Review

Structure-function relationships in the nucleobase-ascorbate transporter (NAT) family

Lessons from model microbial genetic systems

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Nucleobase Transporters

Highly specific nucleobase transport systems exist in all domains of life. A small number of genes encoding such purine and/or pyrimidine carriers have been cloned and studied in great detail, mostly in bacteria, fungi and protozoa, but also in plants and mammals. The sequences of hundreds of other putative proteins extant in databases are homologous to the known nucleobase transporters, but in the majority of cases their physiological functions remain undetermined. Sensu strictu nucleobase-specific transporters belong to four evolutionarily distinct protein families (http://www.membranetransport.org). These are the Nucleobase Cation Symporter family 1 (NCS1), also known as Purine-Related Transporter family (PRT), the Nucleobase-Ascorbate Transporter family (NAT or NCS2), the AzgA-like family and the so called Equilibrative Nucleoside Transporter family (ENT). The NCS1 family includes nucleobase/H+ symporters from prokaryotes, fungi and plants. Most known members are specific for purines or pyrimidines (adenine, hypoxanthine, guanine, uracil, cytosine), but some are also specific for other purine related compounds (allantoin, hydantoin, thiamine, pyridoxal-based compounds and nicotinamide riboside). The NAT/NCS2 family includes bacterial, fungal and plant uric acid/xanthine/uracil-H+ symporters and, surprisingly, the mammalian L-ascorbate/Na+ transporters. The only members of known function of the AzgA-family, those of the ascomycetes Aspergillus nidulans and Aspergillus fumigatus, and the plant Arabidopsis thaliana, are strictly specific for purine/H+ symport (adenine, hypoxanthine, guanine). This family seems not to be present in metazoan. Finally, nucleobase-specific transporters of the ENT family are present in protozoa and mammals. Most seem to be H+ symporters and, as the name of the family indicates, some can also recognize nucleosides. In plants, two more families, PUP and UPS, include members, which transport purine-related metabolites (cytokinin, trans-zeatin, caffeine, ribosylated purine derivatives and allantoin, uric acid, xanthine, respectively), usually coupled with H+ symport. In mammals multiple organic anion transporters, belonging to distinct evolutionary families, have been reported to contribute to net renal excretion of uric acid (URAT1, UAT, Oat1, Oat3, MRP2, MRP4 and Oatv1). Finally, the SLC2A9 transporter gene, considered to encode a fructose transporter, was recently shown, by expression in Xenopus laevis oocytes, to be a uric acid facilitator (an MFS member) with pronounced sex-specific effects related to serum uric acid concentrations, hyperuricemia, gout and possibly cardiovascular disease and diabetes.

Given the metabolic importance of purine salvage and metabolism in all kinds of cells, the complex role of uric acid in mammals and allantoin in plants, but also the fact that purine and pyrimidine analogues are being widely used as antimetabolites against a host of different diseases and infections, ranging from anti-tumor and leukemia chemotherapy and anti-viral compounds to drugs against parasitic disease or for the prevention of organ transplant rejection and the treatment of gout, knowledge on how nucleobase and related transporters work has an obvious scientific interest. However, nearly nothing is known on the structure of any transporter involved in nucleobase transport, a problem often associated with long hydrophobic transmembrane proteins. Only homology threading (Diallinas G, unpublished) shows some evidence that the NCS1 transporters might have structural similarity with the Aquifex aeolicus leucine (LeuT) and the Vibrio parahaemolyticus galactose (vSGLT) transporters, two bacterial carriers, belonging to distinct symporter families, whose structure has recently been solved. NCS1, NAT/NCS2 and AzgA transporters might also be very distantly related, but this is not supported by homology threading. In contrast to the dearth of structural studies, a wealth of genetic and biochemical approaches carried out mostly in the filamentous fungus Aspergillus nidulans and in Escherichia coli have, in the recent years, contributed significantly to our knowledge of nucleobase transporters. In this review, we highlight results specifically concerning structure-function relationships of two prototype microbial NAT members and show how these findings are related to NAT function in general. We will not discuss the regulation of the expression of these microbial permeases, a subject recently reviewed elsewhere.
The NAT Family and its Physiological Role

