Role of Transmembrane Segment 10 in Efflux Mediated by the Staphylococcal Multidrug Transport Protein QacA*

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The staphylococcal multidrug exporter QacA confers resistance to a wide range of structurally dissimilar monovalent and bivalent cationic antimicrobial compounds. To understand the functional importance of transmembrane segment 10, which is thought to be involved in substrate binding, cysteine-scanning mutagenesis was performed in which 35 amino acid residues in the putative transmembrane helix and its flanking regions were replaced in turn with cysteine. Solvent accessibility analysis of the introduced cysteine residues using fluorescein maleimide indicated that transmembrane segment 10 of QacA contains a 20-amino-acid hydrophobic core and may extend from Pro-309 to Ala-334. Phenotypic analysis and fluorimetric transport assays of these mutants showed that Gly-313 is important for the efflux of both monovalent and bivalent cationic compounds, whereas Asp-323 is only important for the efflux of bivalent substrates and probably forms part of the bivalent substrate-binding site(s) together with Met-319. Furthermore, the effects of N-ethyl-maleimide treatment on ethidium and 4',6-diamidino-2-phenylindole export mediated by the QacA mutants suggest that the face of transmembrane segment 10 that contains Asp-323 may also be close to the monovalent substrate-binding site(s), making this helix an integral component of the QacA multidrug-binding pocket.

Multidrug resistance is the phenomenon in which a single transmembrane transport protein or protein complex mediates the export of a wide range of structurally dissimilar toxic compounds. Multidrug efflux pumps are ubiquitous and belong to five distinct transport protein families, including the ATP-binding cassette superfamily, the major facilitator superfamily (MFS), the resistance/nodulation-cell division superfamily, the drug/metabolite transporter family, and the multidrug and toxic compound extrusion family (1). These proteins have increasingly become a major obstacle for the treatment of bacterial infectious diseases and the chemotherapy of human cancers. A detailed understanding of the substrate recognition and transport mechanisms of these transporters is required to overcome the problems associated with multidrug resistance.

The multidrug resistance gene qacA is carried by multiresistance plasmids from clinical isolates of Staphylococcus aureus and other coagulase-negative staphylococci (2, 3). qacA encodes a 514-amino-acid protein, QacA, that possesses 14 α-helical transmembrane segments (TMS) (4). QacA is a member of the MFS and mediates substrate/H+ antiport utilizing the proton motive force as the driving force (5). It confers resistance to >30 structurally dissimilar organic cations, including monovalent cationic compounds, such as quaternary ammonium compounds (Qacs) and intercalating dyes, and bivalent compounds, such as diamidines and biguanidines (6, 7). Fluorimetric analysis of QacA-mediated export suggests that monovalent and bivalent substrates bind to distinct sites on the QacA protein (8). Comparative and mutagenesis studies of QacA and the closely related QacB protein, which differs from QacA by conferring little or no resistance to bivalent compounds, viz. diamidines and biguanidines, have revealed that the presence of an acidic residue at position 323 in TMS 10 of QacA is essential for the high levels of resistance seen to bivalent compounds (4). Competition studies show that QacA utilizes a high affinity binding site(s) for the transport of bivalent substrates that are absent in QacB, implying direct involvement of Asp-323 and hence TMS 10 in the substrate recognition process (7, 8).

To date, very limited functional and structural information of the 14-TMS MFS proteins is available, and crystallographic studies of these proteins are inherently difficult to perform, necessitating molecular and biochemical studies of these proteins. Because QacA contains no intrinsic cysteine (Cys) residues (see Fig. 1), it is an ideal target for Cys-scanning mutagenesis studies (9–11). Therefore, to understand the importance of TMS 10 in QacA-mediated efflux, Cys-scanning mutagenesis was carried out on TMS 10 and its flanking regions. The effect of Cys substitution on protein expression was studied by Western blot analysis. The extents of TMS 10 were examined by analyzing the relative reactivity of the QacA Cys-substituted mutant proteins to two differently labeled maleimido compounds, and functionally important residues were identified by phenotypic analysis and fluorimetric transport assays.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The Escherichia coli strain DH5α (12) was used as the host strain for general cloning purposes, Western blotting, and solvent accessibility analyses. The E. coli strain BHB2600 (13) was used for minimum inhibitory concentration (MIC) analyses and fluorimetric transport assays. Both strains were cultured at 37 °C in Luria broth containing 100 μg/ml ampicillin where appropriate.

