Accessibility of substituted cysteines in TM2 and TM10 transmembrane segments in the Plasmodium falciparum equilibrative nucleoside transporter PfENT1

Infection with Plasmodium species parasites causes malaria. Plasmodium parasites are purine auxotrophic. They import purines via an equilibrative nucleoside transporter (ENT). In P. falciparum, the most virulent species, the equilibrative nucleoside transporter 1 (PfENT1) represents the primary purine uptake pathway. This transporter is a potential target for the development of antimalarial drugs. In the absence of a high-resolution structure for either PfENT1 or a homologous ENT, we used the substituted cysteine accessibility method (SCAM) to investigate the membrane-spanning domain structure of PfENT1 to identify potential inhibitor-binding sites. We previously used SCAM to identify water-accessible residues that line the permeation pathway in transmembrane segment 11 (TM11). TM2 and TM10 lie adjacent to TM11 in an ab initio model of a homologous Leishmania donovani nucleoside transporter. To identify TM2 and TM10 residues in PfENT1 that are at least transiently on the water-accessible transporter surface, we assayed the reactivity of single cysteine-substitution mutants with three methanethiosulfonate (MTS) derivatives. Cysteines substituted for 12 of 14 TM2 segment residues reacted with MTS-ethyl-ammonium-biotin (MTSEA-biotin). At eight positions, MTSEA-biotin inhibited transport, and at four positions substrate transport was potentiated. On an α helical wheel projection of TM2, the four positions where potentiation occurred were located in a cluster on one side of the helix. In contrast, although MTSEA-biotin inhibited 9 of 10 TM10 cysteine-substitution mutants, the reactive residues did not form a pattern consistent with either an α helix or β sheet. These results may help identify the binding site(s) of PfENT1 inhibitors.

In many developing nations, malaria is a major public health problem killing about 500,000 people annually. Infection with obligate intracellular, eukaryotic parasites of the genus Plasmodium causes malaria. Five Plasmodium species, falciparum, knowlesi, malariae, ovale, and vivax, infect humans. The highest mortality is associated with Plasmodium falciparum infection. P. falciparum parasites have developed resistance to drugs that have been used to treat malaria (1, 2). Chloroquine was the mainstay of malaria treatment for over 50 years but its use is now limited by widespread resistance. Artemisinin-based combination therapies (ACT) were designated as the first line treatment in 2006, but resistance against ACT has been documented in South-East Asia since 2011 and more recently in parasites from Africa (3–6). The spread of ACT-resistant malaria strains highlights the importance of developing new anti-malarials that target novel metabolic pathways and proteins in the parasite.

One novel target is the P. falciparum equilibrative nucleoside transporter type 1 (PfENT1) (7, 8). Plasmodium parasites are purine auxotrophic, but can synthesize pyrimidines by de novo synthesis (9–12). PfENT1 is the primary purine transporter for the import of purine nucleobases and nucleosides, necessary for DNA and RNA synthesis, replication, and other metabolic processes (11, 13–15). PfENT1-knockout parasites (pfent1Δ) are not viable in culture with media purine concentrations similar to those found in human blood, below 10 μM (8, 16–18). Recent experiments using random transposon integration inferred that PfENT1 is an essential gene for blood stage parasites (19). Previously, we used a yeast-based high throughput screen to identify small molecule inhibitors of PfENT1 that kill malaria parasites in culture (8). The inhibitors represent six distinct chemical scaffolds. In the present work, we sought to understand more about the structure of the transmembrane domain...
of PfENT1 with the long-term goal of identifying the residues that contribute to the binding site for the inhibitors.

The substituted cysteine accessibility method (SCAM) is an experimental approach to study the structure of membrane transporters, channels, and receptors (20–22). SCAM experiments assess the reactivity of sulfhydryl-reactive reagents with cysteines (Cys) substituted systematically, one at a time, in transmembrane segments. We used methanethiosulfonate (MTS) reagents to assess reactivity. MTS reagents react by an SN2 nucleophilic attack on the Cys sulfhydryl. Their reaction rate is a billion times faster with the ionized thiolate anion \( \text{S}^2/- \) than with the un-ionized thiol (SH) (23, 24). Because only sulfhydryls that are, at least transiently, on the water-accessible protein surface are likely to ionize, reaction with MTS reagents is a proxy for water accessibility of the corresponding wildtype (WT) protein residue (22). Thus, SCAM allows the identification of residues within transmembrane segments that are, at least transiently, on the water-accessible protein surface. We previously used SCAM to investigate the structure of the PfENT1-transmembrane domain (25).

PfENT1 is a member of the SLC29 equilibrative nucleoside transporter gene family (26). A glycosylation site insertion mutagenesis study supported the predicted 11-transmembrane segment transmembrane topology with a cytoplasmic N terminus, a large cytoplasmic loop between transmembrane segments 6 (TM6) and TM7, and an extracellular C terminus (Fig. 1) (27). No high resolution structures are available for PfENT1 or for a homologous ENT. An ab initio model has been constructed for the homologous *Leishmania donovani* LdNT1.1 transporter and validated using disulfide cross-linking and site-directed mutagenesis (28–30). We used this model to choose TM segments for this study. In a previous SCAM study, we identified residues in TM11 that line the purine permeation pathway (25). In the ab initio model TM2 and TM10 are adjacent to TM11. In this study, we used SCAM to identify the water-accessible residues within the PfENT1 TM2 and TM10 segments. Our results indicate that Cys substituted for some TM2 residues reacted with the MTS reagents. We infer that they are water accessible and may line the permeation pathway. Based on the pattern of MTS-reactive residues, much of TM2 appears to form an \( \alpha \) helix. Cys substituted for several TM10 segment residues reacted with MTSEA-biotin. The pattern formed by the TM10 reactive residues was not consistent with either an \( \alpha \) helix or \( \beta \) sheet.

