Role of Intramembrane Polar Residues in the YgfO Xanthine Permease

HIS-31 AND ASN-93 ARE CRUCIAL FOR AFFINITY AND SPECIFICITY, AND ASP-304 AND GLU-272 ARE IRREPLACEABLE

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Using the YgfO xanthine permease of Escherichia coli as a bacterial model for the study of the evolutionarily ubiquitous nucleobase-asorbate transporter (NAT/NCS2) family, we performed a systematic Cys-scanning and site-directed mutagenesis of 14 putatively charged (Asp, Glu, His, Lys, or Arg) and 7 highly polar (Gln or Asn) residues that are predicted to lie in transmembrane helices (TM). Of 21 single-Cys mutants engineered in the background of a functional YgfO devoid of Cys residues (C-less), only four are inactive or have marginal activity (H31C, N93C, E272C, D304C). The 4 residues are conserved throughout the family in TM1 (His-31), TM3 (Asn-93/Ser/Thr), TM8 (Glu-272), and putative TM9a (Asp-304/Asn/Glu). Extensive site-directed mutagenesis in wild-type background showed that H31N and H31Q have high activity and affinity for xanthine but H31Q recognizes novel purine bases and analogues, whereas H31C and H31L have impaired affinity for xanthine and analogues, and H31K or H31R impairs expression in the membrane. N93S and N93A are highly active but more promiscuous for recognition of analogues at the imidazole moiety of substrate, N93D has low activity, N93T has low affinity for xanthine or analogues, and N93Q or N93C is inactive. All mutants replacing Glu-272 or Asp-304, including E272D, E272Q, D304E, and D304N, are inactive, although expressed to high levels in the membrane. Finally, one of the 17 assayable single-Cys mutants, Q258C, was sensitive to inactivation by N-ethylnaleimide. The findings suggest that polar residues important for the function of YgfO cluster in TMs 1, 3, 8, and 9a.

The nucleobase-asorbate transporter (NAT)2 or nucleobase-gation symporter-2 (NCS2) family is an evolutionarily ubiquitous family of purine, pyrimidine, and L-asorbate transporters, with members specific for cellular uptake of uracil, xanthine, or uric acid (microbial and plant genomes) or vitamin C (mammalian genomes) (1, 2). Despite their importance for the recognition and uptake of several frontline purine-related drugs, NAT/NCS2 members have not been studied systematically at the molecular level, and high resolution structures or mechanistic models are missing. More than 1000 sequence entries are known, but few have been functionally characterized to date. The best studied eukaryotic member is UapA, a high affinity uric acid/xanthine: H+ symporter from the ascomycote Aspergillus nidulans (3–7). Studies with chimeric transporter constructs (3), site-directed mutagenesis, second-site suppressors, and kinetic inhibition analysis of ligand specificity have shown that a conserved NAT/NCS2 motif region between putative transmembrane helices 8 and 9 of UapA includes determinants of substrate recognition and selectivity, with at least one residue (Gln-408) implicated in binding with the imidazole moiety of purine (4), whereas a conserved QH motif at the middle of TM1 is important for activity and/or correct targeting to the plasma membrane (5), and an aromatic residue at the middle of TM12 (Phe-528) may act as a purine substrate selectivity filter (6). It has been proposed that TM1, TM12, and the NAT motif region interact functionally to determine affinity and specificity for uric acid (7).

Recently, we characterized the first purine-specific members of the NAT/NCS2 family from a Gram-negative bacterium, namely YgfO and YicE of Escherichia coli K-12 (8), as high affinity xanthine: H+ symporters that cannot use uric acid, hypoxanthine, uracil, or other nucleobases as a substrate and cannot recognize analogues substituted at positions 7 or 8 of the imidazole ring. We launched a systematic series of Cys-scanning and site-directed mutagenesis studies of YgfO to elucidate structure-function relationships in a bacterial NAT (9, 10). In the course of these studies, we showed that the NAT motif sequence region of YgfO includes the essential determinants Gln-324, irreplaceable for high affinity binding and uptake; Asn-325, irreplaceable for active transport; and an α-helical stripe of residues (Thr-332, Gly-333, Ser-336, Val-339), highly sensitive to site-directed alkylation and important for ligand selectivity (9). In addition, we provided evidence that Asn-430 of TM12 is close to the purine binding site and Ile-432 optimizes binding indirectly (10). These studies also show that the bacterial (9, 10) and fungal (4, 6) NAT determinants are strikingly similar, implying that few of the residues conserved within the members of NAT family may be invariably critical for function.

In this report, we have studied the highly polar (Gln or Asn) and putatively charged (Asp, Glu, His, Lys, or Arg) residues of...
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YgfO permease that are predicted to lie in transmembrane helices. Such residues are expected to face other hydrophilic parts of the protein and/or the solvent-accessible environment of the binding pocket and often play crucial roles in substrate binding and the mechanism of energy coupling in active transport (11–14). Employing systematic site-directed mutagenesis of a set of 14 putatively charged and 7 highly polar residues predicted to lie in TMs (Fig. 1) and combining evidence from transport, immunoblotting, sulfhydryl alkyla-
tion, and ligand inhibition assays of a set of 60 site-directed mutants, we have identified four new important determinants in the YgfO mechanism: His-31 and Asn-93, which are crucial for affinity and/or specificity of binding purine ana-
alogues; and Glu-272 and Asp-304, which are irreplaceable for active xanthine transport. The results are discussed in conjunction with our previous findings on the role of TM12 and the NAT motif region and with respect to comparison with the major fungal homolog (UapA).