Chemicals—Benzalkonium, chlorhexidine, 4',6-diamidino-2-phenylindole (DAPI), dequalinium, ethidium (Et), pyronin Y, and fluorescein-5-maleimide from Molecular Probes. Diamidinodiphenylamine (DD) was provided by Rhône-Poulenc Rorer (Dagenham, UK). All other materials were of reagent grade containing 100 μg/ml ampicillin where appropriate.

Site-directed Mutagenesis—Site-directed mutants were constructed per the QuikChangeTM technique (Stratagene) using pairs of mutagenic

* This work was supported by Project Grant 301938 from the National Health and Medical Research Council (Australia). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: MFS, major facilitator superfamily; DAPI, 4',6-diamidino-2-phenylindole; DD, diamidinodiphenylamine; Et, ethidium; MIC, minimum inhibitory concentration(s); NEM, N-ethyl-maleimide; Qac, quaternary ammonium compound(s); TMS, transmembrane segment(s).
Multidrug Substrate-binding Domain of QacA

![Secondary structure model of the QacA multidrug transport protein](image)

FIGURE 1. Secondary structure model of the QacA multidrug transport protein. 14-TMS model based on hydropathy profile and gene fusion analysis (4). TMSs are indicated by boxes and numbered on the top left corner. The residues subjected to Cys substitution in this study, from Ser-308 to Val-342, are shown in black circles. The position of Asp-323, found previously to be important for the function of QacA, is shown as a white circle.

primers and plasmid pSK4322 as a template. This plasmid was constructed by cloning a PCR-derived 1.6-kb qacA-coding fragment from plasmid pSK1 (14) into the EcoRI and BamHI sites of the vector pBluescript II SK(+) A His6 tag was also incorporated into the C terminus of QacA during PCR. All mutated sequences were verified by DNA sequencing.

Labeling of the QacA Cys Mutant Proteins with Fluorescein Maleimide or [14C]NEM—E. coli DH5α cells harboring plasmids encoding QacA Cys-substituted mutants were inoculated into Luria broth supplemented with 100 μg/ml ampicillin, grown to OD₆₅₀ = 0.9 and collected by centrifugation. Bacterial cells were disrupted by passing three times through a French pressure cell (SLM Aminco, Spectronic Instruments) at 20,000 psi. After removal of cell debris by centrifugation, membrane vesicles were collected by ultracentrifugation at 125,000 × g for 1 h and resuspended in 50 mM Tris-HCl buffer (pH 7.0) to a protein concentration of 20 mg/ml. Typically, 30 μl of membrane vesicles were used for each reaction. Fluorescein maleimide or [14C]NEM was added to a final concentration of 0.25 mM and then incubated at 30 °C for 10 min. The reactions were terminated by adding NEM to a final concentration of 20 mM. The membrane vesicles were solubilized with 1% SDS and QacA mutant proteins purified by affinity chromatography using Ni²⁺ nitrilotriacetic acid-agarose. Approximately 2 μg of the purified QacA protein was subjected to SDS-PAGE. For fluorescein maleimide labeling, fluorescein maleimide bound to QacA was visualized by scanning wet gels directly using a Molecular Imager® FX (Bio-Rad). The gels were then stained with Coomassie Brilliant Blue R-250 and scanned with a GS-710 calibrated imaging densitometer (Bio-Rad). For [14C]NEM labeling, the gel was first stained and dried. The dried gel was exposed to a phosphor screen, and the [14C]NEM-bound QacA mutant proteins were visualized by scanning with a Molecular Imager® FX (Bio-Rad).

To determine the effect of Et and DAPI binding on the labeling of the QacA Cys mutant proteins by fluorescein maleimide, membrane vesicles containing QacA mutant proteins were incubated at 30 °C for 5 min in the absence or presence of 1 mM Et or DAPI; labeling of mutant proteins was performed as described above.

Determination of Resistance Profiles and Fluorimetric Analysis of QacA-mediated Et and DAPI Efflux—MIC analyses and transport assays of QacA-mediated efflux of Et and DAPI were performed as described previously (8). Briefly, transport activities of bacterial cells incubated with 12 different concentrations of each substrate were measured fluorimetrically. Initial transport velocities were calculated by averaging the linear part of each fluorescence-decreasing curve and computationally fitted to estimated Vₘₐₓ and Kₘ values. Vₘₐₓ values of each QacA mutant protein were normalized against the protein expression levels obtained by densitometric scanning of Western blots using a QacA-specific antiserum.