The NAT family was defined by the cloning and characterization of the UapA\(^{27,28}\) and UapC\(^{29}\) uric acid-xanthine permeases of the ascomycete Aspergillus nidulans, and the UraA\(^{30}\) and PyrP\(^{31}\) uracil permeases of Escherichia coli and Bacillus subtilis, respectively. At present, the function and specificity of fourteen more NAT proteins is known (Table 1). All six studied mammalian NATs (SVCT1 and SVCT2) transport L-ascorbic acid rather than purines or pyrimidines. Thus, the NAT family is subdivided into three sub-families in respect to substrate specificity: the first, present in bacteria and fungi, is specific for the oxidized purines xanthine and/or uric acid, the second, present only in bacteria, is specific for uracil, and the third present in vertebrates is specific for L-ascorbic acid. The third is currently present in vertebrates is specific for L-ascorbic acid. No function is known for metazoan homologues outside the vertebrates. All known bacterial, fungal and plant NATs are high-affinity \(\text{H}^+\) symporters, while the mammalian SVCT1s use Na\(^+\) for L-ascorbate transport.\(^{4}\) Hundreds of NAT homologues are present in nearly all organisms, a prominent exception being Saccharomyces cerevisiae and several protozoa. All NATs are predicted to consist of twelve putative transmembrane segments (TMS) and cytoplasmically located N- and C-tails, whereas no apparent symmetry or the presence of large cytoplasmic loops is evident.\(^{4,32}\) The high \(\alpha\)-helical content of NATs is suggested by Circular Dichroism studies, showing that two prototype members of the family (UapA and YgFO) are >80–90% helical (Frillingos S, Lemuh N, Diallinas G, unpublished observations). Most divergent members can be recognized by the presence of two highly conserved motifs. These are the so-called NAT signature motif, \([\text{Q/E/P}]\)-\(\text{N}-\text{X-X-X-X-}[\text{R/K/G}]\) located in an amphipathic region just upstream from TMS9,\(^{32-34}\) and the QH motif in the middle of TMS1,\(^{32,35}\) (see Fig. 1). Both motifs have been shown to be critical for the function of fungal and bacterial NATs (see below). Microbial NATs, whether bacterial or fungal, show higher similarity among each other (30–35%) than to plant and metazoan homologues (22–24%). Plants have numerous NAT/NCS2 transporters, the only one with known function being Lpe1, a high-affinity, high-capacity, uric acid-xanthine/\(\text{H}^+\) symporter, necessary for chloroplast development in maize.\(^{36}\) Drosophila melanogaster has a single NAT homologue, Caenorhabditis elegans six and Arabidopsis thaliana twelve, whose function remains unknown.\(^{8}\) The metazoan NAT proteins are 35–40% identical among themselves and 25% identical with plant members. Mammals, besides the two known ascorbate transporters (SVCT1 and SVCT2), also have homologues of unknown function which possess motifs more similar to those present in the characterized nucleobase transporters of bacteria, fungi and plants.\(^{4}\) These proteins might be related to nucleobase-related uptake activities. Most vertebrates, except primates, also include NATs possessing conserved motifs that are more similar to those present in the bacterial, fungal or plant nucleobase carriers, rather than to the mammalian vitamin C transporters.\(^{4}\) As a general comment, it

\[\text{Table 1 NAT proteins of known function}\]

| Organism          | Transporter | Physiological substrates \([K_m/i (\mu M)]\) | Other ligands \([K_m/i (\mu M)]\) | Transport capacity |
|-------------------|-------------|-------------------------------------------|---------------------------------|-------------------|
| Aspergillus       | UapA        | X [7], UA [8]                            | 2TX [63], 3MX [28], 8MX [100],  | H                 |
|                   | UapC        | X [4], UA [136]                          | 1MX [50], 2TX [50], 3MX [100],  | M                 |
|                   |             |                                           | 8MX [60], 2TX [65], 3MX [100],  | H                 |
|                   |             |                                           | 8MX [50], 1MX [60], 2TX [97], 3MX [22], 6TX [84], 8MX [80], 6AZX [55], 0X[4] | H                 |
| Rattus norvegicus | SVCT1       | AA [29], DAA [33]                        |                                 |                   |
|                   | SVCT2       | AA [10–100]                              |                                 |                   |
| Sus scrofa        | SVCT2       | AA [8–62]                                | DC [2,35], AA-DC [0,2], AA-NHDC [24], BrAA [2,7], BrAA-KA [7,4], BrAA-DC [21] | L/H                   |
| Mus musculus      | SVCT1       | AA [29]                                  |                                 |                   |
| Homo sapiens      | SVCT1       | AA [65–237]                              | BrAA [33]                       | H                 |
|                   | SVCT2       | AA [20–115]                              |                                 | L                   |
| Candida albicans  | UapC        | X [6], UA [171]                          | 2TX [30], 2TUA [97], 3MX [22], 6TX [84], 8MX [80], 6AZX [55], 0X[4] | H                 |
|                   | Xut1p       | X [4], UA [50]                           |                                 |                   |
| Escherichia coli  | YgFO        | X [4]                                    | 1MX [36], 2TX [91], 3MX [72], 6TX [41], 9MX [53] | H                 |
|                   | Yice        | X [3]                                    |                                 | L                  |
|                   | UraA        | U                                         |                                 | n.d.               |
| Bacillus subtilis | PucK        | UA, X [9]                                |                                 | n.d.               |
|                   | PbuX        | X                                         |                                 | n.d.               |
|                   | PyrP        | U                                         |                                 | n.d.               |
| Zea mays          | Lpe1        | X [30], Ua [33]                          |                                 | H                 |
| Rattus norvegicus | SVCT1       | AA [29]                                  | DAA [7–700]                     | H                 |
|                   | SVCT2       | AA [10–100]                              |                                 |                   |
| Mus musculus      | SVCT2       | AA [20–115]                              |                                 | L                   |
| Homo sapiens      | SVCT1       | AA [65–237]                              | BrAA [33]                       | H                 |
|                   | SVCT2       | AA [8–62]                                | DC [2,35], AA-DC [0,2], AA-NHDC [24], BrAA [2,7], BrAA-KA [7,4], BrAA-DC [21] | L/H                   |