### Results

**MTS reagent effects on WT PfENT1**

PfENT1 has 11 endogenous Cys residues. Therefore, we first determined whether MTS reagents had an effect on WT PfENT1. We assayed the effect of a 5-min application of increasing concentrations of the three MTS reagents on \([3H]\)adenosine (\([3H]\)Ado) uptake into yeast expressing WT PfENT1. Reagent solubility issues limited the maximum concentration of MTSEA-biotin to 8 mM. We chose to limit the application duration to 5 min to avoid issues related to changing MTS reagent concentration during an experiment due to spontaneous hydrolysis of the MTS reagents. In particular, for MTSET\(^-\) the hydrolysis half-time is 11.2 min in aqueous solution at pH 7.0 (24). The hydrolysis half-time for MTSES\(^-\) is much longer, 370 min, whereas the hydrolysis half-time for MTSEA-biotin has not been determined but is likely to be longer than the MTSET\(^-\) half-time (24). Concentrations of MTS reagents below 1 mM had no effect on \([3H]\)Ado uptake into yeast expressing WT PfENT1 (Fig. 2). However, MTS reagent concentrations above 1 mM caused up to ~25% inhibition of \([3H]\)Ado uptake into yeast expressing WT PfENT1 (Fig. 2, Table 3). We sought to quantify reactivity based on the MTS reagent concentra-
Water-accessible residues in PfENT1 TM2 and TM10 segments

Figure 2. Effects of increasing concentrations of MTS reagents on the function of WT PfENT1. A–C, concentration-dependent effects of application of (A) MTSEA-biotin, (B) MTSET, and (C) MTSES on \([^{3}H]\)Ado uptake into yeast expressing WT PfENT1. Data were normalized to the lowest concentration of the MTS reagent. Each point is the average of three or more independent experiments. Average and S.D. are shown, for some points error bars are smaller than the points and are not shown. Please note that the same WT data are shown in the following figures to provide a point of comparison for the effects of the MTS reagents on the individual TM2 and TM10 Cys mutants. Panel A appears in Figs. 4 and 7, panel B appears in Figs. S1 and S3, and panel C appears in Figs. S2 and S4.

Characterization of Cys-substitution mutants

In the ab initio model of the LdNT1.1, transporter residues from TM2 and TM10 flank TM11 and appear to line the putative purine transport pathway (28–30). The N-terminal ends of both TM2 and TM10 of PfENT1 are predicted to be extracellular and the C-terminal ends are predicted to be cytoplasmic (Fig. 1). We mutated residues from the predicted extracellular end of these two membrane-spanning segments toward the cytoplasmic end. In TM2 we mutated 14 consecutive residues, one at a time, to Cys from Phe-63 through Val-79, except residue 75, which is an endogenous Cys and was left unchanged. In TM10 we mutated 10 consecutive residues to Cys, one at a time, from Ala-348 through Phe-357.

We expressed each Cys-mutant in the purine auxotrophic yeast. The purine auxotrophic yeast can grow in media containing adenine as the sole purine source because adenine can enter the yeast through the endogenous FCY2 transporter (31). In the absence of functional PfENT1, the purine auxotrophic yeast cannot grow in media that contains adenosine as the sole purine source (data not shown), because they lack an endogenous adenosine transporter (8). In contrast, PfENT1-expressing yeast can grow in media with either adenine or adenosine as the sole purine source because PfENT1 can transport adenosine into the yeast (8). Yeast expressing each of the TM2 and TM10 Cys mutants were able to grow in media containing 1 mM adenosine as the sole purine source (data not shown). This implies that all of the Cys mutants formed functional adenosine transporters that imported sufficient adenosine to meet the purine requirements for yeast proliferation.

To determine whether the Cys substitutions affected the transporter’s purine affinity, we measured the affinity of the nucleobase, hypoxanthine, and nucleoside, inosine, by uptake competition experiments. In uptake competition experiments, the purines hypoxanthine or inosine could compete with a radiolabeled purine nucleoside at either the Cys-mutant PfENT1 or at a downstream metabolic enzyme (32). To avoid this potential complication in the interpretation of the experiments, because PfENT1 transports uridine (Urd) (8), we used \([^{3}H]\)uridine (\([^{3}H]\)Urd).

For many of the TM2 and TM10 mutants the IC_{50} values for hypoxanthine and inosine inhibition of \([^{3}H]\)Urd were within 1 order of magnitude of the IC_{50} value for WT PfENT1 (Fig. 3, Tables 1 and 2). For the nucleobase hypoxanthine, only the IC_{50} value for V74C was about 12-fold higher than the hypoxanthine IC_{50} value for WT. In contrast, for inosine the IC_{50} values were more than 10-fold higher than WT for F63C, N66C, Q69C, L73C, V74C, and F352C. At these positions, the Cys substitutions reduced the affinity for inosine by greater than 10-fold.

Reactivity of TM2 Cys-substitution mutants with MTS reagents

We tested the reactivity of the MTS reagents with individual Cys-substitution mutants using procedures similar to those we used for WT. We determined the effect of a 5-min application of increasing concentrations of the three MTS reagents on
Water-accessible residues in PfENT1 TM2 and TM10 segments

Figure 3. Purine nucleobases and nucleosides inhibit [3H]uridine uptake into yeast expressing PfENT1 TM2 (A and B) and TM10 (C and D) Cys-substitution mutants. Increasing concentrations of hypoxanthine (A and C) and inosine (B and D) compete to inhibit uptake of [3H]uridine into yeast expressing the indicated Cys-substitution mutant. Illustrative experiments are shown for each mutant. Solid and dashed lines are variable slope concentration-response fits to the data for each mutant or WT.