Effect of NEM Treatment on the Efflux of Et and DAPI—Aliquots of bacterial cells prepared as previously described (8) were incubated in the presence or absence of 5 mM NEM at 37 °C for 20 min. After removal of NEM by washing with 20 mM HEPES buffer (pH 7.0), the cells were resuspended in the above buffer, loaded with 15 μM Et or 10 μM DAPI, and the QacA-mediated efflux analyzed as previously described (8).

Second-site Suppressor Analysis of the QacA D323C Mutant—To isolate second-site suppressors, E. coli DH5α cells harboring the plasmid encoding the QacA D323C mutant were plated on Luria broth agar plates supplemented with a non-permissive concentration of the bivalent compound Dd (100 μg/ml) and incubated at 37 °C for 48 h. The resulting colonies were purified, their plasmids extracted and transformed into fresh E. coli DH5α cells, and the resistance profiles of the resulting transformants determined by MIC analysis. The 1.6-kb qacA-coding regions of the plasmids able to confer resistance to Dd were then fully sequenced.

RESULTS

Cys-scanning Mutagenesis of QacA TMS 10 and the Expression of Mutant Proteins—Previous studies revealed that a negatively charged Asp at position 323 of TMS 10 of QacA was essential for the recognition of bivalent cationic compounds, implying the direct involvement of this TMS in the substrate recognition process (4, 8). To further understand the importance of this TMS in the function of QacA, 35 amino acid residues in putative TMS 10 and its flanking regions, from Ser-308 to Val-342 of QacA (Fig. 1), were replaced with Cys individually by site-directed mutagenesis and the resulting mutants functionally and biochemically characterized. Western blotting with a QacA-specific antisera revealed that most of the QacA mutant proteins were expressed at levels essentially comparable with that of the QacA wild-type protein (data not shown), indicating that Cys substitution generally did not have a significant effect on the expression of the mutant proteins. However, the QacA mutant proteins K311C, R336C, and K340C were expressed at levels below or equivalent to 50% of that of QacA wild-type protein, suggesting that these basic residues may be important for protein expression, insertion, and/or stability.
that the majority of the mutants conferred resistance to most of the substrates tested at levels comparable with those of QacA wild type (Fig. 3). However, some QacA derivatives, such as L314C, Y315C, G322C, and A327C, were more susceptible to one or more substrates tested but not to an entire category of monovalent or bivalent substrates. The QacA G313C mutant conferred significantly reduced resistance to dequalinium and chlorhexidine and no resistance to all of the other compounds tested, and the QacA D323C mutant lost resistance to all of the bivalent compounds tested, indicating that Gly-313 is important for resistance to all QacA substrates, whereas Asp-323 is essential only for resistance to bivalent compounds, as previously reported (4, 8).

**Et and DAPI Efflux by QacA Mutants**—To verify the phenotypes conferred by these QacA mutants, fluorimetric analyses of the efflux of Et and DAPI, which were chosen as representatives of monovalent and bivalent substrates of QacA, respectively, were performed as previously described (8), and the kinetic parameters of transport, $V_{\text{max}}$ (Fig. 4) and $K_m$ (data not shown), determined.

The results showed that only one of the 35 mutants, the QacA G313C mutant, completely lost Et export activity (Fig. 4A). The remaining QacA mutants mediated significant Et efflux, with activities ranging from $\sim 48$ (M324C) to $231\%$ (R336C) compared with that of the QacA wild type. For DAPI export, both the QacA G313C and D323C mutants completely lost DAPI transport activity. These results further confirmed the importance of residues Gly-313 and Asp-323 in efflux mediated by QacA. Additionally, replacement of Met-319 with Cys resulted in a significant reduction in DAPI transport activity ($\sim 12\%$ of that of QacA wild type; Fig. 4B), indicating that this residue was also important for DAPI transport. The other QacA mutants retained significant DAPI transport activities (Fig. 4B). Transport analyses of the QacA K311C, R336C, and K340C mutants revealed that these derivatives have high Et and DAPI transport activities when taking into account their low expression levels (Fig. 4). It is possible that the reduced amount of these mutant proteins is an adjustment made by the cell so that these highly active derivatives are not present at toxic levels. The $K_m$ values of Et and DAPI transport by the QacA mutants were all $\ll 20 \mu M$ (data not shown), consistent with previous results (8), indicating that QacA interacts with Et and DAPI specifically and these amino acid substitutions do not greatly affect substrate binding.

