Identification and Mutational Analysis of Amino Acid Residues Involved in Dipyridamole Interactions with Human and Caenorhabditis elegans Equilibrative Nucleoside Transporters*

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The equilibrative nucleoside transporters, hENT1 and CeENT1 from humans and Caenorhabditis elegans, respectively, are inhibited by nanomolar concentrations of dipyridamole and share a common 11-transmembrane helix (TM) topology. Random mutagenesis and screening by functional complementation in yeast for clones with reduced sensitivities to dipyridamole yielded mutations at Ile129 in TM 11 of CeENT1 and Met132 in TM 1 of hENT1. Mutational analysis of the corresponding residues of both proteins suggested important roles for these residues in competitive inhibition of hENT1 and CeENT1 by dipyridamole. To verify the roles of these residues in dipyridamole interactions, hENT2, which naturally exhibits low dipyridamole sensitivity, was mutated to contain side chains favorable for high affinity dipyridamole binding (i.e., a Met at the TM 1 and/or an Ile at the TM 11 positions). The single mutants exhibited increased hENT2 sensitivity to inhibition by dipyridamole, and the double mutant was the most sensitive, with an IC50 value that was only 2% of that of wild type. Functional analysis of the TM 1 and 11 mutants of hENT1 and CeENT1 revealed that Ala and Thr in the TM 1 and 11 positions, respectively, impaired uridine and adenosine transport and that Leu142 of hENT1 was involved in permeant selectivity. Mechanistic and structural models of dipyridamole interactions with the TM 1 and 11 residues are proposed. This study demonstrated that the corresponding residues in TMs 1 and 11 of hENT1, hENT2, and CeENT1 are important for dipyridamole interactions and nucleoside transport.

Nucleosides are hydrophilic molecules that require the presence of integral membrane transporter proteins to move across biological membranes (1–3). Nucleoside transporters are also responsible for the cellular uptake of many nucleoside analogs used in the treatment of solid tumors, hematologic malignancies, and viral diseases (4, 5). Extracellular concentrations of adenosine, a signaling molecule that binds to G protein-coupled cell surface receptors, are regulated by nucleoside transporters (6).

Members of the equilibrative nucleoside/nucleobase transporter (ENT) family have been identified in many eukaryotes, and most mediate facilitated diffusion of nucleosides, although some members are proton-coupled (7). All ENT family transporters appear to share a common membrane architecture with 11 transmembrane helices (TMs), a large cytoplasmic loop between TMs 6 and 7, and in many cases a large glycosylated loop between TMs 1 and 2 (8).

Four ENT family members have been identified by molecular cloning from human tissues. The potent transport inhibitor nitrobenzylmercaptopurine ribonucleoside (NBMPR) can be used to functionally distinguish human ENT1 (hENT1), which mediates equilibrative NBMPR-sensitive (es) transport activity, from hENT2, which mediates equilibrative NBMPR-insensitive (ei) transport activity (9–11). Moreover, hENT1 is 2–3 orders of magnitude more sensitive to inhibition by coronary vasodilator drugs such as dilazep and dipyridamole than hENT2 (12). No functional characteristics have been published for hENT3 or hENT4 (7, 13, 14).

Five ENT family members have been identified by sequence homology in the genomic data base for Caenorhabditis elegans (15). C. elegans ENT1 and 2 (CeENT1 and 2) share 94% amino acid sequence identity and have been functionally characterized and determined to be sensitive to inhibition by the coronary vasodilator dipyridamole but insensitive to NBMPR and dilazep (15).

Several studies have addressed the mechanism of dipyridamole binding, which appears to compete with permeants and NBMPR for binding to the outward facing aspect of mammalian es transporters at nanomolar concentrations, although allosteric properties have been observed at micromolar concentrations (16–19). The mechanism of dipyridamole binding to CeENT1 has not been reported.

Knowledge of the amino acid residues involved in dipyridamole interactions with ENTs is limited. In previous work, we screened a library of hENT1 mutants for clones with reduced sensitivity to dilazep, resulting in the identification of residue 33 of hENT1 and hENT2 as important determinants of not only

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**EXPERIMENTAL PROCEDURES**

**Strains and Media**—KY114 (MATa, gal, ura3–52, trp1, lys2, ade2, hisd2000) was the parental yeast strain used to generate KTK, which was transformed with pYPhENT1 or pYPCeENT1 using a lithium acetate method (23). The point mutations resulting in the CeENT1-I49M and -I49A, and hENT1-I33M, -L442I, and -I33M/L442I changes in amino acid sequence were randomly mutated by propagation in the XL-1 RED mutator strain of *E. coli* (In-<150g/ml). hENT1 and CeENT1 (Fig. 1, A–D) were obtained from Sigma, and radiolabeled compounds were obtained from Moravek Biochemicals (Brea, CA). The transport assays were performed in 96-well plates as previously described (27, 28). Yeast cells were grown in CMM/GLU medium to mid-exponential growth phase and transferred to vials for quantification of radioactivity by scintillation counting.