Table 1
Average IC50 values and ratio of mutant to WT IC50 values for hypoxanthine and inosine competition for [3H]uridine uptake by TM2 Cys-substitution mutants

|           | Hypoxanthine IC50 M | Inosine IC50 M | Mut/WT hypoxanthine | Mut/WT inosine |
|-----------|---------------------|----------------|---------------------|---------------|
| WT        | 1.36E-04            | 1.72E-05       | 1.00                | 1.00          |
| F63C      | 6.83E-04            | 5.54E-03       | 3.02                | 322.04        |
| K64C      | 3.08E-04            | 3.20E-05       | 1.14                | 1.86          |
| Y65C      | 4.23E-04            | 1.34E-04       | 3.11                | 7.80          |
| N66C      | 6.70E-05            | ND             | 4.94                | ND            |
| T67C      | 3.06E-05            | 5.04E-05       | 0.22                | 2.93          |
| F68C      | 4.84E-05            | 1.54E-04       | 0.36                | 8.95          |
| Q69C      | 8.64E-06            | 2.39E-04       | 3.64                | 13.90         |
| I70C      | 2.13E-04            | 3.44E-05       | 1.57                | 2.00          |
| T71C      | 5.06E-05            | 3.72E-05       | 0.37                | 2.17          |
| G72C      | 1.20E-03            | 2.83E-05       | 8.84                | 1.65          |
| L73C      | 2.79E-04            | 1.82E-04       | 2.05                | 10.56         |
| V74C      | 1.60E-03            | 2.97E-03       | 11.76               | 172.86        |
| S76C      | 2.15E-05            | 1.25E-04       | 0.16                | 7.30          |
| S77C      | 3.77E-05            | 2.94E-06       | 0.28                | 0.17          |
| I78C      | 6.09E-05            | 2.21E-05       | 0.45                | 1.29          |
| V79C      | 4.93E-05            | 3.57E-05       | 0.36                | 2.08          |

Table 2
Average IC50 values and ratio of mutant to WT IC50 values for hypoxanthine and inosine competition for [3H]uridine uptake by TM10 Cys-substitution mutants

|           | Hypoxanthine IC50 M | Inosine IC50 M | Mut/WT hypoxanthine | Mut/WT inosine |
|-----------|---------------------|----------------|---------------------|---------------|
| WT        | 1.36E-04            | 1.72E-05       | 1.00                | 1.00          |
| A348C     | 1.63E-04            | 3.93E-05       | 1.20                | 2.29          |
| M349C     | 1.77E-05            | 1.71E-04       | 0.13                | 9.93          |
| L350C     | 2.73E-05            | 2.34E-05       | 0.12                | 1.36          |
| A351C     | 3.83E-04            | 3.25E-06       | 2.81                | 0.19          |
| F352C     | 5.09E-03            | 3.14E-04       | 3.78                | 18.25         |
| T353C     | 3.70E-04            | 1.07E-04       | 2.71                | 6.23          |
| N354C     | 1.87E-04            | 7.60E-06       | 1.37                | 0.44          |
| G355C     | 3.41E-05            | 2.49E-05       | 0.25                | 1.45          |
| W356C     | 8.03E-05            | 2.15E-05       | 0.35                | 1.25          |
| F357C     | 7.67E-05            | 3.47E-05       | 0.56                | 2.02          |

* ND, not determined.

[3H]Ado uptake into yeast expressing the individual Cys-substitution mutants. For two of the 16 TM2 Cys-substitution mutants, V74C and S77C, none of the three MTS reagents caused effects that were significantly different from those observed with WT PfENT1 (Fig. 4, Figs. S1 and S2). In contrast, for the other 14 TM2 Cys mutants, application of at least one of the MTS reagents caused a significant concentration-dependent effect on [3H]Ado uptake. MTSEA-biotin application caused inhibition of subsequent [3H]Ado uptake with 10 of the mutants (Fig. 4, Table 3). At three of these mutants, N66C, I70C, and G72C, MTSET+ application also inhibited subsequent [3H]Ado uptake (Fig. 4, Fig. S1). For F68C, only MTSET+ had an effect and it caused potentiation of subsequent [3H]Ado uptake (Fig. 4, Figs. S1 and S2). MTSES− only inhibited subsequent [3H]Ado uptake with the G72C mutant (Fig. 4, Fig. S2). For the G72C mutant, the Hill slope of the concentration-effect relationship was shallow, about −0.6, with all three MTS reagents suggesting that inhibition involved a multistep process (Fig. 4, Figs. S1 and S2, Table 3).

The maximal effect at 8 mM MTSEA-biotin, the highest concentration tested, ranged from 90% inhibition for I70C to about 30 to 40% inhibition for F63C and Y65C (Fig. 4, Table 3). For all of the mutants more cytoplasmic than L73C (Fig. 4), the reaction did not appear to have gone to completion during the 5-min application of 8 mM MTSEA-biotin. Consistent with this observation, the MTSEA-biotin XC50 concentration was <400 μM for all mutants between F63C and I71C (Fig. 4, Table 3). In contrast, the MTSEA-biotin XC50 concentration was >1 mM for all mutants between L73C and V79C (Fig. 4, Table 3).

Surprisingly, with four of the Cys mutants, K64C, T67C, F68C, and T71C, the MTS reagent application potentiated subsequent [3H]Ado uptake (Fig. 5, Table 3). The extent of potentiation of [3H]Ado uptake ranged from about a 2-fold increase...
for K64C (Fig. 5A) to about 20-fold for T71C (Fig. 5D). Except for the K64C mutant, MTSET caused the largest amount of potentiation of [3H]Ado uptake. For the F68C mutant, only MTSEA-biotin affected subsequent [3H]Ado uptake (Fig. 5C). It is notable that at positions K64C, T67C, and T71C all three MTS reagents caused potentiation. In contrast at the other TM2 reactive residues, only MTSEA-biotin caused a functional effect. We do not think that the increased [3H]Ado uptake was