EXPERIMENTAL PROCEDURES

Materials—[8-3H]Xanthine (27.6 Ci mmol−1) and [8-14C]uric acid (51.5 mCi mmol−1) were purchased from Moravek Biochemicals. Non-radioactive nucleobases and ana-
logues were from Sigma. Oligodeoxynucleotides were synthe-
sized from BioSpring GmbH. High fidelity Taq polymerase (Phusion High Fidelity PCR System) was from Finnzymes. Restriction endonucleases used were from Takara. Horseradish peroxidase (HRP)-conjugated avidin was from Amersham Bio-
sciences. HRP-conjugated penta-His antibody was from Qia-
gen. All other materials were reagent grade and were obtained from commercial sources.

Bacterial Strains and Plasmids—E. coli K-12 was trans-
formed according to Inoue et al. (15). TOP10F’ (Invitrogen) was used for the initial propagation of recombinant plas-
mids. T184 (16) harboring pT7-5/ygfO (8) with given
replacements was used for IPTG-inducible expression from the lacZ promoter/operator.

DNA Manipulations—Construction of expression plasmids and BAD (biotin acceptor domain)-tagged versions of YgfO containing a C-terminal tail with the BAD of Klebsiella pneu-
moniae oxaloacetate decarboxylase and the C-terminal 12 pep-
tides of E. coli LacY has been described (8). Construction of the His10-tagged versions has also been described (9). For construction of Cys-less YgfO, the five native Cys codons were replaced simultaneously with Ser codons using two-stage (multiple over-
lap/extension) PCR on the template of wild-type YgfO tagged at
the C terminus with the BAD tag and transferred to the His10-
tagged background by BamHI-Hpal restriction fragment replacement (10). For construction of mutants, two-stage PCR was performed on the template of Cys-less or wild-type YgfO tagged at the C terminus with either BAD or His10 as indicated. The entire coding sequence of all engineered constructs was verified by double-stranded DNA sequencing in an automated DNA sequencer (MWG-Biotech).

Growth of Bacteria—E. coli T184 harboring given plasmids was grown aerobically at 37 °C in Luria-Bertani medium con-
taining streptomycin (0.01 mg/ml) and ampicillin (0.1 mg/ml). Fully grown cultures were diluted 10-fold, allowed to grow to mid-logarithmic phase, induced with IPTG (0.5 mM) for an additional 2 h at 37 °C, harvested, and washed with the appropriate buffers.

Transport Assays and Kinetic Analysis—E. coli T184 were assayed for active transport of [3H]xanthine (1 μM) by rapid filtration, at both 25 and 37 °C, as described (8). For kinetic
uptake measurements, initial rates were assayed at 5–20 s in the concentration range of 0.1 to 100 μM [3H]xanthine. Selected mutants were also assayed for transport of [14C]uric acid (0.04–2 mM) using the paralog YgfU (Fig. 2) as a positive con-
trol. For assaying the effect of N-ethylmaleimide (NEM) on xanthine uptake activity, T184 cells were preincubated with NEM at the indicated conditions, reactions were stopped by the addition of a 20-fold excess of dithiothreitol, excess reagents, and ligands were removed by centrifugation. Transport assays were performed in the presence of phenazine methosulfate (0.2 mM) and potassium ascorbate (20 mM) (10). For ligand competition experiments, uptake of [3H]xanthine (1 μM) was assayed in the absence or presence of unlabeled analogues (1 mM) (8). Competitive inhibition has been revealed with certain ana-
logues (1-methyl-, 2-thio-, and 8-methylxanthine) by assaying their effect on Kmax and Vmax for wild type and selected mutants, showing that Vmax remains unaltered (9, 10).

Immunoblot Analysis—Membrane fractions of E. coli T184 harboring the given plasmids were prepared and subjected to SDS-PAGE (12%) as described (8). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon PVDF; Pall Corp.). YgfO-BAD was probed with avidin-HRP and YgfO-
His10 with penta-His antibody-HRP. Signals were developed with enhanced chemiluminescence (ECL).

In Silico Analysis—Comparative sequence analysis of NAT/ NCS2 homologs was based on a BLAST-p search and ClustalW alignment, and the most recent genome annotations were used
to retrieve sequence data. Based on the functional (8) and phy-
logenetic data, the YgfO permease recently has been renamed XanQ (EcoGene data base of E. coli sequence and function). Initial analysis of transmembrane topology was performed using the program TMHMM (17).