X, xanthine; UA, uric acid; 1MX, 1-methylxanthine; 2TX, 2-thioxanthine; 3MX, 3-methylxanthine; 6TX, 6-thioxanthine; 8MX, 8-methylxanthine; 8AZX, 8-azaxanthine; 9MX, 9-methylxanthine; OX, oxypurinol; ALL, allopurinol; U, Ura; AA, L-ascorbic acid; DAA, D-ascorbic acid; BrAA, 6-Bromo-6-deoxy-L-ascorbic acid; DC, Diclofenamic acid; KA, kynurenic acid; AA-DC, 6-diclofenamic-L-ascorbic acid; AA-KA, 6-Kynurenic-L-ascorbic acid; BrAA-KA, 6-Bromo-6-kynurenic-L-ascorbic acid; BrAA-Dc, 6-Bromo-6-diclofenamic-L-ascorbic acid; AA-NH-DC, AA-NH-DC, 6-amino-6-diclofenamic-L-ascorbic acid. \(\mu\)M lead to allopurinol sensitivity. \(b\)The \(K_m\) values of SVCT2-mediated ascorbate transport in heterologous systems indicate a low capacity transporter. However data from endogenously expressed SVCT1 and SVCT2 propose similar \(V_m\) values.11,16 H, high; M, moderate; L, low; n.d., not done.\(^{11,16}\)
Figure 1. Topology and structure-function relationships in the two NAT prototypes, UapA and YgfO. Putative α-helical TMSs are displayed as cylinders. Residues important for function are shown. Residues absolutely necessary for function are highlighted in dark grey background (UapA: Q85, E356, D388, Q408, N409; YgfO: Q324, N325). Residues affecting specificity or/and affinity are highlighted within squares (UapA: N71, L77, L84, Q113, G111, T416, R417, T526, F528; YgfO: T332, G333, N430, I432). In UapA, H86 in TMS1 affects topogenesis and interacts genetically with M151 (TMS3). In YgfO encircled domains display regions where Cys-scanning mutagenesis has been performed. The residues shown, except I432, are sensitive to NEM alkylation when replaced by Cys. N325C is partially protected against NEM labeling upon substrate addition, while Q324C is highly reactive but not substrate protected, as expected due to impaired substrate binding. N430C is completely protected from alkylation upon 2-thioxanthine addition, while A323C NEM reactivity is enhanced upon substrate addition. A speculative topology model summarizing interdomain synergy in UapA is also shown.
seems that nucleobase-specific NATs seem to be ubiquitous from prokaryotes to fish, but are lost in several mammalian groups, and entirely lost in primates. On the other hand, ascorbic acid-specific NATs seem to appear in fish, amphibians, marsupials, mammals and primates. We find very intriguing that the mammalian L-ascorbate transporters seem to have evolved from uric acid/xanthine transporters of lower eukaryotes, since uric acid and xanthine are very different from ascorbic acid (discussed later). Table 1 summarizes characteristics of all known NAT proteins.

In bacteria, fungi and plants NATs are responsible for high-affinity, high-capacity uptake of xanthine and/or uric acid or uracil from the growth medium. Uric acid can be directly channeled to nucleoside and nucleic acid synthesis, and this seems to serve a back-up scavenging role for pyrimidine salvage. On the other hand, xanthine and uric acid cannot be used to make other purines or nucleosides but can be utilized as nitrogen sources. In plants, inactivation of the uric acid-xanthine transporter ZmLPE1, the unique NAT protein of maize, leads to a defective chloroplast phenotype. The lpe1 gene is expressed in non-photosynthetic tissues, mostly in roots, and seems to be repressed by light. The expression of Lpe1 suggests that this permease might not only be responsible for uric acid/xanthine uptake, but also be involved in plastid function and/or metabolic processes affected by light. We recently obtained evidence for a cryptic role of NAT-mediated uric acid intercellular transport and catabolism in fungal development. In particular, in A. nidulans we showed that metabolically produced uric acid is transported, solely via the major uric acid transporter UapA, from the mycelium to asexually differentiated aerial cells, called metulae, where it is further oxidized to allantoin. (Borbolis F, Karachaliou M, Hatzinikolaou D, Pantazopoulou A and Diallinas G, unpublished). Metulae form the primary sterigmata from where secondary sterigmata (phialidiae) bud to eventually produce chains of external uninucleate conidiospores. Despite the fact that the lack of the uric acid transporter (uapAΔ) does not lead to a major mutant phenotype, total lack of uric acid production in mutants genetically blocked in upstream steps of purine catabolism (e.g., hxA hxnS), leads to increased sensitivity to oxidation and decreased sporulation. Furthermore, when uric acid is supplied externally A. nidulans sporulates more intensely, is more resistant to oxidation and conidiospores show increased survival to UV (Borbolis F, Karachaliou M, Hatzinikolaou D, Pantazopoulou A, Diallinas G, unpublished).

In metazoa, the only NAT members of known function are the mammalian ascorbate/Na+ transporters SVCT1 and SVCT2. Ascorbate (vitamin C) is an effective antioxidant and UV absorbent and plays a key role in many enzymatic reactions as a cofactor. Its varying distribution between extra- and intra-cellular fluids, its presence in a plethora of organs and tissues, and the fact that humans have lost the ability to synthesize it, makes the study of its transport systems to study, using classical and reverse genetics and biochemical approaches, structure-function relationships of NATs. A. nidulans or the Genetic Approach