**Effect of NEM on Et and DAPI Efflux by QacA Mutants**—NEM has widely been used to probe the substrate-binding site/transport pathway of membrane transport proteins (11, 17). For example, residues in the E. coli lactose permease LacY, which have Cys-substituted mutants inactivated by NEM alkylation, are often found to be in close vicinity to the substrate-binding site (18–20). To test whether any of the residues in TMS 10 of QacA were likely to form or be spatially juxtaposed to the substrate-binding site(s)/transport pathway, the effects of NEM treatment on Et and DAPI efflux mediated by the QacA mutants were examined. The QacA G313C and D323C derivatives lost both Et and DAPI transport activities, respectively, and hence could not be tested. Results showed that there were no mutants with an Et efflux capability that was completely abolished by the addition of NEM (Fig. 5A). However, Et efflux mediated by the QacA P309C, A320C, and A327C derivatives was inhibited significantly ($\approx 50\%$) by $\sim 65, 50,$ and $70\%$, respectively, whereas Et efflux by the QacA A334C mutant was enhanced to $250\%$ (Fig. 5A). The magnitudes of NEM inhibition of the above mutants were similar over a range of NEM concentrations ($1–10 \text{ mM}$) or different incubation time periods ($10–40$ min) in the presence of $5 \text{ mM}$ NEM (data not shown), indicating that the incomplete inhibition observed was not due to an incomplete reaction of the Cys residues with NEM. Therefore, the inhibition of QacA-mediated Et efflux by NEM was
probably because of the steric hindrance of substrate binding or translocation resulting from NEM alkylation, as suggested using similar studies that have been performed with other transport proteins (17–19).

The effect of NEM treatment on the DAPI efflux of the QacA mutants was also examined (Fig. 5B). NEM treatment abolished DAPI efflux mediated by the QacA P309C mutant and significantly enhanced that by the QacA A334C mutant by ~10-fold (Fig. 5B). No significant effect of NEM treatment on the DAPI efflux by the other QacA mutants was observed.

**Binding of Et and DAPI Induced a Different Conformational Change to QacA**—To examine whether any amino acids within TMS 10 of QacA were so close to the substrate-binding site(s) that the introduced Cys residues could be allosterically protected from maleimide alkylation by the addition of substrates, or whether substrate binding induced a conformational change of the QacA protein, thereby making the Cys residues more or less accessible to solvent, the effects of pre-incubation with Et and DAPI on the labeling of a number of QacA mutant proteins by fluorescein maleimide, including P309C, L316C, A320C, D323C, M324C, A327C, P328C, A330C, P331C, G332C, A334C, and F337C, were analyzed. Most of the mutants tested possessed a Cys residue predicted to be on the same face of TMS 10 as Asp-323, which was likely to be close to the substrate-binding site, or with relatively high solvent accessibility (Fig. 2), could be tested for substrate protection against maleimide alkylation. The results indicated that, although pre-incubation with Et had no significant effect on the maleimide labeling of all of the mutant proteins tested (data not shown), pre-incubation with DAPI did significantly increase the labeling of mutant proteins A327C and P331C by ~4.2- and 6.7-fold, respectively (Fig. 6). This suggested that the binding of DAPI may have caused a conformational change of the QacA protein, rendering residues P331C and A327C more exposed to the aqueous phase and hence allowing higher degrees of alkylation by fluorescein maleimide.

**Isolation and Characterization of a QacA D323C/A320E Double Mutant**—To determine whether an additional mutation can compensate for the loss of the negative charge at position 323 of TMS 10, a second-site suppressor mutant of the QacA D323C mutant was isolated. After growth on a non-permissive concentration of the substrate Dd, only one resistant QacA mutant was isolated. DNA sequencing of the promoter and coding region of \( qacA \) confirmed that the initial Cys substitution was still present in addition to a second-site mutation, creating a QacA D323C/A320E double mutant. This double mutant regained high levels of resistance to bivalent compounds (Fig. 3) and displayed a much higher DAPI transport activity, ~10-fold higher than that of the QacA wild type, whereas it retained similar Et transport activity as the QacA wild type (data not shown). This indicated that an acidic Glu residue at position 320 of TMS 10, which is one turn away from position 323 and on the same face of the helix (Fig. 7), can compensate for the mutation of D323C. When treated with NEM, DAPI efflux mediated by this mutant was completely abolished, suggesting that Asp-323 is directly involved in bivalent substrate binding.