Table 3

| Cys mutant | Avg. MTSEA-biotin X_{50} (normalized) | S.D. MTSEA-biotin X_{50} (normalized) | Number of experiments | Avg. Hill slope MTSEA-biotin (normalized) | S.D. Hill slope MTSEA-biotin (normalized) | Avg. % of uptake at 8 mM MTSEA-biotin | S.D. of % of uptake at 8 mM MTSEA-biotin |
|-------------|--------------------------------------|--------------------------------------|----------------------|------------------------------------------|------------------------------------------|---------------------------------------|------------------------------------------|
| WT          | >8E-03                               |                                      | 5                    | -1.86                                    | 0.29                                     | 78                                    | 6                                         |
| F63C        | 7.25E-05                             | 3.18E-05                             | 4                    | -1.86                                    | 0.29                                     | 65                                    | 6                                         |
| K64C        | 7.06E-07                             | 1.33E-07                             | 3                    | 3.36                                     | 0.69                                     | 193                                   | 80                                        |
| Y65C        | 6.86E-05                             | 1.35E-05                             | 4                    | -1.88                                    | 0.42                                     | 62                                    | 6                                         |
| N66C        | 1.40E-05                             | 1.59E-06                             | 4                    | -1.08                                    | 0.24                                     | 34                                    | 3                                         |
| T67C        | 2.88E-05                             | 1.95E-06                             | 4                    | 1.49                                     | 0.07                                     | 737                                   | 59                                        |
| F68C        | NE                                   |                                      | 3                    | -1.01                                    | 0.28                                     | 34                                    | 5                                         |
| Q69C        | 3.64E-04                             | 1.14E-04                             | 3                    | -1.71                                    | 0.10                                     | 10                                    | 1                                         |
| I70C        | 1.93E-04                             | 1.09E-05                             | 4                    | -1.23                                    | 0.05                                     | 355                                   | 42                                        |
| T71C        | 4.63E-05                             | 2.52E-06                             | 3                    | -0.57                                    | 0.03                                     | 54                                    | 11                                        |
| G72C        | 3.06E-05                             | 2.19E-05                             | 4                    | -1.17                                    | 0.40                                     | 72                                    | 6                                         |
| L73C        | 5.26E-03                             | 1.34E-03                             | 2                    | -0.99                                    | 0.06                                     | 33                                    | 0                                         |
| V74C        | NE                                   |                                      | 4                    | -0.99                                    | 0.06                                     | 33                                    | 0                                         |
| S76C        | 2.81E-03                             | 4.35E-04                             | 3                    | -1.23                                    | 0.03                                     | 66                                    | 8                                         |
| S77C        | NE                                   |                                      | 4                    | -1.23                                    | 0.03                                     | 66                                    | 8                                         |
| I78C        | 1.06E-03                             | 1.14E-04                             | 2                    | -1.07                                    | 0.07                                     | 40                                    | 2                                         |
| V79C        | 4.90E-03                             | 6.20E-04                             | 2                    | -1.07                                    | 0.07                                     | 40                                    | 2                                         |

a NE, no effect.

Figure 5. Application of MTS reagents potentiated [3H]adenosine uptake by yeast expressing four of the TM2 Cys-substitution mutants by the positively charged MTSET, negatively charged MTSES, and the neutral MTSEA-biotin, into yeast expressing the various TM2 mutants. Each graph is representative of the mean of three experiments carried out on separate days. Error bars represent S.E. Only mutants that show [3H]adenosine uptake potentiation with the different MTS reagents are shown.

Figure 4. Concentration-dependent effects of MTSEA-biotin application on [3H]Ado uptake into yeast expressing WT and the TM2 Cys mutants. Data from each independent experiment was normalized to the lowest concentration of MTSEA-biotin used. Each point is the average of independent experiments. Average and S.D. are shown, for some points error bars are smaller than the points and are not shown. Solid line is a four-parameter variable slope fit to the data by GraphPad Prism 7. The data for WT and the F68C, V74C, and S77C mutants could not be fit unambiguously. X_{50} values, number of independent replicates, Hill slope, and uptake at 8 mM MTSEA-biotin relative to uptake with the lowest MTSEA-biotin concentration are reported in Table 3. Please note that the WT data in Fig. 2A is also shown in Figs. 4 and 7 to provide a point of comparison for the effects of MTSEA-biotin on the individual TM2 and TM10 Cys mutants, respectively.

Water-accessible residues in PfENT1 TM2 and TM10 segments

J. Biol. Chem. (2019) 294(6) 1924 –1935

1929
due to an increase in the number of PfENT1 transporters during the short period of MTS reagent application, however, we did not explicitly test this. More likely, either the affinity for Ado increased or the transporter turnover rate increased. We tested whether MTS modification of these Cys mutants altered the Ado affinity. To determine Ado affinity, we measured the inhibition of \[3H\]Urd uptake into yeast expressing the four Cys mutants by increasing concentrations of cold adenosine (Fig. 6). A 5-min application of 3 mM MTS reagent did not alter the \(IC_{50}\) value for adenosine inhibition of \[3H\]Urd uptake (Fig. 6). This implies that MTS reagent modification of these four Cys mutants most likely increased the transporter turnover rate.

**Reactivity of TM10 Cys-substitution mutants with MTS reagents**

Similar experiments, as for the TM2 mutants, were carried out for the TM10 mutants (Fig. 7, Table 4). For six of the 10 TM10 Cys-substitution mutants, M349C, L350C, A351C, N354C, G355C, and W356C, the MTSEA-biotin reaction went to completion in the concentration–time domain of the experiment as indicated by the extent of reaction plateauing at the highest three concentrations used (Fig. 7). For three of the TM10 mutants, A348C, F352C, and F357C, the MTSEA-biotin reaction did not appear to go to completion in the 5-min period of reagent application (Fig. 7). For A348C and F352C, the calculated \(XC_{50}\) was <1 mM, but for F357C it was >1 mM (Table 4). Finally, the effect of the reagents on T353C appear similar to WT (Fig. 7, Table 4). Application of MTSET\(^+\) or MTSES\(^-\) did not affect subsequent \[3H\]Ado uptake for any of the TM10 mutants except for G355C, where 3 mM MTSET\(^+\) caused about 50% inhibition (Figs. S3 and S4).

**Discussion**

An underlying assumption of SCAM studies is that the Cys substitutions do not significantly alter the native protein structure (22, 24). All 24 of the Cys mutants supported growth of purine auxotrophic yeast on adenosine as the sole purine source. Because these purine auxotrophic yeast lack an endogenous adenosine transporter, their ability to grow with adenosine as the sole purine source provides strong evidence that all of the Cys mutants retain the capacity to transport adenosine. This suggests that the overall structure of the mutants is similar to WT. However, for six mutants, F63C, N66C, Q69C, L73C, V74C, and F352C, the Cys mutation did reduce the inosine affinity by more than an order of magnitude (Tables 1 and 2). Curiously, these five TM2 mutants lie on one side of an α-helical wheel projection of TM2 (Fig. 8B). At four of these positions, except for V74C, the Cys mutation did not have as large an effect on hypoxanthine affinity. Because hypoxanthine is the nucleobase of inosine, this may suggest that the inosine ribose sugar might interact with this region of TM2. Two other residues lie on the same side of the putative TM2 helix, I70C, where MTSEA-biotin caused the greatest inhibition of \[3H\]Ado uptake, and S77C, two helical turns more cytoplasmic where MTS reagents had no functional effect (Tables 1 and 3).

