OxIT, the oxalate-formate antiporter of Oxalobacter formigenes, has a long charged residue, lysine 355 (Lys-355), at the center of transmembrane helix 11 (TM11). Because Lys-355 is the only charged residue in the hydrophobic sector, we tested the hypothesis that lysine 355 contributes to the binding site for the anionic substrate, oxalate. This idea was supported by mutational analysis, which showed that of five variants studied (Lys-355→ Cys, Gly, Gln, Arg, or Thr), residual function was found for only the K355R derivative, in which catalytic efficiency had fallen 2,600-fold. Further insight came from a study of TM11 single-cysteine mutants, using the impermeant, thiol-specific reagents, carboxyethyl methanethiosulfonate and ethylsulfonate methythiosulfonate. Of the five reactive positions identified in TM11, four were at the cytoplasmic or periplasmic ends of TM11 (S344C and A345C, and G366C and A370C, respectively), whereas the fifth was at the center of the helix (S359C). Added study with carboxyethyl methanethiosulfonate and ethylsulfonate methylthiosulfonate showed that the attack on S359C could be blocked by the presence of the substrate, oxalate, and that protection could be predicted quantitatively by a kinetic model in which S359C is accessible only in the unliganded form of OxIT. Parallel study showed that the proteoliposomes used in such work contained OxIT of right side-out and inside-out orientations. This, coupled with the finding of substrate protection, leads us to conclude that S359C lies on the translocation pathway through OxIT. Since position 359 and 355 lie on the same helical face, we suggest that Lys-355 also lies on the translocation pathway, consistent with the idea that the essential nature of Lys-355 reflects its role in binding the anionic substrate, oxalate.

In the anaerobic bacterium, Oxalobacter formigenes, the proton-motive force is generated by the combined action of an internal oxalate decarboxylation system and the electrogenic oxalate/formate antiporter, OxIT (1–4). OxIT therefore plays a pivotal role in construction of a “virtual” proton pump, an organizational scheme with relevance to several aspects of microbial cell biology (4–6). Biochemical study (2, 7, 8) suggests that OxIT, a member of the major facilitator superfamily (MFS) (9), may also serve as a valuable model for more broadly directed studies of membrane transport proteins. For such reasons, further study of OxIT may contribute to ongoing studies of both the biology and biochemistry of membrane transport.

Hydropathy analysis of the OxIT amino acid sequence (3), together with circular dichroism spectroscopy of the purified, solubilized protein (8), suggests the presence of 12 transmembrane α-helices, a characteristic shared by most other members of the major facilitator superfamily (9). This analysis also predicts that Lys-355 is positioned near the center of transmembrane helix 11 (TM11) (3), an expectation verified by site-directed fluorescent labeling of single-cysteine variants in this vicinity (10). Direct evaluation of OxIT topology (8) suggests that Lys-355 does not interact with a nearby anionic residue, thereby raising an apparent paradox. Placement of an uncompensated charge within a hydrophobic environment adds a destabilizing element to membrane protein structure (12, 13), but OxIT can be remarkably stable in lipid-detergent micelles (7, 10). This contradiction might be reconciled if the energetic disadvantage contributed by Lys-355 were used to offset the energetic cost of binding and/or transporting substrate anions. Such compensating effects could be achieved in a simple way if TM11 forms part of the substrate translocation pathway, allowing direct interactions between positively charged Lys-355 and negatively charged substrate. That solubilized OxIT is intensely stabilized by the presence of its substrate anions (7, 10), gives indirect support to this view, but more concrete observations are necessary if this model is to guide further work.

The experiments reported here sought to address such issues by asking if one or more residues on TM11 is involved in substrate binding and/or translocation. To do this, we first examined the role of Lys-355 itself by analysis of site-specific mutants.

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mutants. We then exploited a panel of single-cysteine variants to search throughout TM11 for perturbations attributable to either cysteine substitution or to site-specific chemical modification. Together, these two lines of study provide evidence justifying the idea that as substrate passes through OxlT, it encounters residue(s) along TM11 in the vicinity of Lys-355.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Protein Expression**—Wild-type OxlT was encoded within a 1.4-kilobase XbaI-HindIII fragment in plBluescript II SK + ; expression of OxlT was regulated by the lac promoter (3). This plasmid also served as the vector for site-directed mutagenesis using the double-stranded protocol (Cameleon8, Stratagene) to generate mutants of Lys-355. Similar techniques were used to construct a fully active cysteine-less variant with nine tandem histidine residues added at its C terminus to facilitate protein purification (8, 10). This latter derivative was used as the template for generation of the panel of single-cysteine derivatives spanning TM11 (10). OxlT and its mutants were carried in *Escherichia coli* strain XL-1, together with plasmid pM8421 (Spec+ LacI+) to limit inappropriate basal expression (3). OxlT expression was induced by addition of 0.5 mM isopropyl-1-thio-β-n-galactopyranoside to cells in the mid-exponential phase of growth; cells were harvested after an additional 4 h growth.