**DISCUSSION**

Phenotypic and fluorimetric analyses of the QacA Cys mutants showed that the majority of the residues were not crucial for the

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**FIGURE 3. MIC analyses of QacA Cys mutants.** MIC values for the indicated compounds were determined in \( E. coli \) strain BH182600, harboring plasmids encoding QacA wild-type (wt) or Cys-substituted mutant proteins or pBluescript II SK(+) (vector) (8). All experiments were performed in triplicate; representative results of one experiment are shown here. MICs for Dd could not be determined for all mutants because of limited availability of the compound. Chlorhexidine is abbreviated as Chl and A320E represents the QacA D323C/A320E double mutant.**

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function of QacA (Figs. 3 and 4). However, some Cys substitutions exerted substrate-specific effects; for example, the QacA L314C and Y315C mutants lost resistance to benzalkonium but conferred substantial resistance to other compounds (Fig. 3). Such altered substrate specificity, because of the mutation of a single amino acid, has been well documented for multidrug resistance proteins (4, 21–24) and reflects the poly-specific nature of the multidrug recognition mechanism. By contrast, the QacA D323C mutant lost resistance to all tested bivalent compounds and DAPI efflux capability but retained wild-type levels of resistance to monovalent compounds and mediated Et efflux to a level not significantly different from that of the QacA wild type (Figs. 3 and 4), substantiating the importance of this residue in the recognition of bivalent compounds (4, 8). This postulate was further validated by the analysis of the QacA D323C/A320E double mutant.

The QacA M319C mutant exhibited an altered resistance profile to some compounds (Fig. 3) and substantially reduced DAPI transport activity (Fig. 4B) but retained significant Et transport activity (Fig. 4A), suggesting that Met-319 is also important for the efflux of bivalent compounds. When projected on an α-helical wheel, residues Asp-323 and Met-319 are close on the same face of TMS 10 (Fig. 7). Therefore, Met-319 may also participate in the binding of bivalent substrates, with Asp-323 being more intimately involved and Met-319 on the periphery.

Gly-313 was shown to be crucial for the export of both monovalent and bivalent compounds (Figs. 3 and 4). This residue was originally predicted to be outside the confines of TMS 10, placed on the external loop between TMS 9 and 10 (Fig. 1). However, the results of maleimide labeling demonstrated that a Cys residue in position 313 was non-reactive to either fluorescein maleimide (Fig. 2) or [14C]NEM (data not shown). Although Gly-313 is on the same face of TMS 10 as Asp-323, it is three helical turns removed from the latter residue and located near the extracellularly facing end of TMS 10 (Fig. 7). It is likely that Gly-313 may assume a structurally important role that is required for the function of QacA rather than being directly involved in substrate binding. This is commonly seen (10, 25, 26), as Gly occurs frequently in the TMS of membrane proteins and is often found in interhelical positions (27) that play important roles in mediating interactions between helices (28, 29). The lack of a side chain allows Gly to form close interhelical contacts and provides a good packing surface (30); Gly can also be either the donor or acceptor of an interhelical C–H...O hydrogen bond that may contribute to the stability of interhelical interactions (31). Therefore, Gly can serve as a molecular notch for orienting multiple helices in a folded protein (27). It is tempting to suggest that Gly-313 mediates such interhelical interactions or facilitates dynamic conformational changes that are critical for transport of all QacA substrates.

A striking feature of TMS 10 of QacA is that it contains multiple Gly and Pro residues; in addition to Gly-313, there are two more Gly residues, Gly-322 and Gly-332, and three Pro residues, Pro-318, Pro-328,
and Pro-331 (Fig. 7A). Gly and Pro residues have been shown to be important for the structure and function of membrane transport proteins (10, 32). The presence of Pro residues often disturbs the α-helical geometry of transmembrane helices by introducing kinks or swivels, which are important for the conformational flexibility of membrane transporters (32–34). Furthermore, similar to Gly, Pro residues may also mediate interhelical interactions (28, 29).

It was therefore surprising that Cys substitution of these residues within TMS 10 did not have significant effects on the expression of the QacA mutant proteins nor on QacA-mediated efflux, except for the G313C mutant already described. However, similar findings have been reported with bacteriorhodopsin, where intramembranous Pro residues could be substituted with Ala with little structural consequences (35). An evolutionary scenario was proposed where a kink is first introduced into a helix via mutation of a residue to Pro, thereby driving helix distortions. Further residue substitutions are then selected for leading to packing adjustments to optimize tertiary interactions. Thus, subsequent removal of Pro residues, as performed with QacA, results in no overall change to the structure of the helix, as its structure has already been stabilized and the presence of Pro is no longer necessary to preserve the kink (35).