A. nidulans is probably the organism where purine uptake and catabolism have been studied in most detail. All structural and regulatory genes involved, including five genes encoding purine permeases, have been identified and studied. Most importantly, A. nidulans, unlike S. cerevisiae, utilizes purines as sole nitrogen sources, which in turn means that given a suitable genetic background, any mutation affecting a purine carrier is reflected in a discernable phenotype. Transporter-specific mutants can be selected and characterized by simple growth tests on purines or purine toxic analogues in a genetic background lacking all other purine transporters. Such single- or multiple transporter mutant strains are already available through targeted knockout approaches and genetic crossing. Thus, in principle, different genetic screens or selective approaches can be designed to obtain mutations, in any purine transporter. These mutations can then easily be distinguished into those affecting transporter topogenesis or those affecting several aspects of transporter function, such as transport capacity, substrate specificity, substrate affinity or ion selectivity. Detailed systematic mutational analysis can be employed in parallel to further characterize the role of specific amino acid residues. Subsequent, easily performed, transport kinetic analysis in germinated fungal conidiospores, using radiolabeled purines, and the use of GFP as a silent fluorescent tag allow rigorous conclusions to be drawn on the nature of these mutations (for a graphical summary of these tools see Fig. 2). Two of the five nucleobase permeases in A. nidulans, namely UapA and UapC, belong to the NAT family. One of these, UapA, has become the paradigm for structure-function analysis in the NAT family.

Structure-Function Relationships: The Power of Model Microbial Systems

Despite the fact that high-resolution structures of membrane proteins are now emerging, the hydrophobic and metastable nature of transporters makes them difficult to study by traditional X-ray crystallography or NMR. In addition, even in cases of solved structures, the fact that transporters need to be removed from their natural membrane environment for their structure to be determined, may lead to artifacts, especially if complementary functional data from in vivo studies are missing. In addition, although solved structures provide fundamental molecular details on membrane protein function, alone they cannot give satisfying information on the dynamic mechanism of transport catalysis. Therefore, static structural data should, ideally, be integrated with genetic, biochemical, biophysical studies and other computational methods, to understand how transporters function at the molecular level. The most successful paradigm of integration of multidisciplinary approaches in studying a transporter is that of the lactose permease (LacY) of E. coli. In the next sections of this review, we highlight how the acomycete A. nidulans and E. coli have become unique complementary systems to study, using classical and reverse genetics and biochemical approaches, structure-function relationships of NATs.
UapA is a high-affinity (Km 7–8 μM), high-capacity transporter specific for uric acid and xanthine, able to transport also the analogues 2-thiouric acid, 2-thioxanthine, 3-methylxanthine, and the drugs allopurinol and oxypurinol. The first effort to identify regions critical for its function was the construction of chimeras consisting of UapA and UapC, the second NAT protein of A. nidulans. UapC is a carrier with high-affinity (Km 5 μM) for xanthine, moderate affinity for uric acid (Km 130 μM) and very low affinity (Km >500 μM) for other purines, not recognized by UapA, such as adenine or hypoxanthine. The approach of chimeric protein analysis revealed that the region between TMS8 and TMS9 is necessary and sufficient for determining the basic characteristics of the two permeases. In other words, chimeras including the TMS8-9 region from UapC had functional characteristics similar to UapC, rather than those of UapA. More specifically, they have reduced affinity for uric acid and acquired the capacity of very low binding of purines, other than uric acid and xanthine (see also later). This region included one of the two highly conserved motives of the NAT family, and in particular the NAT signature motif \( \{408[Q/E/P]-N-X-G-X-X-X-T-[R/K/G]\}^{417} \), numbers refer to UapA. Extensive physiological, microscopic and kinetic analyses of systematic site-directed mutations have provided evidence that this motif is part of the substrate translocation pathway. None of the several mutations made affected UapA translocation in the plasma membrane, as this was evidenced using GFP-tagged versions of mutant UapA molecules. In growth tests on purines, mutations in \{Q/E/P\}^{408} and N409 had the most pronounced effect, practically showing that these residues are irreplaceable for UapA function at 25°C. Some mutations in G411 and T416 (G411A, G411P, T416N) alter the specificity of UapA, so that in addition to uric acid and xanthine, the corresponding mutants could also grow on high concentrations (>0.5 mM) of hypoxanthine and adenine. Detailed uptake and competitive inhibition assays have confirmed that these mutations result in a protein able to transport adenine and hypoxanthine. Furthermore, the same analysis showed that Q408 is involved in substrate binding and substrate binding specificity, N409 is necessary for transport catalysis per se, while all other conserved residues of the motif (G411, T416, R417) contribute less, but considerably, to UapA specificity. Competition assays with a more than 30 different purine analogues, substituted each time at a single position in the purine ring, have shown that Q408 might directly be involved, as H+ donor, in interactions with the unprotonated N9 of xanthine or the C8 = O of uric acid. In line with this, mutant Uap-AQ408E could bind purines with protonated N9-H, such as hypoxanthine, guanine or 7-deaza-xanthine. This confirmed that Q408 is a basic element in substrate recognition and thus critical residue determining the substrate affinity and specificity of UapA. More recent mutational analysis has further shown that the TMS8-9 loop includes two more amino acid residues, outside the NAT signature motif, absolutely essential for UapA function. These are the absolutely conserved Ghu356 and the relatively conserved Asp388 (Fig. 1). Even the most conservative substitutions of these amino acids led to almost inactive UapA molecules. With the exception of mutant Uap-A-D388E, which was mostly retained in the Endoplasmic Reticulum (ER), the plasma membrane topology of all other mutants concerning E356 and D388 was unaffected. The residual transport activity of mutant E356D (5%) was exploited, through overexpression of the corresponding allele, to show that this substitution leads to a significant increase (8–17-fold) in the binding affinities of UapA for the physiological substrates and a similar or even greater increase for several xanthine analogues. Finally, in the same analysis it was shown that two more residues in the TMS8-9 segment, Q382 and K439, contribute moderately to the affinity of UapA for xanthine analogues with bulky substitutions.