For dipyridamole concentration-effect relationships, the yeast suspensions were first incubated for 15–30 min with dipyridamole to allow equilibration of the inhibitor with its binding sites before the addition of radiolabeled permeant as previously described (12, 17, 29–31).

**RESULTS**

**Dixon Plot Analysis of Dipyridamole Inhibition of hENT1 and CeENT1**—To determine whether dipyridamole inhibited hENT1 and CeENT1 by a common mechanism, yeast cells producing recombinant hENT1 or CeENT1 were incubated with 10, 25, or 100 μM [3H]adenosine in the absence or presence of increasing concentrations of dipyridamole. Dixon plot analysis (32) of these data suggested that dipyridamole was a competitive inhibitor of adenosine transport for both proteins, with K_i values of 30 ± 1 and 400 ± 20 nM, respectively, for hENT1 and CeENT1 (Fig. 1, A and B, and Table I). In a similar experiment, yeast cells producing recombinant hENT1 or CeENT1 were incubated with 50, 150, or 500 μM [3H]uridine in the absence or presence of increasing concentrations of dipyridamole. Dixon plot analysis of the resulting data suggested that dipyridamole was also a competitive inhibitor of uridine transport for both hENT1 and CeENT1 with K_i values of 40 ± 4 and 310 ± 40 nM, respectively (Fig. 1, C and D, and Table I).

**Random Mutagenesis and Screening of CeENT1**—Growth of yeast cells in the presence of methotrexate and sulfanilamide results in depletion of TTP pools and growth arrest (33). Although yeast lack endogenous plasma membrane transport systems for thymidine (34), heterologous production of hENT1 results in transport of extracellular thymidine, which is subsequently metabolized to TMP by recombinant herpes simplex thymidine kinase in KTK cells, bypassing the methotrexate/sulfanilamide-induced growth arrest (20, 26, 35). The yeast expression plasmids pYPCeENT1 and pYPPhENT1 were randomly mutated by propagation in the XL-1 RED mutator strain.
of E. coli (Stratagene) and screened by complementation in KTK yeast cells for functional mutants with reduced sensitivity to dipyridamole (i.e. for mutants that could salvage extracellular thymidine in the presence of dipyridamole). Screening of a hENT1 cDNA mutant library for dipyridamole resistance resulted in the isolation of 20 clones, all of which contained mutations in codon 33, which corresponds to Met33, a residue previously identified in screens of a hydroxylamine-mutated hENT1 library for resistance to dilazep, an inhibitor of hENT1 and hENT2 (12) that is structurally unrelated to dipyridamole (see Fig. 2 for chemical structures). Four separate screens of the CeENT1 libraries resulted in the isolation of five clones, four of which did not contain mutations in the CeENT1 cDNA, and one of which contained a mutation in codon 429, resulting in the conversion of Ile429 in putative TM 11 to Thr.

Multiple sequence alignments of TM 1 of human, rat, and C. elegans ENT1 and ENT2 revealed that Met is present at residue 33 in dipyridamole-sensitive hENT1, whereas Ile is present in the dipyridamole-insensitive transporters rENT1, rENT2, and hENT2 (Fig. 3). The corresponding TM 1 residue in CeENT1 and CeENT2 (i.e. Ile 49) was the same as in the dipyridamole-insensitive mammalian transporters. TM 1 also contains two highly conserved Gly residues, which are present in all six transporters, that may be involved in helix-helix contacts (7); these residues correspond to Gly22 and Gly24 of hENT1.

Multiple sequence alignments of TM 11 of human, rat, and C. elegans ENT1 and ENT2 revealed that the four mammalian transporters contain a Leu, whereas both CeENT1 and CeENT2 contain an Ile at the corresponding positions (i.e. positions 442 in hENT1 and 429 in CeENT1). This result suggested that the positions occupied by either Leu or Ile were not responsible for the observed differences in dipyridamole sensitivities of the transporters. Because the Leu/Ile residue of TM 11 is the second residue of a highly conserved GXXXG helix-helix interaction motif (7, 36, 37) and although it is not part of the helix-helix contact, mutations at this site may affect the conformation of TM 11 and other parts of the proteins.