RESULTS

Delineation of Polar Residues That Fall in TMs—Based on
prediction algorithms, experimental evidence on the cytoplasmic orientation of the C terminus (8, 17), and SCAM analysis
residue falls in the TMs (Fig. 1). These residues are: His-22, Gln-23, Thr-30, His-31, Glu-55, Gln-90, Asn-93, Lys-164, Asn-
184, Glu-186, His-187, Asp-232, Lys-249, His-257, Gln-258,
Glu-272, Asp-304, Gln-330, Arg-337, Arg-341, Arg-385, Asn-
390, Asp-413, Glu-429, Asn-430, Asn-443. Of them, Asp-304 falls in a putatively transmembrane α-helical segment of ambiguous topology, preceding the NAT signature motif sequence (9), which we designate TM9a (Fig. 1), and Thr-30 is included in the group of the highly polar (Gln/Asn) resi-

4 K. Papakostas and S. Frillingos, manuscript in preparation.
5 Kenn Rudd, personal communication.
6 G. Mermelekas and S. Frillingos, manuscript in preparation.
as all other NAT transporters conserve a Gln at the corresponding position (Fig. 2). Interestingly, the bulk of the 26 residues falls in only six TMs of YgfO (TMs 1, 3, 6, 8, 9a, 9b, and 12); residues falling in TM1 (His-31), TM8 (Glu-272), TM9a (Asp-304), and TM12 (Asn-443) are highly conserved in the NAT family, whereas Asn-93 (TM3) and Asn-430 (TM12) are not conserved per se but are conserved with respect to the polar character of the side chain (Asn, Ser, or Thr, at the corresponding positions) (Fig. 2). Of the cohort of 26 residues, five (Gln-330, Arg-337, Glu-429, Asn-430, and Asn-443) have already been studied by us in the context of previous Cys-scanning analyses (9, 10). Thus, we selected the remaining 21 residues (Fig. 1) for a systematic site-directed mutagenesis study.

FIGURE 1. Proposed topology of YgfO highlighting the polar/charged residues. This model is based on the program TMHMM, evidence that the C terminus is cytoplasmic (10, 25), and our unpublished evidence6 on the accessibility of loops to hydrophilic reagents from SCAM analysis. Putatively charged (K/R/H/D/E) or highly polar (Q/H) residues are enlarged and circled. Residues that fall in transmembrane helices (TMs) or in the NAT motif sequence (residues 323–333), as well as residue Asp-276 (which is discussed under “Discussion”) are shown with a dark background. Residues delineated as important to our studies are numbered and shown in red (this study) or blue (previous studies). The ambiguous topology segment 299–323 upstream of the NAT motif is designated as TM9a, and the transmembrane segment 330–357 that follows is designated as TM9b. SCAM analysis6 of the NAT motif shows that residues 323–333 are accessible to solvent from the outside (light blue-gray area), indicating that this region is topologically dynamic and might constitute a flexible, substrate-accessible (7, 9) reentry loop.

FIGURE 2. Sequence alignment of segments containing putative intramembrane polar residues of YgfO. Shown is a consensus of the 16 characterized NAT/NC2 transporters (with GenBankTM accession numbers in parentheses): E. coli YgfO (XanQ) (P67444), YicE (XanP) (P0AGM9), YgfU (Q46821),6 and UraA (P0AGM7); Bacillus subtilis PbuX (P42086), PucK (O32140), and PucJ (O32139); Clostridium perfringens YcpX (BAB80103); Lactococcus lactis PyrP (AAK05701); A. nidulans UapA (Q07307) and UapC (P487777); Aspergillus fumigatus ARpAp (X746919); Candida albicans Xut1 (AA3222); Zea mays Lpe1 (AAB17501); Homo sapiens SVCT1 (SLC23A1) (AHA50261) and SVCT2 (SLC23A2) (Q9UGH3). The full-length sequences were aligned using ClustalW and part of this alignment including the residues of interest is presented. Cuneiform symbols denote the positions of a highly polar/charged residue in YgfO. Larger cuneiforms indicate residues delineated as important from work presented herein or previous analyses (9, 10). Filled cuneiforms denote highly conserved polar residues. Residues similar to the consensus are shaded, and highly conserved residue positions are indicated with continuous vertical shading. An asterisk in the consensus sequence denotes positions conserving a polar character of the side chain but not the side chain per se. The NAT signature motif sequence (9) is indicated by a bold horizontal bar.
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FIGURE 3. Active xanthine transport activities of single-Cys mutants. E. coli T184 harboring pT7-5/ygfO(C-less-BAD) with the given mutations was grown aerobically at 37 °C in complete medium to mid-logarithmic phase, induced with IPTG (0.5 mM) for 2 h, normalized to 0.7 mg of protein/ml of cell suspension, and assayed for transport of [3H]xanthine (1 μM) at 25 °C. A, initial rates of uptake measured at 5–20 s. Control values obtained from T184 harboring pT7-5 alone (0.015 nmol mg⁻¹ min⁻¹ on average) were subtracted from the sample measurements in all cases. Results are expressed as a percentage of the rate of Cys-less YgfO (1.8 nmol mg⁻¹ min⁻¹ on average) with standard deviations from three independent determinations. B, steady state levels of xanthine accumulation (reached at 1–10 min for most mutants). Control values obtained from T184 harboring pT7-5 alone (0.01 nmol mg⁻¹ on average) were subtracted from the sample measurements in all cases. Results are expressed as a percentage of the level of Cys-less YgfO (0.8 nmol mg⁻¹ on average) with standard deviations from three independent determinations.