In an attempt to suppress the cryosensitivity of the Q408E mutant, a second-site suppressor, F528S, was identified within TMS12. Subsequent kinetic analysis of systematic site-directed replacements of F528 revealed that substitution of Phe with small, aliphatic, amino acids led to transporter molecules that could catalyze the translocation of a series of purines, albeit with low affinity (>1 mM), without affecting the high-affinity (5–10 μM) for its physiological substrates, uric acid and xanthine. This suggested that this residue is not part, sensu strictu, of the substrate binding site. Recently, a different genetic screen to obtain UapA mutants able to transport adenine revealed two more residues that could affect UapA specificity in a similar way. These residues were the partially conserved T526 and the variable Q113, the first lying within TMS12 and the second in the putative extracellular loop between TMS1 and TMS2. Analysis of the kinetic profile of several site-directed mutants supports the idea that these mutations relax the specificity of UapA. Similarly to F528 mutation, T526 and Q113 substitutions should affect specificity indirectly, as they do not affect the high-affinity binding of UapA to its physiological substrates.

The role of the TMS1 region on UapA function and specificity was further studied by systematic mutations of the second most conserved NAT motif (Q^88-H^86) and of three Leu residues forming a Leu repeat (L77, L84, L91) within this α-helix, and finally, of an Asn residue (N71) located just upstream from the α-helix. Leu residues in TMSs might be involved in tight α-helical packing. Q85 mutations showed that this conserved residue is absolutely essential for function. Mutants conserving some transport activity (Q85E, Q85N, Q85L) have shown that the substrate affinity or specificity of UapA is not significantly altered in those strains, suggesting that the corresponding replacements affect transporter catalysis per se, rather than substrate binding. Mutations in H86 affect UapA folding rather than function and are discussed later. Ala substitutions of the Leu residues (L77, L84) of the Leu repeat and of Asn71 led to mutants that could grow on natural substrates, but also on high concentrations of hypoxanthine or adenine. Kinetic analysis showed that these novel substrates are transported through very low binding affinities, while the high affinities for physiological substrates were little or not affected.

Further insight on the role of specific amino acids of the TMS8-TMS9 loop and the other two domains found to affect UapA specificity was gained by characterizing a series of double mutants. Physiological growth tests, GFP microscopy and detailed kinetic analyses showed a plethora of allele-specific phenotypes compatible with a functional, and probably physical, synergy between the TMS8-9 loop, TMS12 and TMS1 (see Fig. 1). Prominent examples from this analysis are the double mutants Q408E/F528S, Q408E/T526M, Q408E/T526L, which, besides from their normal kinetic profile towards their physiological substrates (uric acid and xanthine), also showed UapA mediated high affinity, and relatively high capacity, hypoxanthine transport. This distinguishes them...
The Nucleobase-Ascorbate Transporter family

Figure 2. Summary of the tools used to study structure-function relationships in the UapA permease. (A) Growth phenotypes of a standard wild-type and isogenic strains carrying wild-type (uapA⁺) or mutant alleles of uapA, or lacking entirely uapA (uapAΔ). Besides the wild-type, all the other strains are knocked-out for all purine transporters except uapA. Growth tests shown are on urea (UR), uric acid (UA), hypoxanthine (HX) or adenine (AD), as sole nitrogen sources, as well as NO₃ plus oxypurinol (OX), a toxic xanthine analogue. Unless otherwise stated, purines were supplied at 0.5 mM final concentrations and growth tests were performed at 37°C. Mutant E356D is a high-copy (hc) transformant, as the corresponding single-copy allele has no detectable UapA activity. Notice the cryosensitive nature and low transport rate of Q408E and E356D, the altered specificity of T526M, Q408E/T526M, or the additive nature of Q408E and T526M alleles in respect to adenine or hypoxanthine transport. (B) Epifluorescent microscopy of strains carrying wild-type (WT) and mutant UapA-GFP molecules. D388N is a loss of function mutant with wild-type topology, whereas D388E and H86D show problematic trafficking. Arrows highlight septa and arrowheads ring-like structures corresponding to the nuclear envelope associated Endoplasmic Reticulum. (C) Upper: Different steps in the germination process of A. nidulans. The developmental stage prior germ tube emergence is established as ideal for direct uptake measurements. Lower: % UapA-mediated uptake rate of radiolabeled xanthine in the mutants shown in (A) and an example of dose response curves obtained for four different substrates, from which Ki values are calculated using GraphPad Prism software. (D) UapA-xanthine interactions modelled kinetically. The rationale for identifying positions of the xanthine ring that interact with UapA is explained in the text and in detail in Goudela et al., and Papageorgiou et al. ΔG⁰ values are calculated from Kᵢ's (Kᵢ = IC₅₀/[1 + (L/Kₘ)]) using GraphPad Prism software. R stands for an amino acid in the transporter interacting, via an H-bond, with a specific purine position.
from the corresponding single mutants, which either bind with high affinity but cannot transport (Q408E) or transport with very low affinity (F528S, T526M and T526L) hypoxanthine.