**Membrane Preparation and Assays of Oxalate Transport**—Membrane ghosts, prepared as described (10, 14), were suspended in distilled water; membrane proteins were solubilized by incubating for 20 min on ice in Buffer A (20 mM MOPS/K (pH 7), 20 mM N,N,N-trimethylprropanol, 4.2 mg/ml *E. coli* phospholipid, 10 mM potassium oxalate, and 1.5% (w/v) octylglucoside). Here, and in all work involving thiol labeling (below), the *E. coli* lipid used for solubilization and reconstitution was hydrated with distilled water rather than with the usual 2 mM β-mercaptoethanol (1). Solubilized protein (0.7–1 mg/ml) was reconstituted by detergent dilution (10, 15) to give proteoliposomes loaded with 100 mM potassium oxalate, 50 mM MOPS/K (pH 7).

In most cases, [14C]Oxalate transport was measured by a rapid filtration assay. Triplicate 100–200-μl aliquots of proteoliposomes were placed at the center of Millipore GSTF filters (0.22-μm pore size), washed twice with 5 μl of Buffer B (100 mM potassium sulfate, 50 mM MOPS/K, (pH 7) to remove external (unlabeled) oxalate, and after interrupting the vacuum, proteoliposomes trapped on the filter were overlaid with 300 μl of Buffer B containing 100 μM [14C]oxalate. Unless otherwise indicated, the assay was terminated after 20 s by filtration, followed with two washes using 5 ml of iced Buffer B. Depending on experimental design, oxalate transport is given as a fraction of the maximum incorporation or as relative to that of the cysteine-less parental protein, after correction for differences in protein expression (10). This assay provides a wider dynamic range than available earlier (3, 8, 10) and is especially suited to the parallel study of a large number of samples.

[14C]Oxalate transport was also monitored by a traditional filtration assay (3, 10). In these cases, washed proteoliposomes suspended as a concentrated stock in Buffer B were diluted 10-fold into this same buffer at 23 °C, and the transport reaction was initiated by addition of either 100 μM or 1 mM labeled substrate, as specified. At timed intervals, samples were withdrawn for filtration and washing.

**Thiol Labeling**—To screen for thiol reactivity in the TM11 panel of single-cysteine derivatives, membrane ghosts were placed with excess probe (2 mM MTSET or 1 mM MTSES) in 20 mM potassium phosphate (pH 8) for 1 h at 23 °C. Unreacted probe was removed either by a quench using 5 mM β-mercaptoethanol, or by four cycles of washing with 20 mM potassium phosphate (pH 7), as noted. Subsequently, membrane protein was solubilized and reconstituted as described above. These conditions allow labeling of residues on both intra- and extracellular surfaces (10).

**OxlT Orientation after Reconstitution**—The orientation of OxlT in proteoliposomes was deduced from the pattern of thiol labeling of cysteines placed at either end of TM11 and also by monitoring the effects on substrate transport of external trypsin. For thiol labeling, the target protein was solubilized and reconstituted as described above. These con-
Fig. 1. Lys-355 is essential to normal OxlT function. A, proteoliposomes were prepared from extracts of wild type OxlT and from mutants in which Lys-355 was replaced by Arg, Thr, and Gin. (The K355C mutant is described below.) Oxalate-loaded proteoliposomes suspended in Buffer B were given 1 mM labeled substrate. Samples were taken at the indicated times for filtration and washing to determine \([^{14}C]\text{Oxalate accumulation.} B,\) cartoon showing the organization of TM 11; amino acids are given using the single letter code.

**RESULTS**

Two classes of experiments explored the idea that Lys-355 and other residues on TM11 might be associated with substrate binding and translocation. In one group of studies, described immediately below, the role of Lys-355 itself was examined by noting the behavior of its mutants. In a second group of experiments, cysteine-scanning mutagenesis identified a residue, near to Lys-355, which served as an informative target for cysteine-directed agents. Both lines of study provided evidence consistent with the idea that Lys-355 and residues in its vicinity normally serve to aid in the translocation of OxlT substrates.