Active Xanthine Transport of Single-Cys Mutants—Using a functional YgfO devoid of Cys residues (C-less), we replaced each of the 21 putative intramembrane polar amino acid residues (Fig. 1) individually with Cys. After verification of the sequence, each single-Cys mutant was transformed into E. coli T184 and assayed for its ability to catalyze active xanthine transport. As shown in Fig. 3A, of the 21 single-Cys mutants, 13 transport xanthine at rates between 45 and 100% or more of C-less permease, N390C transports at a rate of 35% of C-less, and R385K (wt) are highly active (80% of wild type), H31C (wt), D232C (wt), and R304C (wt) display low but significant activity (25–40% of wild type), N93C (wt) transports marginally (5% of wild-type), whereas replacement with Asp results in a mutant with marginally detectable xanthine uptake rate and a very low level of accumulation reached after 10 min (Fig. 4). The compromised expression (~25–45% of C-less) displayed by mutants K164C, D232C, Q258C, and R385C reflects their relatively low transport rates (15–45% of C-less) (Fig. 3). The inactive N93C also displays low but significant expression in the membrane (25% of C-less).

Expression and Transport Analysis of Mutants in Wild-type Background—From the Cys-scanning transport analysis described above, eight positions of inactive, low activity, and/or low expression mutants were delineated. We analyzed these positions further by (a) transferring single-Cys mutations to the wild-type YgfO background and/or (b) engineering a most conservative site-directed replacement mutant to introduce an amino acid other than Cys. Thus, we constructed and assayed mutants (a) H31C (wt), N93C (wt), K164C (wt), D232C (wt), Q258C (wt), E272C (wt), D304C (wt), and R385C (wt) and (b) N93Q (wt), K164R (wt), D232E (wt), Q258N (wt), E272D (wt), D304E (wt), and R385K (wt) (Fig. 5). Of the Cys-replacement mutants, K164C (wt) is highly active (80% of wild type), H31C (wt), D232C (wt), Q258C (wt), and R304C (wt) display low but significant activity (25–40% of wild type), N93C (wt) transports marginally (5% of wild-type), while E272C (wt) and D304C (wt) display negligible activity (Fig. 5A). Of the second set of mutants, K164R (wt) displays higher activity than wild type (160%), D232E (wt), Q258N (wt), and R385K (wt) are highly active (50–90% of wild type), E272D (wt) and D304E (wt) display very low rates (5% and 2%, respectively) and levels of accumulation (30 and 10%, respectively), and N93Q (wt) is inactive (Fig. 5B). All mutants of the two sets are expressed in the E. coli membrane to high levels comparable with those of the wild type (Fig. 5).

Transport Analysis of Site-directed Mutants at Positions His-31, Asn-93, Glu-272, and Asp-304—The conserved (Fig. 2) positions His-31, Asn-93, Glu-272, and Asp-304, where replacements of Glu-272 and Asp-304 display very low rates (5 and 2%, respectively) and levels of accumulation (30 and 10%, respectively), and N93Q (wt) is inactive (Fig. 5B). All mutants of the two sets are expressed in the E. coli membrane to high levels comparable with those of the wild type (Fig. 5).

Replacements of Glu-272 and Asp-304—Our data show that replacement of Glu-272 with Gln or Cys yields negligible activity, whereas replacement with Asp results in a mutant with marginally detectable xanthine uptake rate and a very low level of accumulation reached after 10 min (Fig. 6, B and C). Replacement of Asp-
304 with Asn, Cys, or Glu also results in marginally active or inactive mutants. Kinetic analysis shows that mutants replacing Glu-272 or Asp-304 display very low or negligible rates of xanthine uptake at any of the concentrations tested, in the range 0.1 to 100 \mu M (Table 1). No xanthine uptake activity was detected for any of these mutants at pH ranging from 4.0 to 10.0 at either 25 or 37 °C, and no uptake of uric acid (0.04–2 mM) could be detected either (data not shown). Although inactive, these mutants are expressed in the membrane to wild-type levels (Fig. 6). In addition, no activity of the inactive single-Cys mutants E272C and D304C (Fig. 3) was rescued by incubation with the negatively charged (2-sulfonatoethyl)methanethiosulfonate (19), and no uptake activity could be detected with double-Cys replacement mutants (20) E272C/H31C, D304C/H31C, E272C/R385C, and D304C/R385C or double replacement mutants harboring a conservative change of Glu-272 or Asp-304, including E272D/N430C, D304E/N430C, E272Q/N430C, D304N/N430C, E272D/N325C, D304E/N325C, E272Q/N325C, and D304N/N325C. All of these inactive mutants are expressed in the membrane to high levels (Fig. 6 and data not shown).

Replacements of His-31—Site-directed replacement of His-31 with a putatively positively charged residue, Arg or Lys, results in negligible activity, corroborated by negligible or marginally detectable expression in the membrane (Fig. 7). Replacement of His-31 with Leu or Cys results in intermediate activity (xanthine uptake rates and levels of 40–50% relative to wild type) and slightly compromised but high expression in the membrane (Fig. 7). Replacement of His-31 with Asn in approximately wild-type levels of expression and xanthine uptake activity, and replacement with Gln results in 1.2–1.5-fold higher expression and xanthine uptake activity than wild type (Fig. 7). Kinetic transport analysis shows that mutants with Asn or Gln at position 31 display a \( K_m \) approximating wild type and a higher \( V_{max} \), whereas mutants with Cys or Leu display high \( V_{max} \) but very low affinity, with a 10- or 5-fold increased \( K_m \), respectively (Table 1).