Concentration-Effect Relationships for Dipyridamole Inhibition of hENT1, CeENT1, and Various Mutants—To determine the effects of mutations at Met33 and Leu442 of hENT1 and Ile49 and Ile429 of CeENT1, site-directed mutagenesis was undertaken at these positions. Eight of the resulting mutants (hENT1-M33I, -M33A, -L442T, and -L442I and CeENT1-I49M, -I49A, -I429L, and -I429T) exhibited transport activity, whereas two (hENT1-M33T and -L442A) did not (data not shown). Yeast producing CeENT1, hENT1, or one of the various functionally active mutants were incubated in the absence (controls) or presence of increasing concentrations of dipyridamole. The data were analyzed by linear regression using GraphPad Prism version 4.0 software, the slope and y intercept of which were used to calculate the K_i values as the average x value of the point of intersection for all lines on the graph. The average K_i values from three separate experiments are presented in Table I.

![Fig. 1. Dixon plot analysis of dipyridamole inhibition of hENT1- and CeENT1-mediated uridine and adenine transport.](http://www.jbc.org/Downloaded from http://www.jbc.org)
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The average IC₅₀ values (±S.E.) from three separate experiments were determined using GraphPad Prism version 4.0 software by nonlinear regression analysis. Some representative curves are presented in Fig. 3. The Kᵢ values were determined from the Dixon plots presented in Figs. 1 and 7. ND, not determined.

| Protein | TM  | IC₅₀ | Ratio | p value | Kᵢ  |
|---------|-----|------|-------|---------|------|
| hENT1   | 11  | 30 ± 2| 1.0   | 1⁰     | 40 ± 4|
| M33I    | 1   | 810 ± 140| 27    | 0.005b | 760 ± 1|
| M33A    | 1   | >30,000| >>>   | ND      |      |
| L442T   | 11  | 22 ± 2| 0.75  | 0.06⁷  |      |
| L442I   | 11  | 10 ± 1| 0.34  | 0.001b |      |
| M33I/L442I | 1/11 | 100 ± 10| 3.5   | 0.007⁷ |      |
| M33A/L442I | 1/11 | 600 ± 70| 20    | ND      |      |
| CeENT1  | 11  | 180 ± 10| 1.00  | 1⁴     | 310 ± 40|
| I49M    | 1   | 150 ± 10| 0.84  | 0.1³³  |      |
| I49A    | 1   | 140 ± 20| 0.75  | 0.07⁵  |      |
| I429L   | 11  | 730 ± 110| 4.05  | 0.009⁴ |      |
| I429T   | 11  | 3600 ± 600| 20    | 0.006⁴ |      |
| I49MI429L | 1/11 | 170 ± 20| 0.96  | 0.008³ |      |
| I49MI429T | 1/11 | 770 ± 260| 4.2   | 0.01² |      |
| rENT1   | 11  | 10500 ± 2300| 1.0   | 1.0000 |      |
| hENT2   | 1   | 4400 ± 500| 1.0   | 1⁰     |      |
| I33M    | 1   | 480 ± 50| 0.11  | 0.002⁶ |      |
| L442I   | 11  | 1500 ± 100| 0.34  | 0.005⁶ |      |
| M33I/L442I | 1/11 | 88 ± 21| 0.02  | 0.001⁶ |      |

⁰ Ratio = Mutant IC₅₀/Wildtype IC₅₀.

CeENT1-I49M/I429L displayed an IC₅₀ value that was 13% of that of CeENT1-M33I and 4-fold higher than that of CeENT1-I429T and -I429L. CeENT1-I49M/I429L and -I49M/I429T were then determined (Table I). hENT1-M33I/L442I displayed an IC₅₀ value that was much lower than that of CeENT1. The partial and complete restoration of sensitivity to dipyridamole in the two residue 429 mutants by the presence of a Met residue at position 49 suggested a functional interaction between TMs 1 and 11 in binding of dipyridamole by CeENT1.

To complement these observations on CeENT1, the hENT1 TM 1 mutants, which were less sensitive to dipyridamole than wild type hENT1, were combined with the L442I mutation. The concentration-effect relationships for dipyridamole inhibition of uridine transport by the resulting double mutants (CeENT1-I49M/I429L and -I49MI429T) in experiments summarized in Table I. CeENT1-I49M/I429L displayed an IC₅₀ value that was 22% of that of CeENT1-I429T and 4-fold higher than that of CeENT1 (see also Fig. 4B). CeENT1-I49M/I429T displayed an IC₅₀ value that was 24% of that of CeENT1-I429L and similar to that of CeENT1. The partial and complete restoration of sensitivity to dipyridamole in the two residue 429 mutants by the presence of a Met residue at position 49 suggested a functional interaction between TMs 1 and 11 in binding of dipyridamole by CeENT1.

