalent TM domain in all nucleobase/ascorbate transporters, exhibits an amphipathic character with many polar residues falling on the same surface of an α-helical wheel. These observations suggest that this region (L8-TM9-L9 or L9-TM10-L10) may in fact form part of a polar environment which is involved in purine binding and translocation through the lipid bilayer (figure 8).

Despite the physiological, medical and pharmacological importance of nucleobase transporters in mammals, and in humans in particular, genes encoding these proteins have not been identified and their molecular characteristics remain unknown (Kraupp and Marz 1995). While this work was being written, Tsukaguchi *et al.* (1999) and Rajan *et al.* (1999) reported the sequence of two mammalian ascorbate transporters which present significant similarities with microbial nucleobase transporters and conserve the functional motif analysed herein. It is believed that knowledge obtained through structure-function analyses of the *A. nidulans* purine transporters will eventually permit the use of this easily manipulated fungus as a prototype microbial system for studying the role of several putative nucleobase/ascorbate transporter sequences identified in databases.

## Experimental procedures

### *Media, growth conditions and strains for A. nidulans*

Minimal (MM) and complete (CM) media for *A. nidulans* have been previously described (Cove 1966, Scazzocchio *et al.* 1982). Supplements were added when necessary. In growth tests, all purines were used at a final concentration of 0.1 mg/ml. Wild-type and all mutant *uapA* genes were introduced and studied in strain *yA2 argB2 uapC*-201 *uapC*401 *uapA*24 *azgA*4 (ACZ). *biA*1, *pabaA*1, *argB*2 indicate auxotrophies for biotin, p-aminobenzoic acid, and arginine, respectively. *yA*2 results in yellow conidia. These markers do not affect the regulation of gene products involved in purine catabolism. *uapA*24, *azgA*4 and *uapC*401 are genetically obtained loss-of-function mutations in the corresponding genes. *uapC*-201 is a promoter mutation (Diallinas *et al.* 1995).

### In silico *analyses*

Different topological models shown in figure 7 were generated by a combination of the following programs: 'SOSUI', Tokyo University of Agriculture and Technology, Japan, www.tuat.ac.jp/%7Emitaku/Adv_sosui; 'PhdTopology' Predict-Protein, EMBL-Heidelberg, Germany, www.embl-heidelberg.de/tmap/tmap_sin.html; 'Tmpred' Prediction of Transmembrane Regions and Orientation, ulrec3.unil.ch/software/TMPRED_form.html; 'TOPREDII', www.biokemi.su.se/~server/topred2. Other graphical figures were performed with the program Canvas 3.5 for Apple computers.

### *DNA manipulations and plasmid construction*

Plasmid isolation from *E. coli* strains and DNA manipulations were performed as described by Sambrook *et al.* (1989). Total genomic DNA isolation from *A. nidulans* strains has been previously described by Lockington *et al.* (1985). Fragments carrying site-directed *uapA* mutations were entirely sequenced using a sequencing kit as instructed by the supplier (United States Biochemical, Cleveland, Ohio). A series of oligonucleotides specific for both strands of the *uapA* gene (Diallinas *et al.* 1995), and the T3 and T7 primers (Pharmacia), were used for sequencing. Control plasmids pIJL16 and pAN510, including the *argB* or the *argB* and *uapA* genes, respectively, were described previously (Diallinas *et al.* 1998). Plasmids were introduced into *E. coli* by standard transformation techniques (Sambrook *et al.* 1989).

### *Construction of uapA mutations*

Specific *uapA* mutations were constructed *in vitro* by oligonucleotide-directed targeted mutagenesis using the pALTER-exp1 system according to manufacturer instructions (Promega). Mutations were initially constructed into a 460 bp *Pst*I-*Pst*I restriction fragment from *uapA* cloned in pALTER-exp1. Constructs were confirmed by sequencing the entire *Pst*I-*Pst*I restriction fragment. The mutated fragment was subsequently used to replace the corresponding *Pst*I-*Pst*I restriction fragment in plasmid pAN510, a pBLUESCRIPT derivative carrying a full-length *uapA* gene and an *argB* gene which allows selection of transformants in an *argB*$^-$ mutant; Diallinas *et al.* 1998). The oligonucleotides used to construct the mutations F447Y, Q449E, N450D, N450Q and T457S were the following; N450Q: 5′-CCTTTGCGCAGCAGAACGGCGTGATTGCCC-3′; N450D: 5′-GACGACCTTTGCGCAGGACAACGGCGTG-3′ T457S: 5′-GTGATTGCCCTCAGTCGCTGCGCAAACCGC-3′; F447Y; 5′-CCCATGACGACCTATGCGCAGAACAACG-3′; Q449E: 5′-GACGACCTTTG-CGGAGAACAACGGCGTG-3′.

