SUMMARY

DEVELOPMENT OF A MODEL BACTERIAL SYSTEM FOR STUDYING STRUCTURE-FUNCTION RELATIONSHIPS IN THE NUCLEOBASE – ASCORBATE TRANSPORTER FAMILY (NAT/NCS2)

DOCTORATE THESIS BY
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The coding potential of the genome of *E. coli* K-12 includes *ygfO* and *yicE*, two members of the evolutionarily conserved NAT/NCS2 transporter family that are highly homologous to each other (45% residue identity) and closely related to UapA of *Aspergillus nidulans*, a most extensively studied microbial member of this family. *YgfO* and *yicE* were cloned from the genome, over-expressed extrachromosomally and assayed for uptake of [^{3}H]xanthine and other nucleobases, in *E. coli* K-12, under conditions of negligible activity of the corresponding endogenous systems. Alternative, essentially equivalent functional versions of YgfO and YicE were engineered by C-terminal tagging with ten consecutive His codons (His_{10} version) or with an epitope from the *E. coli* lactose permease and a biotin-acceptor domain from *Klebsiella pneumoniae* (BAD version) or with green fluorescent protein (GFP version) to allow immunodetection and affinity purification.

Both YgfO and YicE were shown to be present in the plasma membrane of *E. coli* and function as specific, high-affinity transporters for xanthine (K_m 4.2 μM for YgfO, or 2.9 μM for YicE), in a proton motive force-dependent manner. They display no detectable transport of uracil, hypoxanthine, or uric acid at external concentrations of up to 0.1 mM. Using transport inhibition experiments with 64 different purines and purine-related analogues, YgfO and YicE-mediated [^{3}H]-xanthine transport was efficiently inhibited by 1-, 3- and 9-methylxanthine, 2-thioxanthine and 6-thioxanthine. Both YgfO and YicE are inefficient in recognizing uric acid or xanthine analogues modified at positionς 7 and 8 of the purine ring (7-methylxanthine, 7-deazaxanthine, 8-methylxanthine, 8-azaxanthine, allopurinol), which distinguishes them from their fungal homologues UapA and Xut1.

To engineer genetic backgrounds appropriate for Cys-scanning mutagenesis, we have replaced each native Cys of YgfO and YicE with a Ser. Mutations were made on a functional

YgfO or YicE tagged at C terminus with an epitope from *E. coli* lactose permease and either a biotin-acceptor domain or a His_{10}. All single-replacement mutants (YgfO C203S, C204S, C219S, C227S, C433S and YicE C40S, C58S, C141S, C333S) were found to express in the membrane at wild-type levels and catalyze high [^{3}H]xanthine uptake. Combinatorial replacements yielded functional Cys-less versions for both YgfO and YicE. Relative to wild-type, Cys-less YgfO displays an increased V_{max} for xanthine uptake and increased plasticity for bulky substituents at the imidazol part of purines. There are no other significant changes, as determined by transport kinetics and inhibition analysis with 18 different purines and purine-related analogues.

The nucleobase-ascorbate transporter (NAT) signature motif is a conserved sequence motif of the ubiquitous NAT/NCS2 family, implicated in defining the function and selectivity of purine translocation pathway in the major fungal homolog UapA. To analyze the role of NAT motif more systematically, we employed Cys-scanning mutagenesis of the *E. coli* homolog YgfO. Using the functional mutant devoid of Cys residues (C-less), each amino acid residue in sequence ^{315}GSIPITTFAQNNGVIQMTGVASRYVG^{340} including the NAT motig (motif in underlined boldface) was replaced individually with Cys. Of the 26 single-Cys mutants, 16 accumulate xanthine to ≥50% of the steady state observed with C-less YgfO, T320C, T321C, I319C and T332C accumulate to low levels (10-25% of C-less), F322C, N325C and N326C accumulate marginally (5-8% of C-less), P318C, Q324C and G340C are inactive. When transferred to wild-type, F322C(wt) and N326C(wt) are highly active, but P318G(wt), Q324C(wt), N325C(wt) and G340C(wt) are inactive and G340A(wt) displays low activity. Immunoblot analysis shows that replacements at Pro318 or Gly340 are associated with low or negligible expression in the membrane. All other mutants are expressed to high or moderate levels.

Inhibition analysis with *N*-ethylmaleimide (NEM) shows that the single-Cys mutants fall to three regions: positions 315-322 are insensitive to *N*-ethylmaleimide, with IC_{50}s ≥0.4 mM, positions 323-329 are highly sensitive, with IC_{50}s of 15-80 μM, and sensitivity of positions 330-340 follows a periodicity, with mutants sensitive to inactivation clustering on one face of an alpha-helix. Sulfhydryl alkylation analysis of the single-Cys mutants using an engineered carboxyl-terminal His_{10} tag and Ni^{2+}-IDA affinity chromatography reveals that most of the single-Cys mutants of region 330-340 are accessible to NEM and selective inactivation at the inhibition-sensitive positions should be attributed to severe blocking effects of the maleimidyl adduct that are associated with the conformational changes of turnover.

In order to understand the contribution of the key conserved residues of the NAT motif to the mechanism and ligand recognition profile of YgfO, positions Gln324, Asn325, Gly327, Thr332 and Gly333 were subjected to extensive site-directed mutagenesis and mutants were analyzed for active transport, kinetics and inhibition of [^{3}H]xanthine uptake by a series of purines and synthetic analogues. Our mutant library included individual replacements of Gln324 with Asn, Glu, Pro, Ala or Cys, of Asn325 with Asp, Gln, Ser, Thr, Cys or Ala, of Gly327 with Ala, Pro or Cys, of Thr332 with Asn, Ala, Asp or Cys, and of Gly333 with Cys, Arg or Lys. Analysis of these mutants indicates that Gln324 is critical for high affinity uptake and ligand recognition, Asn325 is fully irreplaceable with respect to active xanthine transport, Gly327 is not essential for either active xanthine transport or specificity, while Thr332 and Gly333 are important determinants of the ligand specificity profile.

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Comparative substrate recognition by bacterial and fungal purine transporters of the NAT/NCS2 family.

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We compared the interactions of purines and purine analogues with representative fungal and bacterial members of the widespread Nucleobase-Ascorbate Transporter (NAT) family. These are: UapA, a well-studied xanthine-uric acid transporter of A. nidulans, Xut1, a novel transporter from C. albicans, described for the first time in this work, and YgfO, a recently characterized xanthine transporter from E. coli. Using transport inhibition experiments with 64 different purines and purine-related analogues, we describe a kinetic approach to build models on how NAT proteins interact with their substrates. UapA, Xut1 and YgfO appear to bind several substrates via interactions with both the pyrimidine and imidazol rings. Fungal homologues interact with the pyrimidine ring of xanthine and xanthine analogues via H-bonds, principally with N1-H and =O6, and to a lower extent with =O2. The E. coli homologue interacts principally with N3-H and =O2, and less strongly with N1-H and =O6. The basic interaction with the imidazol ring appears to be via a H-bond with N9. Interestingly, while all three homologues recognize xanthines with similar high affinities, interaction with uric acid or/and oxypurinol is transporter-specific. UapA recognizes uric acid with high affinity, principally via three H-bonds with =O2, =O6 and =O8. Xut1 has a 13-fold reduced affinity for uric acid, based on a different set of interactions involving =O8, and probably H atoms from positions N1, N3, N7 or N9. YgfO does not recognize uric acid at all. Both Xut1 and UapA recognize oxypurinol, but use different interactions reflected in a nearly 26-fold difference in their affinities for this drug, while YgfO interacts with this analogue very inefficiently.

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