Fig. 2. Chemical structures of dilazep and dipyridamole. The structures of dilazep (N₂,N₂-bis[(3,4,5-trimethoxybenzoxazol-2-yl)homopiperazine] and dipyridamole (2,2',2''-tris-[4,8-dipiperidinylpyrimido[5,4-d]pyrimidine-2,6-diyl]dinitrilo)tetraethanol) were generated using ChemDraw Ultra version 6.0 software.

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of hENT1-L442T and -L442I were, respectively, similar to and 34% of the hENT1 value. These results showed that the dipyridamole sensitivity of CeENT1 was affected by the TM 11 but not the TM 1 mutations, whereas the sensitivity of hENT1 was affected by both TM 1 and 11 mutations.

rENT1, which is relatively insensitive to dipyridamole (38), contains an Ile at the TM 1 position and a Leu at the TM 11 position. The concentration-effect relationship for dipyridamole inhibition of rENT1 yielded an IC₅₀ value of 10500 ± 2300 nM, which was 58- and 350-fold higher than the values for CeENT1 and hENT1, respectively (Table I). Although much less sensitive to dipyridamole inhibition than either hENT1 or CeENT1, rENT1 was capable of interacting with dipyridamole. That both hENT1 and rENT1 contain a Leu residue at the TM 11 position suggested that this residue contributed to dipyridamole binding by both transporters rather than being responsible for their differences in sensitivity.

For both hENT1 and CeENT1, the presence of a Met at the TM 1 and an Ile at the TM 11 positions was consistent with their high sensitivities to dipyridamole. These residues were further investigated by combining the CeENT1 TM 11 mutants with reduced sensitivities (I429L and I429T) with the TM 11 mutant with high sensitivity (I49M) and assessing the concentration-effect relationships for dipyridamole inhibition of uridine transport by the resulting double mutants (CeENT1-I49M/I429L and -I49MI429T) in experiments summarized in Table I. CeENT1-I49M/I429L displayed an IC₅₀ value that was 22% of that of CeENT1-I429T and 4-fold higher than that of CeENT1 (see also Fig. 4B). CeENT1-I49M/I429T displayed an IC₅₀ value that was 24% of that of CeENT1-I429L and similar to that of CeENT1. The partial and complete restoration of sensitivity to dipyridamole in the two residue 429 mutants by the presence of a Met residue at position 49 suggested a functional interaction between TMs 1 and 11 in binding of dipyridamole by CeENT1.

To complement these observations on CeENT1, the hENT1 TM 1 mutants, which were less sensitive to dipyridamole than wild type hENT1, were combined with the L442I mutation. The concentration-effect relationships for dipyridamole inhibition of uridine transport by hENT1-M33I/L442I and -M33A/L442I were then determined (Table I). hENT1-M33I/L442I displayed an IC₅₀ value that was 13% of that of hENT1-M33I and 4-fold higher than that of hENT1 (see also Fig. 4A). hENT1-M33A/L442I displayed an IC₅₀ value that was much lower than that of the highly insensitive hENT1-M33A and 20-fold higher than that of hENT1. The observation that the introduction of the
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The average IC50 values from three separate experiments were S.E. values are not shown where the size of the data point is larger than various mutants. Yeast cells producing recombinant hENT1 (Leu442I-M33I/L442I). The concentration-effect relationships for dipyridamole inhibition of uridine transport by hENT1, CeENT1, hENT2, and Various Mutants—

hENT1-M33I/L442I. The concentration-effect relationships for dipyridamole inhibition by hENT1, CeENT1, and Various Mutants—

kinetic parameters of uridine transport for hENT1, CeENT1, and Various Mutants—To assess the effects of mutations at either the TM 1 or 11 positions on permeant transport characteristics, the apparent Km and Vmax values of uridine transport by yeast cells producing hENT1, CeENT1, or one of the mutants were determined by assaying transport rates at increasing concentrations of [3H]uridine (Table II and Fig. 5). Recombinant hENT1- and CeENT1-mediated uridine transport conformed to simple Michaelis-Menten kinetics (Fig. 5) and displayed apparent Km values of 36 ± 6 and 100 ± 10 μM, respectively, and apparent Vmax values of 540 ± 20 and 955 ± 35 pmol/mg/min, respectively. That CeENT1 displayed a lower apparent affinity for uridine than hENT1 was consistent with the results generated by production of these proteins in X. laevis oocytes (15). The apparent Km value for hENT1 reported here was lower than that reported previously (110 μM) (12) because the uptake contribution by the endogenous uracil permease, FUR4, in our previous study (12) was subtracted in the current study by determining background uptake in the presence of 10 μM unlabeled thymidine.