### *Isolation of transformants carrying uapA mutations integrated into the argB gene*

Plasmids carrying wild-type (pAN510) or mutated (pAN510-F447Y, pAN510-Q449E, pAN510-N450Q, pAN510-N450D, pAN510-T457S) *uapA* genes and control plasmid pIJL16 were introduced into the *A. nidulans* strain *yA2argB2uapC-201uapC401uapA24azgA4* (ACZ) by a standard transformation technique (Diallinas and Scazzocchio 1989). Transformants (20–200 per $\mu$g DNA) were selected for their ability to grow in the absence of arginine from MM supplemented with 5 mM urea as unique nitrogen source. Total genomic DNA was isolated from purified transformants, digested with *Eco*RI, and analysed by Southern blotting (see above). *uapA*- and *argB*-specific probes were prepared by isolating internal restriction fragments from these genes and $P^{32}$ labelling with a random priming kit (Amersham). Southern blot analysis permitted the identification of homologous integration events in the *argB* or *uapA* loci, as well as of heterologous integrations at unknown loci. Tranformants arising form integration events at the *argB* locus were selected for further studies. Densitometry readings were used to estimate the number of plasmid copies integrated into the genome.

### *Purine transport assays*

Purine initial uptake rates were assayed in germlings (Diallinas *et al.* 1995, Tazebay *et al.* 1995). These are conidiospores incubated for a short time in nutrient media and, thus, committed to germination and nuclear division, but where the germinal tube is not yet visible. Preliminary work demonstrated that germlings showed the same purine transport systems as mycelia. This method avoids all the complications of uptake measurements in a mycelial mat. The appropriate strains of *A. nidulans* were grown for 3 days on complete media at 37°C. Conidiospores were collected and used to innoculate liquid cultures of 50 ml minimal medium with 5 mM urea as sole nitrogen source and 0.1 mg/ml of uric acid as an inducer. Innoculated cultures were incubated at 120 rev/min for either 4.5 h at 37°C or for 9 h at 25°C. At the end of this incubation step, conidiospores were filtered through blutex membrane and washed three times with minimal medium without purines. Washed conidia were resuspended in 2 ml minimal medium. This gives a concentration of approximately $2 \times 10^8$ conidia/ml. Aliquots (0.1 ml) of this suspension were distributed in 2 ml Eppendorf tubes and equilibrated for 10 min at either 37 or 25°C. Uptake was initiated by the addition of radiolabelled 8-$C^{14}$-xanthine (10 $\mu$M), 8-$C^{14}$-uric acid (10 $\mu$M) or 8-$C^{14}$-hypoxanthine (50 $\mu$M). Uptake assays were terminated by the addition of 'cold' xanthine, uric acid or hypoxanthine, at a final concentration of 0.6 mM, after 60 s. Previous experiments have shown that purine accumulation increases linearly for at least 2 min at 37°C or for 4 min at 25°C; unpublished results). Conidiospores were then washed three times with 2 ml ice-cold minimal medium and radioactivity was measured by liquid scintillation counting. It has also been determined that, under the above conditions, uptake is directy proportional with the number of conidiospores in the suspension. The actual viable conidiospore number was determined, for each assay, by removing and plating on complete medium, serial dilutions of an aliquot obtained before the final washing step. Initial uptake rates are expressed in pmoles substrate incorporated/min $10^7$ viable conidiospores. Competition assays were performed in the simultaneous presence of 10 $\mu$M 8-$C^{14}$-xanthine and 300 $\mu$M 'cold' purines, purine analogues or pyrimidines. All transport assays were carried in at least three different experiments, each experiment in triplicate. Radiolabelled 8-$C^{14}$-hypoxanthine (specific activity 56 mCi/mmol), 8-$C^{14}$-xanthine (specific activity 50 mCi/mmol) and 8-$C^{14}$-uric acid (specific activity 50 mCi/mmol) were obtained from Moravek, Biochemicals (USA).

### *Determination of kinetic parameters*

The apparent Michaelis constant (*K*m) and maximal velocity (*V*) values for 8-C14-xanthine or 8-$C^{14}$-hypoxanthine were determined from double reciprocal plots of the initial uptake rate against substrate concentration. Initial rates were corrected by subtracting background uptake values, evident in the triple *uapA*$^-$ *uapC*$^-$ *azgA*$^-$ mutant, which are due to as yet genetically undefined minor purine transporters present in all strains used in uptake assays. For *K*m and *V* determination, an optimized substrate concentration range was chosen. For xanthine uptake this was 1, 2, 5, 10, 20, 50, 100 $\mu$M and for hypoxanthine uptake this was 5, 10, 20, 40, 80, 150, 300 $\mu$M. To calculate the concentration of purines resulting in 50% inhibition of 8-$C^{14}$-xanthine uptake, a constant 8-$C^{14}$-xanthine concentration (10 $\mu$M) and a concentration range of unlabelled competitors (5, 10, 20, 50, 100, 200 $\mu$M) were used. All transport assays were carried out in triplicate.

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