**Mutations of Lys-355 Disrupt OxlT Function**—If Lys-355 is essential to OxlT function, substitutions at this position should compromise activity. To test this prediction, we used site-directed mutagenesis to replace Lys-355 with alternate residues, including cysteine, glycine, glutamine, arginine, and threonine. In the usual assays of transport by OxlT (see “Experimental Procedures”), these mutants displayed little or no function. In one case (K355G) this was attributable to lack of expression; all other mutants were found in membranes in amounts comparable to the wild type (data not shown; see Ref. 10), so that lack of function was due to failure of OxlT itself. We considered the possibility that such null responses might reflect a poor substrate affinity, and for that reason we also measured transport by oxalate-loaded proteoliposomes using external \([^{14}C]\text{oxalate at 1 nM rather than the usual 0.1 mM. In only one case (the K355R variant) did this added test reveal a significant activity (Fig. 1), although the time course of transport by the mutant was greatly extended relative to that of the parental wild type protein. In separate experiments, we performed a further, kinetic analysis of the K355R derivative. That work showed this mutant to have both an elevated Michaelis constant \((K_m, 5.4 \text{ versus } 0.15 \text{ mM})\) and a reduced maximal velocity \((V_{max}, 66 \text{ versus } 4,700 \text{ nmol/min/mg protein})\), leading to a 2,600-fold reduction in catalytic efficiency \((V_{max}/K_m)\) relative to the parental protein. Accordingly, because mutants of Lys-355 retained little or no function, we conclude this residue is essential for normal OxlT function.

**Functionally Significant Residues in TM11**—The behavior of Lys-355 mutants (Fig. 1) suggests that OxlT requires (at least) the presence of positive charge at position 355. To ask whether nearby residues might also have functional significance, we analyzed a panel of TM11 single-cysteine variants that had earlier been used to establish topological relationships in this region (10). We first tested \([^{14}C]\text{Oxalate transport under initial rate conditions during the oxalate self-exchange reaction (Fig. 2A). The N347C, A368C, and G363C variants were too poorly expressed (\(<5\%\) the parental level) for this analysis, but all others were present at levels high enough (\(>40\%\) normal) (10) to assess functional status. Among the 27 expressed proteins, two (G349C and K355C) gave undetectable levels of \([^{14}C]\text{oxalate transport;} three others (A354C, G362C, G363C) gave marginal responses (0.2–1\% residual activity). In all other cases, we found activity corresponding to at least 1\% of the cysteine-less parental protein, yielding a signal-to-noise ratio of at least 20. Since the turnover number for OxlT is at least 1000-fold greater than usually observed for membrane transporters (1, 4, 8), even these low relative levels of activity might reflect significant rates of anion exchange. Such findings, including the null behavior of K355C, are consistent with earlier studies (10) and with the results noted above (Fig. 1).

In earlier work with this same set of single-cysteine variants, we showed that the TM11 core region (positions 351–361; Fig. 2A) is inaccessible to Oregon Green maleimide, a hydrophilic thiol-reactive agent of moderate size (\(<500\) daltons) (10). The present work shows that, although cysteine substitutions giving reduced function (\(<10\%\) parental) are broadly represented throughout TM11, a striking distribution is found in this inaccessible region. Within this core, cysteine substitutions yielding low specific activity are restricted to the helical face containing Lys-355 (Fig. 2B), indicating a distinct functional asymmetry in this area.

**Accessibility to MTSES and MTSET**—The high velocity of OxlT-mediated reactions ensures that even cysteine substitution mutants with low residual activity (Fig. 2A) show satisfactory signal-to-noise ratios. For this reason we pursued a study of these variants using the thiol-specific probes, MTSES and MTSET. These agents were chosen for their specificity in modification of cysteine (17), because their polar and linear character increases the probability they might have access to regions within OxlT near a substrate binding region (e.g. possibly near Lys-355), and because they generate reaction products of
a clearly distinct character-MTSET implants a fixed positive charge (RS-SCH$_2$CH$_2$N(CH$_3$)$_2$), whereas the reaction with MTSCE results in appearance of a carboxyl group that would carry negative charge at pH 7 (RS-SCH$_2$CH$_2$COO$^-$).