Replacements of Asn-93—Although replacement of Asn-93 with Gln leads to inactivation of YgfO and replacement with Cys results in strikingly low activity (Fig. 5), replacement of Asn-93 with Asp or Thr yields low but significant xanthine uptake (10–15% of wild type), replacement with Ala yields high activity (60–70% of wild type), and replacement with Ser yields activity higher than wild type (1.8-fold higher rate and 1.2-fold higher level of accumulation) (Fig. 8, lower panel). There are no significant differences in the expression levels of these mutants in the membrane (Fig. 8, upper panel). Kinetic transport analysis reveals that mutants with Ser, Ala, or Asp at position 93 display higher affinity than wild type (2-fold decreased \( K_m \) with higher, equivalent, or very low \( V_{max} \), respectively, whereas mutants N93T displays lower affinity (2-fold increased \( K_m \) and wild-type \( V_{max} \) (Table 1).

Ligand Recognition Profiles of Site-directed Mutants at Positions His-31 and Asn-93—To understand whether His-31 or Asn-93 contributes to the substrate and ligand recognition profile of YgfO, we assayed the active His-31 and Asn-93 mutants for inhibition of \(^{3} H \) xanthine uptake in the presence or absence of a series of purines and purine analogues (Table 2). Of
FIGURE 5. Expression and xanthine uptake activities of Cys-replacement (A) and most conservative replacement mutants (B) in the wild-type permease background. E. coli T184 harboring pT7-5/ygfO(wild-type-BAD) with given mutations were grown, induced, and subjected to immunoblot analysis of membrane fractions or assayed for transport of [3H]xanthine (1 μM, 25 °C) exactly as described in the legends for Figs. 3 and 4. Open and closed histogram bars represent initial rate and steady state values, respectively, with standard deviations shown from three independent experiments.
TABLE 1

| Permease | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|----------|-------|-----------|--------------|
| YgfO(wt) | 4.6  | 6.4       | 1391         |
| YgfO(C-less) | 5.5  | 10.2      | 1858         |
| H31C(wt) | 47.4 | 33.9      | 715          |
| H31L(wt) | 26.1 | 23.9      | 919          |
| H31N(wt) | 2.7  | 16.2      | 5945         |
| H31Q(wt) | 3.4  | 12.1      | 3558         |
| N93A(wt) | 2.4  | 5.0       | 2083         |
| N93S(wt) | 2.1  | 5.0       | 431          |
| N93T(wt) | 2.3  | 0.5       | 217          |
| N93C(wt) | ND   | ND        | ND           |
| N93Q(wt) | ND   | ND        | ND           |
| E272D(wt) | 2.7  | 4.8       | 1778         |
| E272Q(wt) | 2.5  | 4.7       | 1880         |
| D304E(wt) | ND   | ND        | ND           |
| D304N(wt) | ND   | ND        | ND           |

FIGURE 6. Expression and xanthine uptake activities of site-directed mutants at positions 272 and 304. E. coli T184 harboring pT7-5/ygfO (wild-type-BAD) or pT7-5/ygfO(C-less-His10) with given mutations were grown, induced, and subjected to immunoblot analysis of membrane fractions (A) or assayed for transport of [3H]xanthine (1 μM, 25 °C) (B and C) exactly as described in the legends to Figs. 3 and 4. Open and closed histogram bars (in B) represent the initial rate and steady state values, respectively, with standard deviations shown from three independent experiments. C, representative time courses of xanthine uptake by mutants E272D(wt), D304E(wt), D304N(wt), and E272Q(wt) in comparison with wild-type YgfO. Although not shown, values for E272Q(wt) were indistinguishable from negative controls (cells harboring pT7-5 alone) or double replacement mutants with a change at Glu-272 or Asp-304 (see under "Results").
inhibitory effect was evident in the presence of 1 mM xanthine (data not shown). On the other hand, activity of Q23C or K249C, which was about 50% of C-less initially (Fig. 3), is enhanced in the presence of NEM almost 2-fold (Fig. 9), i.e. it is restored to C-less YgfO levels. The activity of the remaining 14 mutants is essentially unaltered in the presence of NEM (<1.2-fold enhancement or inhibition).

Site-directed Mutagenesis of Gln-258—The position of the low activity (Fig. 3) and NEM-sensitive mutant Q258C (Fig. 9) was subjected to more extensive site-directed mutagenesis in the wild-type YgfO background. Replacement of Gln-258 with Asn or His results in high xanthine uptake activity comparable with that of wild type (Fig. 5 and data not shown), whereas replacement with Cys results in lower activity (40% of wild type). Kinetic analysis shows that Q258N(wt) and Q258H(wt) transport xanthine with a $K_m$ and $V_{max}$ approximating the wild type (Table 1D). On transport inhibition experiments, Q258H(wt) was found to follow essentially wild-type selectivity for purine ligands, and Q258N(wt) was found to follow wild-type selectivity but also to recognize uric acid and guanine (Table 2). No transport of [14C]uric acid was detected with these mutants at 2 mM (data not shown).