Although there were no differences between the kinetic values obtained for hENT1 and hENT1-M33I, hENT1-M33A displayed a modestly increased Km value and a reduced Vmax value. A similar result was observed for CeENT1 in that CeENT1-I49M displayed a modestly increased Km value and a modestly reduced Vmax value relative to those of CeENT1-I49M. These data suggested that the presence of an Ala residue at the TM 1 position resulted in reduced apparent affinities and transport capacities of both hENT1 and CeENT1.

Like the mutations in TM 1, conservative substitutions of the hydrophobic Leu and Ile residues at the TM 11 positions in hENT1 and CeENT1, respectively, had only minor effects on uridine transport activity. hENT1-L442I displayed a modestly lower apparent Km value than hENT1, and CeENT1-I492L and CeENT1 displayed similar Km values (Table II). In contrast, the less conservative substitution of these residues by Thr markedly reduced the apparent affinities of the transporters for uridine, i.e. hENT1-L442T displayed a Km value that was 10-fold higher than that of hENT1 (Fig. 5A and Table II), and CeENT1-I492T displayed a Km value that was 3-fold higher than that of CeENT1 (Fig. 5B and Table II).

The kinetic properties of the TM 1/11 double mutants of hENT1 and CeENT1 were consistent with those of the corre-
The $K_m$ and $V_{max}$ values were determined using GraphPad Prism version 4.0 software by nonlinear regression analysis. Representative plots for uridine transport by hENT1, hENT2-L442T, CeENT1, and CeENT1-I429T are presented in Fig. 5. Representative plots for adenosine transport by hENT1 and hENT1-L442I are presented in Fig. 6. ND, not determined.

### Table II

**Kinetic properties of uridine and adenosine transport for hENT1, CeENT1, and the respective TM1 and TM11 mutants**

| Permeant | Protein        | Apparent $K_m$ | Apparent $V_{max}$ | $V_{max}/K_m$ |
|----------|----------------|---------------|--------------------|---------------|
|          | $\mu$M         | pmol/mg/min   | pmol/mg/min/\muM   |               |
| Uridine  |                |               |                    |               |
| hENT1    | 36 ± 6         | 537 ± 18      | 15                 |               |
| M33I     | 30 ± 3         | 411 ± 8.7     | 14                 |               |
| M33A     | 58 ± 11        | 263 ± 13      | 4.6                |               |
| L442I    | 66 ± 6         | 715 ± 17      | 11                 |               |
| L442T    | 360 ± 50       | 847 ± 46      | 2                  |               |
| M33I/L442I | 28 ± 5     | 658 ± 23      | 24                 |               |
| M33A/L442I | 64 ± 10    | 424 ± 17      | 6.7                |               |
| CeENT1   | 100 ± 10       | 955 ± 35      | 9.6                |               |
| I489M    | 64 ± 13        | 978 ± 48      | 15                 |               |
| I49A     | 160 ± 13       | 809 ± 21      | 5.2                |               |
| I429L    | 86 ± 8         | 925 ± 24      | 11                 |               |
| I429T    | 340 ± 50       | 655 ± 48      | 1.9                |               |
| I49M/I429L | 140 ± 20   | 892 ± 50      | 6.6                |               |
| I49M/I429T | 960 ± 140    | 1680 ± 130    | 1.8                |               |
| Adenosine|                |               |                    |               |
| hENT1    | 19 ± 2         | 1280 ± 30     | 69                 |               |
| M33A     | 21 ± 9         | 216 ± 19      | 11                 |               |
| M33I     | 16 ± 1         | 1450 ± 20     | 89                 |               |
| L442I    | 180 ± 10       | 2260 ± 70     | 13                 |               |
| L442T    | >500           | ND            | ND                 |               |

**Fig. 5. Concentration dependence of uridine transport by hENT1, hENT1-L442T, CeENT1, and CeENT1-I429T.** Yeast cells containing pYPhENT1 (○) or pYPhENT1-L442I (▲) were incubated for 20 min with increasing concentrations of [3H]uridine. The Eadie-Hofstee plots are presented in the insets. The transport rates presented were derived from the difference between uptake observed in the absence and presence of 10 mM unlabeled thymidine at each uridine concentration. The $K_m$ and $V_{max}$ values were obtained by nonlinear regression analysis using GraphPad Prism version 4.0 software and are presented in Table II. Each point is presented as the mean ± S.E. ($n = 4$), and where the size of the point is larger than the S.E., it is not shown.