Besides from the role of TMS1 in the functional synergy between UapA domains, this α-helix contains elements affecting protein folding and topogenesis. Replacements of the nearly absolutely conserved residue H86 with different amino acid residues resulted in severely affected UapA-mediated transport, due to increased UapA turnover or/and problematic trafficking towards the plasma membrane. Two extreme cases were mutations H86A and H86D, the first leading to rapid vacuolar degradation of UapA, while the second to significant ER-retention. All H86 mutants retained partial activity, which was used to show that the kinetic characteristics of UapA were not affected in these strains. Mutant H86D was used in a genetic screen to get two second-site suppressors, both concerning the same residue (Met151) within TMS3 (Pantazopoulou A and Diallinas G, unpublished). Suppression was allele non-specific, as all M151 changes also suppressed other H86 mutations, while M151 mutations by themselves had nearly wild-type kinetic profiles (Pantazopoulou A, Taraslia V and Diallinas G, unpublished). This genetic analysis suggests the close physical proximity of TMS1 and TMS3.

Up to date, it has been possible to genetically convert UapA from a highly specific uric acid/xanthine transporter to various versions with enlarged or altered specificity. Combination of two mutations (Q408E in the NAT motif and T526L, or T526M in TMS12) was sufficient to make UapA able to bind with high affinity and transport efficiently hypoxanthine and guanine. However, all UapA adenine-transporting mutants recognized this aminopurine with very low affinity, showing that the presence of an amine group at C6 of the purine ring does not permit high-affinity binding. In our lab, we designed another genetic screen in order to get UapA specificity mutations that might be able to transport adenine with high affinity. In principle, this might have led as to UapA residues interacting with the pyrimidine moiety of purines. We used the F528S (TMS12) specificity mutant which has normal kinetics for physiological substrates, but also low affinity (>1 mM) for binding and transporting adenine. This strain can grow on media containing 2 mM but not on 0.5 mM adenine as sole nitrogen source. After UV mutagenesis we obtained 55 uapA-specific mutations growing on 0.5 mM adenine. Preliminary analysis has shown that ~90% of them map in the loop between TMS10-TMS11 and the rest within TMS10 or in the NAT motif. This result shows that positioning of TMS10-TMS11 is critical for the function and specificity of UapA, and further confirms the functional synergy between the UapA domains (Papageorgiou I, Kosti V and Diallinas G, unpublished).

**E. coli or Biochemistry Made Easy**

NAT structure-function relationships have also been studied using the *E. coli* homologue YgfO, a high affinity and capacity xanthine/H+ symporter. Despite the lack of discernable YgfO-associated phylogenotypes and the use of classical genetic approaches, as a consequence of the fact that *E. coli* cannot use purines as sole nitrogen sources, *E. coli* provides some methodological advantages complementary to the *Aspergillus* system. The first arises from the fact that YgfO, unlike fungal NATs, has a minimum number of Cys residues, which has allowed the application of cysteine scanning mutagenesis and other methodologies related to the presence of single Cys at specific sites of a transporter. Secondly, transmembrane protein purification in large amounts, necessary for downstream biochemical and biophysical methodologies, is much easier with bacterial than with fungal cells. Finally, the observation that UapA and YgfO differ significantly in their ability to recognize uric acid and purine analogues other than xanthine, makes the two systems ideal to address questions on the molecular basis of substrate specificity within the NAT family.

Classical systematic mutational analysis of the NAT motif in YgfO, analogous to that performed in *A. nidulans*, has first showed that conserved residues Q324, N325 and G327, T332, G333 have an impressively analogous role to the corresponding to residues in UapA (Q408, N409, G411, T416, R417). Explicitly, Q324 is absolutely required for transport catalysis due to its key role in binding of xanthine and other analogues, N325 is irreplaceable for transport catalysis, T332 and G333 have a fine role in determining YgfO kinetics and specificity, while G327 substitutions with Pro, Ala or Cys resulted in 2–5 fold increased $V_{max}$. Cys-scanning mutagenesis of a 25-amino acid region covering the NAT motif (see Fig. 1) and NEM alkylation analysis showed that most single Cys mutants in the sequence 323–336 undergo strong inhibition upon NEM modification. Interestingly, NEM reactivity is greatly enhanced upon xanthine addition for the A323C mutant. The most plausible explanation for these observations is that this region is part of a conformationally active substrate translocation pathway. The same Cys-scanning analysis showed that the region 329–339 had an alkylation pattern compatible with an α-helical 3 turn structure.

Site-directed and Cys-scanning mutagenesis was also employed in the sequence 419–450 (see Fig. 1), containing the TMS12 of YgfO. Again, results obtained were in full agreement with those found in the *Aspergillus* system (UapA), regarding the role of two key residues. These residues in YgfO are I432 (equivalent to F528 in UapA) and N430 (equivalent to T526 in UapA). None of these residues is absolutely essential for function, but several specific mutations alter YgfO transport kinetics and specificity. More specifically, I432 substitutions with small or bulky amino acids display very low or no activity, whereas substitutions with intermediate amino acids have nearly wild-type transport activity, but altered substrate affinities. More importantly, mutant N430C was found to be highly sensitive to NEM alkylation, while 2-thioxanthine, a high affinity YgfO ligand, was able to completely protect it from NEM inactivation. These results support the idea that N430 is at the vicinity of the substrate binding site, probably posing steric constraints to substrate binding, while the role of I432 is indirect and probably transmitted to the binding pocket through N430 by unfavorable rearrangement of TMS12. Thus, the picture we can draw on the role of TMS12 in YgfO is very similar to that obtained from the work in *Aspergillus*. The similarity in the role of the NAT motif and the TMS12 region for the function and specificity of YgfO and UapA is further supported by a series of double mutants strengthening the idea of a highly specific cross-talk between these domains (discussed in more detail in the next section).