Although Asp-323 and Met-319 have been suggested to form part of the bivalent substrate-binding site of QacA, no other residues in TMS 10, except for Gly-313 (see previous paragraph), were found to be essential for monovalent substrate efflux. NEM treatments significantly inhibited Et efflux by the QacA A334C mutant and significantly enhanced Et and DAPI efflux by the QacA A334C mutant (Fig. 5), suggesting that these residues may be close to the monovalent substrate-binding site/transport pathway of QacA with Ala-334 at the intercept or entrance of the monovalent and bivalent binding sites. With Ala-334, the increase in transport activities may reflect a preference for a bulky hydrophobic side chain (viz. the maleimido group of NEM) at this position for efflux by creating a more favorable environment for substrate binding, or providing an additional interaction site for substrates, thereby stabilizing substrate-QacA interactions.

When projected on an α-helical wheel, these four residues, together with Asp-323 and Met-319, are located on the same face of TMS 10 (Fig. 7). Therefore, this side of TMS 10 of QacA may form part of a multifaceted substrate-binding pocket, which is located in a central cavity as has
been found in other MFS proteins (10, 19, 36, 37), directly interacting with bivalent substrates and also in close proximity to the monovalent substrate-binding site(s). However, the two substrate-binding sites, although close, appear to be somewhat dissimilar (8). This is illustrated by the different effects on substrate profiles attributable to the introduced mutations, the addition of NEM on efflux, and the addition of DAPI and Et on the maleimide labeling of the QacA A327C and P331C mutant proteins (Fig. 6), supporting the earlier suggestion that monovalent and bivalent substrates bind to distinct binding sites (8) and confirm the complex nature of the multidrug-binding site of the QacA protein.

Although the hydrophobic core of TMS 10 is proposed to comprise 20 amino acid residues from Phe-310 to Ile-329, based on solvent-accessibility analysis, the actual length of the TMS may be longer. NEM treatment abolished DAPI and significantly inhibited Et export mediated by the QacA P309C mutant (Fig. 5), implying that NEM alkylation caused steric hindrance to both DAPI and Et transport. This QacA derivative also had lower than wild-type export activities (Fig. 4). Additionally, Pro-309 is located on the same face of TMS 10 as Ala-320, Asp-323, and Ala-327 (Fig. 7B). Taken together, it is quite likely that Pro-309 is on the external periphery of TMS 10 and hence close to the substrate transport pathway. However, the intracellular terminus of TMS 10 cannot be definitely assigned based on the current data.

Originally, TMS 10 was predicted to extend to Leu-333 (4, 38). The QacA A330C, P331C, and G332C mutant proteins were moderately reactive to fluorescein maleimide, whereas P329C and L333C were barely reactive (Fig. 2). A similar reactivity pattern, in which three residues reactive with maleimide are flanked by two non- or weakly reactive residues, has been observed at several ends of TMS 10 of the E. coli tetracycline transporter TetA(B), making the exact extents of these TMS also difficult to be determined (10, 17). However, one clue that can help to identify the cytoplasmic boundary of TMS 10 comes from the positions of highly conserved MFS-specific amino acid sequence motifs (38, 39) in the crystal structure of the E. coli glycerol-3-phosphate transporter GlpT (36). In GlpT, the four conserved positively charged residues of motif A, RXXR, and RXXR from a less well conserved motif were found to delineate the ends of loops 2–3 and 8–9, respectively (36).

In the 14-TMS QacA protein, these motifs are also present, with residues Arg-336 and Lys-340 in loop 10–11 corresponding to the conserved basic residues of the second duplicated motif of the 12-TMS transporters (39–41). It is reasonable to speculate that these conserved MFS-specific motifs play an analogous role in the 14-TMS family of proteins and are therefore located at similar positions. Accordingly, TMS 10 in QacA may extend from Pro-309 on the external facing side to at least Ala-334 on the cytoplasmic side (Fig. 7).

In summary, the studies presented here show evidence that TMS 10 forms an integral part of the substrate-binding pocket of QacA, with residues Asp-323 and Met-319 directly involved in the binding of bivalent substrates. Gly-313 has also been identified as a functionally important residue that appears to assume a structurally important role common to the transport process. Furthermore, mutagenic studies clearly demonstrate the multifaceted nature and flexibility of the multidrug-binding sites that can easily adapt to modifications and that appear to be a common trend among multidrug-binding proteins (42–46).

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