**Fig. 6. Concentration dependence of adenosine transport by hENT1 and hENT1-L442I.** Yeast cells containing pYPhENT1 (●) or pYPhENT1-L442I (▲) were incubated for 10 min with increasing concentrations of [3H]adenosine. The Eadie-Hofstee plots are presented in the inset. The transport rates presented were derived from the difference between uptake observed in the absence and presence of 10 mM unlabeled thymidine at each uridine concentration. The $K_m$ and $V_{max}$ values were obtained by nonlinear regression analysis using GraphPad Prism version 4.0 software and are presented in Table II. Each point is presented as the mean ± S.E. ($n = 4$), and where the size of the point is larger than the S.E., it is not shown.

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Yeast cells containing pYPhENT1 (●) or pYPhENT1-L442I (▲) were incubated for 10 min with increasing concentrations of [3H]adenosine. The Eadie-Hofstee plots are presented in the inset. The transport rates presented were derived from the difference between uptake observed in the absence and presence of 10 mM unlabeled thymidine at each uridine concentration. The $K_m$ and $V_{max}$ values were obtained by nonlinear regression analysis using GraphPad Prism version 4.0 software and are presented in Table II. Each point is presented as the mean ± S.E. ($n = 4$), and where the size of the point is larger than the S.E., it is not shown.

**Fig. 6. Concentration dependence of adenosine transport by hENT1 and hENT1-L442I.** Yeast cells containing pYPhENT1 (●) or pYPhENT1-L442I (▲) were incubated for 10 min with increasing concentrations of [3H]adenosine. The Eadie-Hofstee plots are presented in the inset. The transport rates presented were derived from the difference between uptake observed in the absence and presence of 10 mM unlabeled thymidine at each uridine concentration. The $K_m$ and $V_{max}$ values were obtained by nonlinear regression analysis using GraphPad Prism version 4.0 software and are presented in Table II. Each point is presented as the mean ± S.E. ($n = 4$), and where the size of the point is larger than the S.E., it is not shown.

**Kinetic Parameters of Adenosine Transport for hENT1 and Various Mutants—To determine the effects of the hENT1 TM 1 and 11 mutations on kinetic parameters of adenosine trans-
port, the concentration dependence of adenosine transport was determined (Table II). hENT1-M33A displayed an apparent V\text{max} value that was about 16% of that of wild type hENT1 whereas hENT1-M33I displayed apparent kinetic parameters similar to those of hENT1. hENT1-L442I displayed an apparent K\text{m} value that was 10-fold higher than that of hENT1 (Fig. 6). Adenosine transport by hENT1-L442T did not saturate at concentrations ≥500 μM, suggesting that the affinity of this mutant for adenosine was low, which was consistent with the relatively high K\text{m} value observed for uridine transport by this mutant (Table II).

**Dixon Plot Analysis of Dipyridamole Inhibition of hENT1 and CeENT1 Mutants**—To investigate the effects of mutating the TM 1 and 11 residues on the apparent competitive nature of dipyridamole inhibition of hENT1 and CeENT1, Dixon plot experiments were performed for uridine transport by hENT1-M33I and CeENT1-I429L (Fig. 7, A and C). These mutants were chosen because they displayed decreased dipyridamole sensitivities without marked effects on uridine transport efficiencies (Tables I and II). Dipyridamole inhibited hENT1-M33I and CeENT1-I429L competitively with apparent K\text{i} values that were similar to the corresponding IC\text{50} values determined in the concentration-effect relationship experiments (Table I), suggesting that the mechanism of inhibition was unaffected by these mutations. When the double mutants (hENT1-M33I/L442I and CeENT1-I49M/I429L) were also subjected to Dixon plot analysis (Fig. 7, B and D), they were inhibited competitively, with “recovered” dipyridamole sensitivity compared with the single mutants assessed in Fig. 7 (panels A and B) (Table I) and also exhibited uridine transport efficiencies that were similar to those of the corresponding wild type transporters (Table II).