The residues in TM2 are poorly conserved among equilibrative nucleoside transporter homologues from different species (Fig. S5). In contrast, Gly-355 in TM10 is absolutely conserved.

**Figure 6.** MTS treatment does not affect adenosine affinity in the four TM2 mutants where MTS application caused potentiation of subsequent PfENT1 function. Adenosine affinity was calculated by measuring the effect of increasing concentrations of adenosine on the inhibition of \[3H\]Urd uptake into yeast expressing the K64C, T67C, F68C, and T71C mutants. Each graph represents the mean of three experiments carried out on separate days. Error bars represent S.E.
Figure 7. Effect of MTSEA-biotin application on [3H]Ado uptake into yeast expressing WT and the TM10 Cys-substitution mutants. Data from each independent experiment were normalized to the lowest concentration of MTSEA-biotin. Each point is the average of independent experiments. Average and S.D. are shown, for some points error bars are smaller than the points and are not shown. Solid line is a four-parameter variable slope fit to the data by GraphPad Prism 7. The data for WT and the T353C mutant could not be fit unambiguously. $X_{50}$ values, number of independent replicates, Hill slope, and uptake at 8 mM MTSEA-biotin relative to uptake with the lowest MTSEA-biotin concentration is reported in Table 4. Please note that the WT data in Fig. 2A is also shown in Figs. 4 and 7 to provide a point of comparison for the effects of MTSEA-biotin on the individual TM2 and TM10 Cys mutants.
from human to *Arabidopsis* to *Plasmodium* and the two residues on either side of Gly-355 are highly conserved. Thr-353 is either a threonine or serine, and Asn-354 is absolutely conserved except in a *Culex* ENT homologue where there is a serine in the aligned position (Fig. S6). Thus, despite the conservation of these residues, the Cys substitutions were tolerated.

A goal of SCAM studies is to determine the water-surface accessibility of the corresponding WT residues. In most SCAM studies, covalent reaction between an MTS reagent and a substituted Cys residue is detected by a change in the protein’s function. In the present study, reaction was detected by its effect on the rate of radioactive substrate uptake into yeast expressing the Cys mutant. The inference that MTS reactive Cys residues are on the water-accessible protein surface is based on: 1) MTS reagents react with the ionized thiolate (–SH) form of Cys >10⁹ times faster than with the un-ionized thiol (–SH) (22–24), 2) in free solution, the Cys thiol pKₐ is ~8.5, although in a protein, the thiol pKₐ can depend on the local electrostatic environment, and 3) the MTS reagents used in these experiments have very limited membrane permeability (22). Cys thiols in a membrane-spanning segment will only ionize to any significant extent if they are, at least transiently, on the water-accessible surface of the protein, which could include being in the substrate-translocation pathway through the interior of a membrane-transport protein or ion channel or might be through a crevice extending from the extracellular solution into the membrane-spanning domain. It is important to recognize that as a membrane-transport protein goes through its conformational changes, residues may move from the water-accessible surface to a buried or lipid-facing position depending on the conformational state of the protein. So MTS reagent reactivity does not imply that the corresponding WT residue is always on the water-accessible surface.

Somewhat surprisingly, there were only three positions, V74C, S77C, and T353C, at which we did not detect any functional effect of the MTS reagents (Fig. 8). For positions where MTS reagent application has no effect, one cannot assume that the Cys is nonreactive because covalent modification might have no detectable effect on protein function (33). Alternatively, local steric factors may prevent the MTS reagent from reacting, but the Cys might still be, at least transiently, on the water-accessible surface. So lack of functional evidence of MTS reaction must be interpreted with care.

For the remaining 21 Cys mutants that appeared to react with at least one of the MTS reagents, we can assume that at least transiently the substituted Cys residue was on the water-accessible surface. So lack of functional evidence of MTS reagent application does not imply that the corresponding WT residue is always on the water-accessible surface.

Table 4

| Table 4 | Effects of MTSEA-biotin on TM10 Cys-substitution mutant mediated [{³}H]adenosine uptake |
|---------|----------------------------------------------------------------------------------|
| Avg. MTSEA-biotin | S.D. MTSEA-biotin | Number of experiments | Avg. Hill slope MTSEA-biotin | S.D. Hill slope MTSEA-biotin | Avg. % of max uptake at 8 ms MTSEA-biotin | S.D. of % of max uptake at 8 ms MTSEA-biotin |
| WT        | >8E-03               | 5                      | 1.17                      | 0.07                      | 78                                 | 6                                       |
| A348C     | 4.49E-04             | 3                      | 3.72E-04                  | 4                        | 1.28                      | 0.56                                    | 68                                 | 5                                       |
| M349C     | 1.67E-04             | 2                      | 1.25E-04                  | 4                        | 1.48                      | 0.77                                    | 75                                 | 5                                       |
| L350C     | 3.01E-04             | 4                      | 7.40E-05                  | 4                        | -1.95                     | 0.77                                    | 75                                 | 5                                       |
| A351C     | 7.95E-04             | 4                      | 4.65E-04                  | 4                        | -0.61                     | 0.31                                    | 70                                 | 3                                       |
| F352C     | 4.32E-02             | 3                      | 3.12E-02                  | 4                        | -0.64                     | 0.11                                    | 81                                 | 17                                      |
| N354C     | 9.36E-05             | 4                      | 5.21E-05                  | 4                        | -1.32                     | 0.27                                    | 60                                 | 9                                       |
| G355C     | 1.11E-04             | 4                      | 1.70E-05                  | 4                        | -1.82                     | 0.18                                    | 58                                 | 6                                       |
| W356C     | 6.98E-05             | 3                      | 1.68E-05                  | 4                        | -1.68                     | 1.48                                    | 58                                 | 2                                       |
| F357C     | 1.94E-03             | 4                      | 0.76E-03                  | 4                        | -0.76                     | 0.19                                    | 70                                 | 6                                       |