**How Specificity Is Determined in NATs**

The most dramatic difference in the specificity of NATs was the ‘shift’ from nucleobase/H+ to ascorbate/Na+. The shift in ion selectivity is not a surprise, as it represents a common adaptive change...
from microbial or plant transporters to mammalian carriers. The specificity difference however is extreme as nucleobase and ascorbate are structurally unrelated metabolites. Mutations replacing specific amino acids in the NAT motif of nucleobase transporters with residues found in the ascorbate transporters and vice versa, led to non-functional transporters (unpublished observations). In addition, none of the relaxed specificity mutants obtained with the fungal and bacterial NATs can transport or even bind ascorbate, even at very high concentrations.\(^3\) In other words, the evolutionary change of NATs from nucleobase to ascorbate might be associated with a high mutational barrier. Thus, at present, we cannot draw conclusions on specific molecular elements that are critical for this substrate specificity difference. Below however, we are going to try to give some answers to the question of how NAT specificity towards different nucleobases is determined.

A very detailed specificity profile for nucleobase-specific NATs exists mostly for UapA, YgfO and several of their mutant versions. Analogous profiles exist for the wild-type versions of UapC-like transporters from \(A.\) nidulans (UapC) and \(A.\) fumigatus (AfUapC),\(^4\) and for the Candida albicans Xut1 permease.\(^44\) For several other NATs (see Table 1), we just know their basic physiological substrates. From these data it is obvious that bacterial nucleobase-selective NATs are all high-affinity (\(K_m < 5\ \mu M\) ) scavengers, extremely specific for either xanthine (YgfO, PbuX),\(^48,52\) or uracil (PyrP, UraA).\(^30,31\) Based on physiological tests or operon organization, some bacterial members have also been reported to be specific for uric acid (PucK, PucJ),\(^53\) but in those cases rigorous evidence from transport studies is missing. All known fungal NATs are high-affinity (\(K_m = 4-8\ \mu M\) ) transporters for xanthine, but differ significantly in respect to their affinity for uric acid (\(K_m = 8-130\ \mu M\) ) and in respect to their capacity to recognize other purines with very low affinity (\(>2\ mM\) ). UapA lies at one extreme, showing equally high affinities for xanthine and uric acid and totally excluding other purines (adenine, hypoxanthine or guanine) from its substrate binding site. In addition, in UapA, several xanthine analogues with bulky substitutions at positions seemingly not involved in direct interactions with the substrate binding residues are recognized with significantly reduced affinities. On the other end of the spectrum, UapC-like proteins and Xut1 have an affinity for uric acid that is 12–16 fold reduced compared to that for xanthine. In addition, these permeases recognize with very low affinity other purines (\(>2\ mM\) ).

Based on the binding affinities of functional mutants towards several possible substrates, we propose that UapA specificity is mechanistically achieved by both size hindrance and electrostatic repulsion.\(^3\) The importance of size in substrate-fitting is strongly suggested by the fact that specificity mutations in TMS1 and TMS12, which do not change the affinity for xanthine, allow increased binding affinity of xanthine analogues with bulky groups at positions C2 = O, N3-H, C6 = O, N7-H or C8/C8 = O.\(^3\) Some mutants in fact bind xanthine analogues with bulky substitutions at these positions equally well to xanthine or uric acid. Thus, these positions must not be involved or contribute little to substrate binding. In contrast, the same specificity mutations do not increase the affinities for xanthine analogues with modifications at positions N1-H and N9, strongly suggesting that N1-H and N9 are directly involved in binding. Based on the above, we recently proposed a revised kinetic model (Fig. 2D) where the interaction of xanthine with UapA occurs via H bonds with N1-H and N9, plus a weak bond with C2 = O (\(-2\ kJ/mol\) ), while there is no energetic contribution from N3-H, C6 = O, N7-H or C8. This model allows us to hypothesize that in the interaction of UapA with uric acid, the role of the non-protonated N9 as a proton acceptor is replaced by C8 = O.\(^3\)

Steric effects could, in principle, partially account for the exclusion of adenine or guanine from the UapA binding pocket, due to the presence of amino groups at positions C6 and C2, respectively. Size effects cannot however explain how hypoxanthine is excluded from the UapA binding site. The preferred tautomers of hypoxanthine and xanthine have inversely protonated N atoms at positions 3, 7 and 9 (xanthine has N3-H, N7-H and N9; hypoxanthine has N3, N7 and N9-H).\(^54\) We have proposed that the inverse protonation state of the N atoms in hypoxanthine, compared to xanthine, might repel the former from the substrate binding pocket of the wild-type UapA.\(^3\) A similar mechanism might contribute to the ‘filtering-out’ of adenine and guanine, which have N3, N7 and N9 protonation states identical to those of hypoxanthine. Repulsive electrostatic forces might also explain the reduced binding of the wild-type UapA for oxypurinol, which in its preferred tautomers possess a protonated N8-H. In the specificity mutants, loosening of the binding pocket might allow the accommodation of only bulkier non-natural substrates, but also would reduce any repulsive electrostatic forces that exclude totally or partially non-physiological substrates. Once size or electrostatic restrictions are removed, different purines can be transported by UapA on the basis of their ability to make specific H-bonds within the substrate binding residues in the TMS8-9 region.