DISCUSSION

We have studied the role of 21 highly polar and/or charged residues in E. coli YgfO, the major xanthine-specific bacterial homolog of the NAT family and found that 4 of them are important for the function of the YgfO permease in various aspects. In summary, His-31 (TM1) and Asn-93 (TM3) are crucial for affinity and/or specificity against purine analogues, whereas Glu-272 (TM8) and Asp-304 (TM9a) are irreplaceable for active xanthine transport. Taken together with our previous findings on the irreplaceable role of Gln-324 and Asn-325 in the NAT motif sequence (9) and on Asn-430 in TM12 being in the vicinity of the purine binding site (10), these data suggest that polar residues critical for the mechanism of YgfO cluster in TMs 1, 3, 8, 9a, and 12 and the NAT motif sequence (Fig. 1).
Transport assays were performed in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate. Rates are presented as percentages of the rate measured in the absence of NEM with standard deviations.

![Effect of NEM on the xanthine transport activity of single-Cys mutants.](image)

| Competitor | H31N | H31Q | H31L | H31C | N93A | N93S | N93T | N93D |
|------------|------|------|------|------|------|------|------|------|
| None       | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  |
| HX         | 99   | 98   | 99   | 98   | 60   | 49   | 115  | 73   |
| Uric acid  | 96   | 61   | 52   | 99   | 128  | 89   | 113  | 116  |
| Adenine    | 110  | 95   | 89   | 133  | 120  | 62   | 118  | 80   |
| Guanine    | 89   | 68   | 46   | 91   | 102  | 67   | 65   | 80   |
| Uracil     | 101  | 97   | 48   | 98   | 99   | 95   | 92   | 120  |
| 1-MX       | 45   | 44   | 39   | 113  | 117  | 43   | 38   | 123  |
| 2-SX       | 14   | 4    | 3    | 91   | 91   | 16   | 7    | 43   |
| 3-MX       | 28   | 19   | 16   | 70   | 63   | 41   | 16   | 49   |
| 4-MX       | 20   | 24   | 4    | 123  | 102  | 24   | 11   | 84   |
| 5-MX       | 100  | 76   | 42   | 162  | 84   | 62   | 54   | 121  |
| 6-MX       | 91   | 35   | 25   | 87   | 84   | 9    | 9    | 69   |
| 7-MX       | 100  | 76   | 42   | 162  | 84   | 62   | 54   | 121  |
| 8-MX       | 91   | 35   | 25   | 87   | 84   | 9    | 9    | 69   |
| Allopurinol| 101  | 91   | 98   | 80   | 84   | 110  | 84   | 161  |
| Oxyurinol  | 30   | 5    | 4    | 82   | 74   | 2    | 16   | 13   |

| % [3H]Xanthine uptake rate retained |
|-------------------------------------|
| H31C | H31Q | H31L | H31C | N93A | N93S | N93T | N93D |
|------|------|------|------|------|------|------|------|
| None | 100  | 100  | 100  | 100  | 100  | 100  | 100  |
| HX   | 99   | 98   | 99   | 98   | 60   | 49   | 115  | 73   |
| Uric acid | 96   | 61   | 52   | 99   | 128  | 89   | 113  | 116  |
| Adenine    | 110  | 95   | 89   | 133  | 120  | 62   | 118  | 80   |
| Guanine    | 89   | 68   | 46   | 91   | 102  | 67   | 65   | 80   |
| Uracil     | 101  | 97   | 48   | 98   | 99   | 95   | 92   | 120  |
| 1-MX       | 45   | 44   | 39   | 113  | 117  | 43   | 38   | 123  |
| 2-SX       | 14   | 4    | 3    | 91   | 91   | 16   | 7    | 43   |
| 3-MX       | 28   | 19   | 16   | 70   | 63   | 41   | 16   | 49   |
| 4-MX       | 20   | 24   | 4    | 123  | 102  | 24   | 11   | 84   |
| 5-MX       | 100  | 76   | 42   | 162  | 84   | 62   | 54   | 121  |
| 6-MX       | 91   | 35   | 25   | 87   | 84   | 9    | 9    | 69   |
| 7-MX       | 100  | 76   | 42   | 162  | 84   | 62   | 54   | 121  |
| 8-MX       | 91   | 35   | 25   | 87   | 84   | 9    | 9    | 69   |
| Allopurinol| 101  | 91   | 98   | 80   | 84   | 110  | 84   | 161  |
| Oxyurinol  | 30   | 5    | 4    | 82   | 74   | 2    | 16   | 13   |

**TABLE 2**

Specificity profile of YgfO mutants at positions His-31, Asn-93, and Gln-258

Values show expressed the percentage of [3H]xanthine (1 μM) uptake rate in the presence of 1000-fold excess (1 mM) of unlabeled competitors. The uptake value obtained in the absence of competitor was taken as 100%. Values represent the means of at least three determinations ± S.D. (always <20%). Most significant differences from the wild-type profile are highlighted in bold. All mutants as well as the wild-type version used in these experiments contained a C-terminal BAD. All mutants assayed, including H31C, were in the wild-type YgfO background (WT). The purine bases or analogues used as potential competitors are: hypoxanthine (HX); uric acid; adenine; guanine; uracil; 1-methylxanthine (1-MX); 2-thioxanthine (2-SX); 3-methylxanthine (3-MX); 6-thioxanthine (6-SX); 7-methylxanthine (7-MX); 8-methylxanthine (8-MX); allopurinol; oxyurinol.