Figure 8. α Helical wheel and net projections of TM2 (A and B) and TM10 (C and D). The extent of each transmembrane segment is based on previous transmembrane topology predictions (13, 14) and our own shown in Fig. 1. The TM2 face where the Cys mutations alter inosine affinity is to the left of the dotted line in panel B; these residues are highlighted in bold text. The TM2 face with the Cys mutants where MTS reagent modification causes potentiation of subsequent [³H]Ado uptake is above the dashed line in panel B. Symbols are defined in the figure legend.
Access to that side of TM11 (absolutely conserved G) formational structure of the transporter. Alternatively, the differences in membrane lipid composition might affect the con- 34, 35 34, 35

Water-accessible residues in PfENT1 TM2 and TM10 segments

Experimental procedures

PfENT1 constructs and Cys substitution mutants

All mutants were made in a yeast codon-optimized pfent1 gene with a C-terminal hemagglutinin epitope tag in the pCM189m S. cerevisiae yeast expression vector as described previously (8). The gene encodes the 3D7 parasite strain PfENT1 amino acid sequence (PlasmoDB ID PF3D7_1347200), subsequently referred to as wildtype (WT) PfENT1. In one set of mutants, each of the 11 endogenenous Cys residues was mutated to alanine, one at a time. Single Cys substitution mutants were generated, one at a time, in TM2 from positions 63 to 79, and in TM10 from positions 348 to 357 in the WT PfENT1 background. TM2 residue 75 is an endogenous Cys and was left unchanged. All mutations were generated using the PCR-based QuikChange II Site-directed Mutagenesis kit (Agilent). Primers were designed using the online QuikChange primer design tool. Mutations were verified by DNA sequenc- ing of the entire coding region (Genewiz).

All PfENT1 constructs were expressed in purine auxotrophic S. cerevisiae BY4741 with FLII1 and ADE2 gene deletions (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; fui1Δ::KanMX4; ade2Δ::hphNT1) as described previously (8). The purine auxo- trophic yeast were transformed with 1.0–2.0 μg of plasmid DNA using the lithium-acetate/DMSO method (36).

Yeast growth media

Purine auxotrophic yeast were grown in synthetic defined media (SDM) that contained 2% (w/v) glucose, 0.5% (w/v) ammonium sulfate, 0.17% yeast nitrogen base (U.S. Biologicals), 0.02% (w/v) yeast dropout mix lacking uracil, adenine, histidine, and tryptophan (U. S. Biologicals), 40 mg/liter of tryptophan, and 40 mg/liter of histidine. Media was supple- mented with 1 mM adenosine (Ado) as the sole purine source. For initial plating, yeast transformations were plated on solid media plates that contained SDM (lacking uracil) plus 1 mM adenosine in 2% agar. For selection and propagation of PfENT1- expressing yeast, solid media plates contained SDM (lacking uracil) with 1 mM adenosine in 2% agar. For all uptake experiments, PfENT1 Cys mutant expressing yeast were grown to early mid-log phase, shaking overnight at 225 rpm at 30 °C in SDM containing 1 mM adenosine. Cells were pelleted and washed three times in PBS + glucose (150 mM NaCl, 10 mM KH2PO4, 40 mM KH2PO4, 11 mM glucose, pH 7.4). For all the assays described below, cells were resuspended in PBS + glucose to a suspension density of 2 × 10⁸ cells/ml. Yeast cell density was determined by measuring optical density at 600 nm (A600) (Bio- Rad Benchmark Plus).

Characterization of hypoxanthine and inosine transport by the Cys mutants

To ascertain whether the individual Cys substitutions altered transporter function, we determined the purine affinity of each Cys-mutant. We measured the concentration-dependent inhibition of [3H]Urd uptake by the purine nucleobase, hypoxan- thine, and nucleoside, inosine. PfENT1 transports Urd. We used competition with [3H]Urd uptake to avoid the potential for competition at downstream purine metabolic enzymes (37).
Water-accessible residues in PfENT1 TM2 and TM10 segments

A 96-well plate was preloaded with 50 μl/well of 1:4 serially diluted hypoxanthine or inosine (highest concentration 25 or 10 mM, respectively) in 1× PBS + glucose. 50 μl of 100 nM [3H]Urd ([5,6-3H]uridine, 40 Ci/mmol, Moravek Biochemicals) in PBS + glucose was added to each well. 100 μl of yeast suspension (2 × 10^6 cells/ml) was added and uptake was measured at 15 min. Uptake was terminated by harvesting cells onto glass fiber filters (Filtermat, GF/C; PerkinElmer Life Sciences) using a TomTec 96-well cell harvester (96-3-469). Filtermats were dried for >1 h and sealed in plastic bags containing 5 ml of Betaplate Scint LSC (PerkinElmer Life Sciences). Filtermats were counted using a 1450 Microbeta Trilux (PerkinElmer Life Sciences). IC_{50} values were calculated using Prism 7 (GraphPad Software). All experiments were repeated at least three times on different days.

For most of the Cys mutants, we also characterized inosine uptake by competition with [3H]Ado uptake. A 96-well plate was preloaded with 50 μl/well of serially diluted (1:2) cold inosine (highest concentration, 10 mM). 50 μl of 200 nM [3H]Ado ([2,8-3H]adenosine, 35 Ci/mmol, Moravek Biochemicals) was added to each well. 100 μl of yeast suspension (2 × 10^6 cells/ml) was added and uptake was measured at 15 min. At the end of each experiment, cells were harvested and counted as described above. All experiments were repeated at least three times on different days.

**Substituted cysteine accessibility experiments**

We assayed the reactivity of the TM2 and TM10 segment Cys-substitution mutants with MTS reagents. The MTS reagents used, in order of increasing size, were MTSET^+, MTSES^−, and MTSEA-biotin (Biotium). To ensure that the MTS reagents did not hydrolyze, MTSEA-biotin was prepared as an 800 mM stock solution in DMSO, and MTSET^+ and MTSES^− were prepared as 300 mM stock solutions in DMSO.