The observed low affinity binding of hypoxanthine in the specificity mutants is in agreement with the idea that hypoxanthine interacts principally with UapA only through a strong interaction at N1-H, as it lacks a non-protonated N9. This assumption predicts that, in the specificity mutants, guanine and uracil, which also have a protonated N1-H, might also be transported with affinities similar to hypoxanthine. This is indeed the case. The relatively lower affinity for adenine uptake in specificity mutants might be explained assuming that the H donor role of N1-H in the interaction with UapA might only partially be replaced by the neighboring C6-NH\(_2\). Our assumptions are further supported by double mutants, where specificity mutations are combined with Q408E, a mutation that allows better binding of several non-natural substrates which have a protonated N9-H (hypoxanthine, guanine, 7-deazaxanthine).\(^3\) According to our model (see Fig. 2D), in these mutants, hypoxanthine (or guanine) can now make two H-bonds, one at N1-H simply due to loosening of the binding pocket, and one with N9-H because of the presence of the Glu residue at position 408. In contrast, adenine, which lacks a protonated N1-H, could make only one strong H-bond at N9-H.

We have shown that the unique UapA transporter kinetics and specificity profile necessitate a specific synergy between distant, in primary amino acid sequence, domains. Using UapA-UapC or UapA-YgfO chimeric molecules, the synergistic interactions of different NAT domains was further shown to be highly protein-specific.\(^3\) In UapA-UapC chimeras, replacing, each time any of the three UapA domains involved in functional synergy (TMS8-9 loop, TMS12 or TMS1) with that of the UapC, led to chimeric transporters resembling either UapC, or having a new kinetic and specificity profile. The fact that several mutations in these three domains relax the
substrate binding site of UapA in a manner that allows the transport of all purines and some of them also lower the affinity of UapA for uric acid, in other words making UapA behave like UapC transporters, is a strong indication that specific interdomain synergy in UapA forms the basis of the unique substrate binding characteristics of this carrier, and that this synergy has evolved through gain-of-function interactions not present in other NATs. This idea is further supported by synteny (the physical co-localization of genetic loci on the same chromosome within an individual or species) analysis showing that the *uapA* gene has evolved from duplication of a *uapC*-like gene. YgfO-UapA chimeras involving the TMS12 domain result in transporters with very low transport activity. This is mainly due to increased protein turn-over, implying that synergy between NAT domains is highly protein-specific and necessary for normal transporter folding. Interestingly however, in the chimeric YgfO protein carrying the TMS12 domain from UapA, the mutation of the two specificity-important residues of the *A. nidulans* TMS12 (Thr, Phe) back to the original residues present in the YgfO TMS12 (Asn, Ile), not only reestablished protein stability and transport activity, but led to a transporter that has altered affinity for several xanthine analogues. This observation shows that these two residues, T526 and F528 in UapA or N430 and I432 in YgfO, are key elements for the protein-specific synergy between the NAT motif and TMS12. Furthermore, this specific synergy determines which purines can have access to the substrate binding of UapA or YgfO.

**Conclusions and Perspectives—NATs as Antifungal Drug Gateways?**

Given that purine and pyrimidine analogues are widely used as antimetabolites against a host of diseases and infections, work on NAT structure-function relationships can in the long run be proved extremely useful in predicting and/or designing specific purine-based antifungals or antibacterials that will not be taken up by the host’s analogous transport systems. The NAT homologues from the two most common pathogenic fungi, *Aspergillus fumigatus* and *Candida albicans*, have also been studied kinetically, and models on how they bind their substrates have been proposed. Such models can support the design of nucleobase-related analogues that can be used to target fungi. Comparison between fungal transporters analyzed and analogous human transporters revealed major differences in involvement of N3 in substrate binding. Fungal NATs do not seem to use N3 to bind uric acid, xanthine or their analogues, leading to the prediction that xanthine analogues with N3 substitutions might be good substrates specifically for fungal NATs. N3-substituted purine analogues are channeled to nucleic acid synthesis and can be used at low concentrations as specific antiviral inhibitors. Similarly, 3-deazapurines and their analogues might also be efficient drugs against pathogenic fungi. *A. nidulans* is a candidate for the characterization, through heterologous expression, of novel eukaryotic NATs from more complex animal systems. Its ability to use purines as sole nitrogen sources gives the advantage of immediate discernable phenotypic observation, since all purine transporter genes are known, cloned and knocked out. A successful example is the only plant NAT of known function, the maize Lpe1, which has been successfully expressed and characterized in *A. nidulans*. However, heterologous expression of transmembrane proteins is most often problematic, resulting in defective topogenesis and trafficking. The only *D. melanogaster* NAT homologue, encoded by the *CG6293* locus, was expressed in *A. nidulans* but never reached the plasma membrane despite a series of overexpression strategies and the construction of several chimeric UapA-CG6293 molecules (Gournas C, Vlanti A, Amillis S and Diallinas G, unpublished). Work from *S. cerevisiae* proposes that the stabilization of transmembrane proteins through partial disruption of downregulation mechanisms, like the ubiquitin pathway, can have a positive impact on heterologous expression. Similar genetic tools are at present being developed and tested in *A. nidulans*.

The strict high affinity of UapA for uric acid transport was proposed to be an evolutionary recent gain-of-function modification. Fine changes in interdomain interactions were shown to be responsible for the apparent flexibility of NAT specificity. However, the completely different chemical structure of ascorbic acid must have required more complex structural changes in the NATs of primates. The fact that uric acid and ascorbic serve similar and complementary antioxidant roles in many organisms raises intriguing thoughts about the possible evolution of ascorbate transporters from pre-existing uric acid permeases. This could have been an adaptation of antioxidant strategies of metazoa in response to novel life styles.

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