![Effect of NEM on the xanthine transport activity of single-Cys mutants.](image)

Intramembrane Polar Residues of YgfO (XanQ)

Residue His-31, at the middle of TM1, is almost completely conserved in the NAT family (2) and is preceded by a polar residue, which is Thr-30 in YgfO and Glu in all other NAT transporters (Fig. 2). From our results, it is apparent that His-31 is replaceable for YgfO function, but the presence of a hydrophobic uncharged residue at this position is essential for high affinity substrate binding and (b) Asp304 is highly sensitive to the conformational changes following binding and/or coupling with the permease turnover. It is conceivable, therefore, that Asp-304 (TM9a), 20 residues upstream of the NAT motif region, is situated close to but not at the purine binding site of YgfO and may be essential for the conformational changes following binding and/or coupling with proton translocation. Similar assumptions could be made for Glu-272 (TM8), because the middle of TM8 (His-257) appears to be in proximity with TM12 and the NAT motif region, based on cadmium sensitivity assays with single- and double-Cys mutants. In this vein, site-directed alkylation shows that D304C reacts slowly with NEM in right-side-out membrane vesicles, and reactivity is enhanced in the presence of xanthine, implying that (a) replacement of Asp-304 is compatible with high affinity substrate binding and (b) Asp304 is highly sensitive to the conformational movements associated with the permease turnover. Further experiments to elucidate the role of Asp-304 and Glu-272 in more detail are under way in our laboratory.

mid of TM12, which contains Asn-430, another putative substrate-binding residue (10). It is conceivable that Asp-304 (TM9a), 20 residues upstream of the NAT motif region, is situated close to but not at the purine binding site of YgfO and may be essential for the conformational changes following binding and/or coupling with proton translocation. Similar assumptions could be made for Glu-272 (TM8), because the middle of TM8 (His-257) appears to be in proximity with TM12 and the NAT motif region, based on cadmium sensitivity assays with single- and double-Cys mutants. In this vein, site-directed alkylation shows that D304C reacts slowly with NEM in right-side-out membrane vesicles, and reactivity is enhanced in the presence of xanthine, implying that (a) replacement of Asp-304 is compatible with high affinity substrate binding and (b) Asp304 is highly sensitive to the conformational movements associated with the permease turnover. Further experiments to elucidate the role of Asp-304 and Glu-272 in more detail are under way in our laboratory.

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for xanthine uptake is retained upon replacement with Asn or Gln, whereas a hydrophobic (Leu) or low polarity (Cys) replacement impairs affinity for substrate, and introduction of a positive charge through side-specific mutagenesis (Lys, Arg) or alkylation with (2-aminoethyl)methanethiosulfonate abolishes expression and/or activity. Interestingly, the same conclusion has been reached on the role of the corresponding residue, His-51, in the mammalian, non-purine-transporting homolog hSVCT1 (21), whereas hydrogen bonding of His-86 at the corresponding consensus position of the fungal UapA has been suggested as crucial for proper folding and targeting to the plasma membrane (5). On the other hand, we find that introduction of a more flexible hydrogen-bonding side chain (Gln) in lieu of His-31 leads to a novel specificity profile with broader recognition of purine bases and analogues (Table 2). A specificity change leading to a novel property (recognition of the oxidized form of L-ascorbic acid) has also been reported with mutant H51Q in hSVCT1 (21), and the corresponding H86Q mutant has not been made in UapA (5). We found no evidence for such a role from the mutagenesis of the corresponding residue of YgfO (Thr-30) in this study.

Residue Asn-93, at the middle of TM3, is not conserved as an amidic side chain in the other members of the NAT family, but the nucleobase-transporting members retain a hydrogen-bonding Thr or Ser, and the mammalian SVCTs have an Ala at this position. Replacement of Asn-93 with a small hydrophobic (Ala) or hydrophilic (Ser) residue yields very high xanthine uptake activity, which is comparable (Ala) or higher (Ser) than wild type, but changes the purine selectivity of YgfO by relaxing constraints for binding at the imidazole moiety of substrate. Thus, these mutants are very efficient in recognizing 8-methylxanthine and, to a lesser extent, 7-methylxanthine and uric acid (8-oxy-xanthine), and they even transport [14C]uric acid with low capacity or affinity, properties that are not pertinent to

**FIGURE 10. Substrate recognition properties of mutants replacing Asn93.** Shown is the side chain of residue 93 for wild type (top), N93T (middle), and N93S (bottom) with respect to their purine substrate usage and constraints and characteristic changes in the specificity profile (data from Table 2). Shaded circular areas denote numbered positions of the purine ring where a bulkier substitution abolishes recognition (>70% xanthine uptake activity retained in the presence of the putative competitor) (black) or results in poor recognition (45–60% activity retained) (gray). The purine bases or analogues shown are: 2-thioxanthine (2-SX); 3-methylxanthine (3-MX); 6-thioxanthine (6-SX); 7-methylxanthine (7-MX); 8-methylxanthine (8-MX); uric acid (8-oxy-xanthine) (8-OX).
wild-type YgfO. On the other hand, replacement with a polar residue of comparable volume (Thr) yields low affinity, possibly due to loss of a bi-dentate hydrogen-bonding interaction of Asn-93 (Fig. 10). Introduction of a more bulky carboxyl amide (Gln) or a sulfur-containing, less polar side chain (Cys) in lieu of Asn-93 leads to inactivation, suggesting that both steric and polarity constraints at position 93 are important for function. Finally, the low activity observed with mutant N93D suggests that introduction of a negative charge at this position may also be unfavorable for function. It is interesting to note that replacement of Asn-93 with amino acids encountered at the corresponding residue position of the other NAT transporters (Fig. 2) yields YgfO versions with modified specificity (Fig. 10). Replacement with Ser, in particular, which is conserved in the uric-acid/xanthine transporters Xut1, UapA, UapC, AFUapA, and Lpe1, yields efficient recognition of analogues at the imidazole moiety of substrate, mimicking in part the fungal or plant members (Fig. 10).