A 96-well plate was preloaded with 100 μl of yeast suspension (2 × 10^6 cells/ml). 1 μl of MTS reagent serially-diluted 1:3 in DMSO was added. Plates were incubated for 5 min at room temperature. 100 μl of 100 nM [3H]Ado was then added. After a 15-min uptake, cells were harvested and counted as above. Data were normalized to the lowest concentration of MTS reagent applied after background subtraction. All experiments were repeated at least three times on different days.

**Effect of MTS treatment on Cys mutant adenosine affinity**

For some Cys-mutants, tritiated substrate uptake was potentiated after MTS reaction. To determine whether the potentiation was a consequence of increased substrate affinity or increased rate of uptake, we measured the adenosine affinity. We measured the effect of increasing concentrations of cold adenosine on the uptake of [3H]Urd before and after MTS modification. A 96-well plate was preloaded with 50 μl of 100 nM [3H]Urd, and 50 μl/well 1:2 serially diluted adenosine (highest concentration, 10 mM). A yeast suspension (2 × 10^6 cells/ml) was split into two aliquots. To one aliquot 3 mM MTS reagent was added. An equal volume of buffer without MTS reagent was added to the second aliquot. After 3 min, 100 μl of yeast suspension was added to each well. After 15 min, yeast were harvested and counted as described above to measure uptake.

Adenosine IC_{50} values for the inhibition of [3H]Urd uptake were calculated as described above for hypoxanthine and inosine. All experiments were repeated at least three times on different days.

**Author contributions**—S. N. N., J. R., and M. H. A. formal analysis; S. N. N., A. A., and J. R. investigation; S. N. N., A. A., J. R., and M. H. A. writing-review and editing; A. A. and M. H. A. conceptualization; A. A., J. R., and M. H. A. methodology; M. H. A. supervision; M. H. A. funding acquisition; M. H. A. writing-original draft.

**Acknowledgments**—We thank Drs. I. J. Frame and Roman Deniskin for advice during the initiation of this project and David Pierce for technical assistance.

**References**

1. Sia, J. M., Chong, J. L., and Wellens, T. E. (2011) Malaria drug resistance: new observations and developments. Essays Biochem. 51, 137–160 CrossRef Medline
2. Blasco, B., Leroy, D., and Fidock, D. A. (2017) Antimalarial drug resistance: linking Plasmodium falciparum parasite biology to the clinic. Nat. Med. 23, 917–928 CrossRef Medline
3. Arieux, F., Witkowski, B., Amarantunga, C., Beghain, J., Langlois, A. C., Khim, N., Kim, S., Duru, V., Boucher, C., Ma, L., Lim, P., Leang, R., Duong, S., Suon, S., Cao, J., et al. (2014) A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 505, 50–55 CrossRef Medline
4. Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amarantunga, C., Lim, P., Suon, S., Sueng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chiu, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., et al. (2014) Spread of artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. 371, 411–423 CrossRef Medline
5. Sutherland, C. J., Lansdell, P., Sanders, M., Muwanguzi, J., van Schalkwyk, D. A., Kaur, H., Nolder, D., Tucker, J., Bennett, H. M., Otto, T. D., Berrieman, M., Patel, T. A., Lynn, R., Gkrania-Klotsas, E., and Chiodini, P. L. (2017) pK13-independent treatment failure in four imported cases of Plasmodium falciparum malaria treated with artemether-lumefantrine in the United Kingdom. Antimicrob. Agents Chemother. 61, e02382-16 Medline
6. Lu, F., Culleton, R., Zhang, M., Ramaprasad, A., von Seidlein, L., Zhou, H., Zhu, G., Tang, J., Liu, Y., Wang, W., Cao, Y., Xu, S., Gu, Y., Li, J., Zhang, C., Gao, Q., Menard, D., Pain, A., Yang, H., Zhang, Q., and Cao, J. (2017) Emergence of indigenous artemisinin-resistant Plasmodium falciparum in Africa. N. Engl. J. Med. 367, 991–993 CrossRef Medline
7. Frame, I. J., Deniskin, R., Arora, A., and Akabas, M. H. (2015) Purine import into malaria parasites as a target for antimalarial drug development. Ann. N.Y. Acad. Sci. 1342, 19–28 CrossRef Medline
8. Frame, I. J., Deniskin, R., Rinderspacher, A., Katz, F., Deng, S. X., Moir, R. D., Adjalley, S. H., Coburn-Flynn, O., Fidock, D. A., Willis, I. M., Landry, D. W., and Akabas, M. H. (2015) Yeast-based high-throughput screen identifies Plasmodium falciparum equilibrative nucleoside transporter 1 inhibitors that kill malaria parasites. ACS Chem. Biol. 10, 775–783 CrossRef Medline
9. Landfear, S. M., Ullman, B., Carter, N. S., and Sanchez, M. A. (2004) Nucleoside and nucleobase transporters in parasitic protozoa. Eukaryot. Cell 3, 245–254 CrossRef Medline
10. Downie, M. J., Kirk, K., and Mamoun, C. B. (2008) Purine salvage pathways as targets in Plasmodium falciparum. Eukaryot. Cell 7, 1231–1237 CrossRef Medline
11. Downie, M. J., Saliba, K. J., Bröer, S., Howitt, S. M., and Kirk, K. (2008) Purine nucleobase transport in the intraerythrocytic malaria parasite. Int. J. Parasitol. 38, 203–209 CrossRef Medline
12. Cunnane, M. B., Zhang, Y., Hazleton, K. Z., and Schramm, V. L. (2011) Purine and pyrimidine pathways as targets in Plasmodium falciparum. Curr. Top. Med. Chem. 11, 2103–2115 CrossRef Medline
Water-accessible residues in PfENT1 TM2 and TM10 segments

13. Carter, N. S., Ben Mamoun, C., Liu, W., Silva, E. O., Landfeur, S. M., Goldberg, D. N., and Ullman, B. (2000) Isolation and functional characterization of the PfNT1 nucleoside transporter gene from Plasmodium falciparum. J. Biol. Chem. 275, 10683–10691 CrossRef Medline