**DISCUSSION**

In this study, we found that, despite their limited amino acid sequence identity (26%), hENT1 and CeENT1 both bound dipyridamole with high affinities and competitively with respect to the physiological permeants uridine and adenosine. This common mechanism of inhibition suggested that the residues involved in dipyridamole binding by hENT1 and CeENT1 may be conserved and was consistent with the results of previous studies that had suggested that the dipyridamole-binding site overlaps with the exofacial permeant binding site of ENT transporters (16, 18, 39). Random mutagenesis and screening for dipyridamole resistance by functional complementation in yeast identified Met33 in TM 1 of hENT1 and Ile429 in TM 11 of CeENT1 as key residues. Multiple sequence alignments of the high dipyridamole sensitivity transporters (hENT1, CeENT1, and CeENT2) versus the low dipyridamole sensitivity transporters (rENT1, rENT2, and hENT2) revealed that the TM 1 and 11 positions were always occupied by residues with large hydrophobic side chains, namely either Met or Ile in TM 1 and either Leu or Ile in TM 11 (Fig. 3). Interestingly, although in the mammalian transporters a Met at position 33 is associated with high dipyridamole sensitivity (12), CeENT1 and CeENT2 contain an Ile at the corresponding position. Conversely, although the mammalian transporters contain a Leu at the TM 11 position, regardless of their dipyridamole sensitivities, the corresponding residue in CeENT1 and CeENT2 is Ile. The multiple sequence alignments therefore suggested that binding of dipyridamole is complex and involves contributions from multiple residues from different parts of ENT proteins. The possession of a common, complex dipyridamole-binding site in...
The mutagenesis studies also suggested that the TM 1 residue conformational changes that move the helices farther apart bring the two helices closer together whereas changing the TM 11 residue to Ile induced conformational changes that studies suggested that changing the TM 1 residue to Met and the conformations of the TM 1 and 11 helices. The mutagenesis I49M/I429T (F).

Because TMs 1 and 11 contain highly conserved glycine residues that may be involved in helix-helix contacts (Fig. 3) (7), it is likely that mutation of the residues in question (Met33 and Leu442 of hENT1 and Ile49 and Ile429 of CeENT1) affected the conformations of the TM 1 and 11 helices. The mutagenesis studies suggested that changing the TM 1 residue to Met and the TM 11 residue to Ile induced conformational changes that bring the two helices closer together whereas changing the TM 1 residue to Ala, and the TM 11 residue to Leu or Thr induced conformational changes that move the helices farther apart. The mutagenesis studies also suggested that the TM 1 residue is a primary site of interaction of dipyridamole with wild type hENT1, whereas the TM 11 residue is too far away to interact with dipyridamole (Fig. 8A). The model predicts that the L442I mutation would have no effect on dipyridamole binding to hENT1 (Fig. 8B), and the L442I mutation would bring TMs 1 and 11 closer together such that both residues can simultaneously contribute to dipyridamole binding (Fig. 8C). That Met was favored over Ile at the TM 1 position is likely due to its relatively high degree of conformational flexibility and its highly polarizable sulfur atom (41). Therefore, in hENT1-M33I and hENT1-M33I/L442I, the Ile residue in the TM 1 position likely interacted with dipyridamole, although the strength of the interaction was reduced. That the M33I mutation did not affect the conformation of TM 1 is supported by the observation that this mutant was functionally similar to wild type (Table II). In M33A/L442I, TM 1 would be farther away from TM 11, and the primary site of dipyridamole interaction with hENT1 would become TM 11 (Fig. 8D).

In CeENT1, the observation that TMs 1 and 11 were unable to simultaneously contribute to dipyridamole binding suggested that these two helices are farther apart in CeENT1 than in hENT1 and hENT2 and that TM 11, not TM 1, is a primary site of dipyridamole interaction in the wild type protein (Fig. 8E). The I49M and I49A mutations would be predicted to have no effect on the dipyridamole sensitivity of CeENT1 (Fig. 8F). Mutation of the TM 11 residue to Leu or Thr appeared to move this helix farther away from TM 1 because dipyridamole bound to the resulting mutants with markedly reduced affinities (Fig. 8G). The dipyridamole sensitivity of the CeENT1 TM 11 mutants was “rescued” by the I49M mutation (Table I), which evidently generated a surrogate point of inhibitor interaction (Fig. 8H).

Analysis of uridine and adenosine transport by the TM 1 and 11 mutants of hENT1 and CeENT1 revealed that, for both proteins, an Ala residue at the TM 1 position resulted in a greatly reduced Km value and reduced Vmax values, yielding reduced Vmax/Km ratios (a measure of transporter efficiency) (Table II). A Thr at the TM 11 position resulted in greatly increased Km values for uridine and adenosine transport by both proteins (Table II and Fig. 5). These results supported a common role for the TM 1 and 11 positions in uridine transport by hENT1 and CeENT1.

hENT1-L442I displayed a 10-fold higher apparent Km value compared with that of hENT1 (Fig. 6). That the L442I mutation impaired adenosine transport and not uridine transport suggested that the shape of the permeant binding pocket was specifically altered to a conformation less favorable for adenosine transport and that this residue may line the permeant translocation pathway. Although a Leu residue was favored for adenosine transport, an Ile residue was favored for dipyridamole binding, suggesting different structural requirements at residue 442 for interaction with adenosine and dipyridamole. It is possible that the relatively “open” conformation of wild type hENT1 depicted in Fig. 8A is favored for adenosine transport, whereas the relatively “closed” conformation of hENT1-L442I depicted in Fig. 8C is favored for dipyridamole binding.