Because a high-resolution x-ray structure of a NAT prototype is not available to date (22), to understand the putative spatial association of the important residues delineated herein with the ones reported previously (9, 10) we performed a detailed sequence analysis of NAT homologs, and in conjunction with available biochemical data and the assumption that the conserved highly polar/charged residues (18) should face away from the lipid, we deduced a naive helix packing model for YgfO (Fig. 11). In this model, the NAT motif sequence is shown as a putative reentry loop with Gln-324 and Asn-325 pointing toward the solvent-accessible internal cavity. Residues conserved either per se or with respect to their side-chain character in the known members of the family are shown enlarged (see also Fig. 2). Sites conserving a hydrophobic side chain in the known members are indicated by a larger light gray area facing outside the helix. Sites conserving hydrophobic residues in the 67 closest sequence homologs of YgfO (sequence identity > 43%) are indicated by an additional small oval at the outside of the helix with a number denoting the number of different amino acid replacements found at each position. Sites conserving a hydrophilic residue or a small side chain in the cohort of the 67 closest homologs are shown as hatched circles or targets, respectively. The site of Thr-30, almost invariably conserving a Gln or Thr throughout the family, is indicated with a bolder periphery.
Intramembrane Polar Residues of YgfO (XanQ)

alkylation (9) and accessible to solvent, with at least one of them, Gln-324 (9) or its counterpart in the fungal UapA (4), implicated directly with substrate binding. Placement of the NEM-sensitive face of helix TM9a close to the stripe of small and/or hydrophilic residues of TM12 is supported by functional evidence for interaction between TM12 and the NAT motif region in both a bacterial (10) and a fungal (7) transporter. As shown with other transporters (23–25), such NEM-sensitive faces are expected to be conformationally active or to line the substrate translocation pathway and be critical for the mechanism. Placement of TM8 close to both the NAT motif and TM12 is supported by cadmium-sensitivity assays. Placement of TM1 close to TM12 as well as to the NAT motif region has been proposed from extensive second-site suppressor analysis in the fungal UapA (7). Finally, although placement of TM3 is tentative, an interaction between the fungal TM3 (M151) and TM1 (H86) is indicated from a second-site suppressor study.8 Of the important residues described, His-31 and Asp-304 fall in polar/small side-chain and/or alkylation-sensitive faces of amphipathic α-helical segments (Fig. 11) with extensive lipid-facing stripes of residues on the other side. A similar, but less prominent, effect is seen with Asn-430 in TM12 and the NEM-sensitive face of TM9b. On the other hand, Asn-93 (TM3) is on the opposite side of a polar face of the helical wheel, and Glu-272 (TM8) is not connected to a contiguous polar stripe, suggesting that TM3 and TM8 could be discontinuous helices or tilted with respect to the plane of the membrane and/or participate in multiple hydrophilic contacts with other helices. With respect to TM8, Cys-scanning analysis9 shows that a broader face of positions of NEM-sensitive and/or low activity mutants is formed at the cytoplasmic side, including Ser-269, Glu-272, Ala-273, Gly-275, and Asp-276. Thus, TM8 might extend by one additional helical turn (Fig. 1), introducing Asp-276 as another putative intramembrane charged residue, one turn above Glu-272. Mutagenesis of Asp-276 indicates that a carboxylic acid group at this position may also be important for function.9 In any event, it is clear that all of the helices in Fig. 11 contain important residue sites, including Gln-324 (9) and Asn-430 (10), which interfere with substrate binding. It would be reasonable to test whether the newly identified determinants of affinity and/or specificity, His-31 and Asn-97, are also proximal to the binding site or act by indirect, long-range effects. Site-directed sulfhydryl alkylation cannot be used as a tool to this end, because the corresponding Cys replacement mutants are either inactive or grossly impaired for substrate affinity (Fig. 3; Tables 1 and 2). For example, we found that H31C is alkylatable with NEM in situ and H31C(wt) is sensitive to inactivation by NEM, but no protective or other effect is evident in the presence of xanthine,7 most probably because of the inability of these mutants to bind the xanthine substrate with high affinity.

In conclusion, we have provided evidence for four new important players in the mechanism of the YgfO permease and shown that their role is probably conserved in the NAT/NCS2 family of transporters. Ongoing work on the bacterial (YgfO) and fungal (UapA) NAT prototypes, in conjunction with potential future structural studies (22), is expected to shed light on the mechanism and evolution of purine substrate recognition and selectivity in this important, evolutionarily ubiquitous family of nucleobase-ascorbate transporters.

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