14. Parker, M. D., Hyde, R. J., Yao, S. Y., Mrcobert, L., Cass, C. E., Young, J. D., McConkey, G. A., and Baldwin, S. A. (2000) Identification of a nucleoside/nucleobase transporter from Plasmodium falciparum, a novel target for anti-malarial chemotherapy. Biochem. J. 349, 67–75 Medline

15. Downie, M. J., Saliba, K. J., Howitt, S. M., Bröer, S., and Kirk, K. (2006) Transport of nucleosides across the Plasmodium falciparum parasite plasma membrane has characteristics of PiENT1. Mol. Microbiol. 60, 738–748 CrossRef Medline

16. Traut, T. W. (1994) Physiological concentrations of purines and pyrimidines. Mol. Cell Biochem. 140, 1–22 CrossRef Medline

17. El Bissati, K., Downie, M. J., Kim, S. K., Horowitz, M., Carter, N., Ullman, B., and Ben Mamoun, C. (2000) Genetic evidence for the essential role of PiNT1 in the transport and utilization of xanthine, guanine, guanosine and adenosine by Plasmodium falciparum. Mol. Biochem. Parasitol. 161, 130–139 CrossRef Medline

18. El Bissati, K., Zufferey, R., Witola, W. H., Carter, N. S., Ullman, B., and Ben Mamoun, C. (2006) The plasma membrane permease PiNT1 is essential for purine salvage in the human malaria parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A. 103, 9286–9291 CrossRef Medline

19. Zhang, M., Wang, C., Otto, T. D., Oberstaller, I., Liao, X., Adapa, S. R., Udenze, K., Bronner, I. F., Casandra, D., Mayho, M., Brown, J., Li, S., Swanson, J., Rayner, J. C., Jiang, R. H. Y., and Adams, J. H. (2018) Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. Science 360, eaap7847 CrossRef Medline

20. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Acetylcholine receptor channel structure probed in cysteine-substitution mutants. Science 258, 307–310 CrossRef Medline

21. Akabas, M. H., Kaufmann, C., Archdeacon, P., and Karlin, A. (1994) Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the α subunit. Neuron 13, 919–927 CrossRef Medline

22. Akabas, M. H. (2015) Cysteine modification: probing channel structure, function and conformational change. Adv. Exp. Med. Biol. 869, 25–54 CrossRef Medline

23. Roberts, D. D., Lewis, S. D., Ballou, D. P., Olson, S. T., and Shafer, J. A. (1986) Reactivity of small thiolate anions and cysteine-25 in papain toward methyl methanesulphonate. Biochemistry 25, 5595–5601 CrossRef Medline

24. Karlin, A., and Akabas, M. H. (1998) Substituted-cysteine accessibility method. Methods Enzymol. 293, 123–145 CrossRef Medline

25. Riegelhaupt, P. M., Frame, I. J., and Akabas, M. H. (2010) Transmembrane segment 11 appears to line the purine permeation pathway of the Plasmodium falciparum equilibrative nucleoside transporter 1 (PiENT1). J. Biol. Chem. 285, 17001–17010 CrossRef Medline

26. Baldwin, S. A., Beal, P. R., Yao, S. Y., King, A. E., Cass, C. E., and Young, J. D. (2004) The equilibrative nucleoside transporter family, SLC29. Pflugers Arch. 447, 735–743 CrossRef Medline

27. Sundaram, M., Yao, S. Y., Ingram, J. C., Berry, Z. A., Abidi, F., Cass, C. E., Baldwin, S. A., and Young, J. D. (2001) Topology of a human equilibrative, nitrobenzylthionine (NBMPR)-sensitive nucleoside transporter (hENT1) implicated in the cellular uptake of adenosine and anti-cancer drugs. J. Biol. Chem. 276, 45270–45275 CrossRef Medline

28. Valdés, R., Arastu-Kapur, S., Landfeur, S. M., and Shinde, U. (2009) An ab initio structural model of a nucleoside permease predicts functionally important residues. J. Biol. Chem. 284, 19067–19076 CrossRef Medline

29. Valdés, R., Shinde, U., and Landfeur, S. M. (2012) Cysteine cross-linking defines the extracellular gate for the Leishmania donovani nucleoside transporter 1.1 (LdNT1.1). J. Biol. Chem. 287, 44036–44045 CrossRef Medline

30. Valdés, R., Elferich, I., Shinde, U., and Landfeur, S. M. (2014) Identification of the intracellular gate for a member of the equilibrative nucleoside transporter (ENT) family. J. Biol. Chem. 289, 8799–8809 CrossRef Medline

31. Yao, S. Y., Ng, A. M., Vickers, M. F., Sundaram, M., Cass, C. E., Baldwin, S. A., and Young, J. D. (2002) Functional and molecular characterization of nucleobase transport by recombinant human and rat equilibrative nucleoside transporters 1 and 2: chimeric constructs reveal a role for the ENT2 helix 5–6 region in nucleobase translocation. J. Biol. Chem. 277, 24938–24948 CrossRef Medline

32. Kirk, K., Howitt, S. M., Bröer, S., Saliba, K. J., and Downie, M. J. (2009) Purine uptake in Plasmodium: transport versus metabolism. Trends Parasitol. 25, 246–249 CrossRef Medline

33. Bera, A. K., Chatav, M., and Akabas, M. H. (2002) GABA(A) receptor M2-M3 loop secondary structure and changes in accessibility during channel gating. J. Biol. Chem. 277, 43002–43010 CrossRef Medline

34. Russ, W. P., and Engelman, D. M. (2000) The GxxG motif: a framework for transmembrane helix-helix association. J. Mol. Biol. 296, 911–919 CrossRef Medline

35. Harrington, S. E., and Ben-Tal, N. (2009) Structural determinants of transmembrane helical proteins. Structure 17, 1092–1103 CrossRef

36. Hill, J., Donald, K. A., and Donald, G. (1991) DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res. 19, 5791 CrossRef Medline

37. Riegelhaupt, P. M., Cassera, M. B., Frölich, R. F., Hazelton, K. Z., Hefter, J. J., Schramm, V. L., and Akabas, M. H. (2010) Transport of purines and purine salvage pathway inhibitors by the Plasmodium falciparum equilibrative nucleoside transporter PiENT1. Mol. Biochem. Parasitol. 169, 40–49 CrossRef Medline