The Dixon plot analysis of several of the mutants (Fig. 7) revealed that the competitive nature of dipyridamole inhibition was unaffected by the mutations, suggesting that the TM 1 and 11 residues identified in this study form part of, or lie close to, the dipyridamole-binding site that overlaps with the permeant binding site. In the mechanistic model proposed in Fig. 8, it is likely that, although dipyridamole may be bound to either TM 1 or 11 via hydrophobic contacts, the two helices may be sufficiently close together for dipyridamole to directly block access to the permeant binding site.

To further examine the possibility that the TM 1 and 11 members of the ENT family is supported by the demonstration in this study that not only hENT1 and CeENT1 but also hENT2 and rENT1 exhibited dipyridamole sensitivity, albeit to very different extents. The observed differences were consistent with the findings of previous studies (9, 10, 15, 38, 40).

The results of the mutagenesis and dipyridamole concentration-effect relationship studies (Table I and Fig. 4) suggested that residues in both TM 1 and 11 contributed to dipyridamole binding by hENT1, CeENT1, and hENT2. We propose here a mechanistic model to explain the results of the mutagenesis studies (Fig. 8). This model is based on experimental evidence that TMs 1 and 11 are transmembrane helices (8) and the hypothesis that TMs 1 and 11 are in close proximity to each other, a proposal that will be tested in future cross-linking studies. Although only the contributions of the TM 1 and 11 residues are considered in the model, it is likely that dipyridamole interactions are complex, involving contributions from several residues on different parts of the protein.

The Dixon plot analysis of several of the mutants (Fig. 7) revealed that the competitive nature of dipyridamole inhibition was unaffected by the mutations, suggesting that the TM 1 and 11 residues identified in this study form part of, or lie close to, the dipyridamole-binding site that overlaps with the permeant binding site. In the mechanistic model proposed in Fig. 8, it is likely that, although dipyridamole may be bound to either TM 1 or 11 via hydrophobic contacts, the two helices may be sufficiently close together for dipyridamole to directly block access to the permeant binding site.

To further examine the possibility that the TM 1 and 11
residues identified in this study might line the permeant translocation pathway, we have constructed three-dimensional models of TMs 1 and 11 of hENT1 (Fig. 9). Analysis of the patterns of hydrophobicity and residue conservation in these two helices in alignments of ENT family sequences (7) allows for speculation on the function of these residues and provides a guide for future experimentation. Although the position corresponding to Leu442 in hENT1 and Ile429 in CeENT1 is always occupied by a hydrophobic residue, it lies on a face of the putative TM 11 helix that is sometimes occupied by hydrophilic residues (Fig. 9B). In contrast, the opposite face of the TM 11 helix is predominantly hydrophobic and is occupied by poorly conserved residues (Fig. 9A). It is therefore likely that this face of the helix is in contact with the core of the lipid bilayer, whereas the face containing Leu442 and Ile429 potentially faces the permeant translocation pathway of the transporter. The effects of mutating Leu442 on permeant selectivity support its proposed location in the permeant translocation pathway (Fig. 6).

TM 1 is less amphipathic than TM 11, with positions occupied by hydrophilic residues on all sides, including the one in which Met33 and Ile49 are situated (Fig. 9, C and D). Although many hydrophobic residues are present in TM 1, it differs from TM 11 in that several of these are relatively well conserved, examples being hENT1 positions 22 and 24, which in the ENT family are exclusively or predominantly occupied by Gly residues (Fig. 3) (7). Such conserved positions are likely to be involved in helix-helix packing, and their presence in TM 1 suggests that TM 1 is located internally within a bundle of helices rather than being exposed to the lipid bilayer. Given the importance of hENT1 residue Met33 for dipyridamole binding, we hypothesize that the dipyridamole-binding site involves close packing of TMs 1 and 11, thus bringing Met33 close to Leu442. This hypothesis will be tested in future site-specific cross-linking experiments.

In conclusion, we have identified two residues, one each in TMs 1 and 11 of hENT1 and CeENT1, that are functionally important, contributing to dipyridamole binding and permeant transport. The role of these residues in dipyridamole interactions with ENT family proteins was further confirmed in mutational analysis of hENT2. The data are consistent with the TM 1 and 11 residues forming part of the dipyridamole binding site and lining the permeant translocation channel. The direct validation of these conclusions will require additional studies, such as a substituted cysteine accessibility analysis or acquisition of detailed structural data on the different conformational states of the protein.

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Identification and Mutational Analysis of Amino Acid Residues Involved in Dipyridamole Interactions with Human and Caenorhabditis elegans Equilibrative Nucleoside Transporters

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