Membranes containing TM11 single-cysteine variants were exposed to excess MTSET or MTSCE, with and without tests for reversibility by later exposure to β-mercaptoethanol. Subsequent to this in situ labeling, protein was solubilized, and assays of transport by proteoliposomes recorded residual function. MTSET and/or MTSCE gave significant inhibition at five positions, two each in the TM11 cytoplasmic (S344C, A345C) and periplasmic (G366C, A370C) domains (Fig. 3, top), and one (S359C) within the TM11 core region (Fig. 3, bottom). In each of these cases, there was unambiguous reversal of inhibition by β-mercaptoethanol, suggesting an unrestricted access to the target cysteines by the probes (despite their differing charge), and an equally free access to their modification products by the mercaptoethanol. In two instances MTSCE, but not MTSET, gave an irreversible block (A354C, A358C), but the partial nature of this inhibition (~40%) did not suggest further study would be helpful. Finally, we noted that I360C, also within the TM11 core, responded to MTSET with prominent inhibition but incomplete reversibility, and to MTSCE with a partial inhibition that was similarly not reversed.

Modification of the four peripheral residues (S344C, S345C, G366C, A370C) might have been predicted, since earlier work characterized these regions as accessible to (and reactive with) Oregon Green maleimide or rhodamine maleimide (Ref. 10, and experiments not shown). On the other hand, prominent and readily reversible effects at S359C were unexpected, since this position lies in an “inaccessible” domain (10). We therefore analyzed in more detail the reactivity of this position.

**Oxalate Protects against Modification of S359C**—Because positions 355 and 359 are found on the same helical face (Fig. 2B), evidence that position 359 is on the substrate translocation pathway through OxlT could strengthen the idea that this region forms part of a substrate-binding center. Given the sensitivity of S359C to MTS-linked probes (Fig. 3), such evidence would be provided, in part, by demonstration that oxalate (substrate) protects against inhibition by such thiol-directed agents.

In exploring this possibility, use of MTSET was preferred over MTSCE for reasons of probe selectivity (see below) and stability (half-lives of minutes versus hours in aqueous solution at room temperature, respectively (Ref. 17)). Trials to establish a dose-dependence for probe inhibition of the S359C protein showed that a 5-min treatment of proteoliposomes with 10–30 μM MTSCE decreased OxlT function by about 50% (data not given; see Fig. 4A). We also confirmed that such inactivation took place with an exponential time course (data not shown) and that the presence of substrate markedly reduced such inhibition (see below). Since MTSET had no effect on oxalate transport by cysteine-less OxlT, parent to S359C, we attributed the action of MTSET to a modification of S359C itself. Further, such inhibition appeared relatively specific to the reaction at position 359 and to the anionic nature of MTSET. Partial inhibition of the A358C and I360C variants required a ~5-min treatment with 1–2 mM MTSET (not shown; see Fig. 3), and while MTSET inhibition of S359C (e.g. Fig. 2) was found, comparable effects required 5–10-fold greater concentrations of MTSET than MTCE (data not given).

These preliminary findings, based on reconstitution of crude extracts, led us to develop a simple kinetic model as a framework for interpreting the effects of MTS-linked agents on S359C (see “Experimental Procedures”). To test this model explicitly, we then moved to work with the purified S359C protein. That work indicated the reduced activity of S359C relative to its parent stemmed largely from an increased Michaelis constant (1 mM versus 0.1 mM oxalate), and not a marked change in maximal velocity (150 versus 220 μmol of oxalate/min/mg of protein, respectively). In tests of MTSET inhibition of the reconstituted material, we found a simple exponential relationship between probe concentration and the extent of inhibition (Fig. 4A), as predicted by the kinetic model noted earlier. In a more stringent test, we chose a probe concentration that yielded near-maximal inhibition, and then asked how much inhibition was altered in the presence of oxalate. In that case (Fig. 4B), we recorded a ~95% inhibition by 300 μM MTSET alone, with progressively diminishing effects as oxalate was introduced. At 10–20 mM oxalate, the MTSET block was reduced by half, and at 100 mM oxalate there was a nearly complete rescue. More important, throughout this range, the degree of protection was predictable by the model, given an OxlT dissociation constant ($K_d$) of 2.4 mM (Fig. 4C).

In four other trials of similar design, using either purified protein or crude extracts, we calculated $K_d$ values of 1.5–3.3 mM (mean ± S.E. of 2.2 ± 0.4 mM) for both MTSET (at 30 or 300 μM) and MTSES (at 300 μM). This derived $K_d$ value is compatible with the elevated Michaelis constant for oxalate exchange by the S359C mutant (see above), and is also consistent with an
independent estimate of \( K_d \) (2.4 mM), based on use of oxalate to stabilize against thermal denaturation (as described in Refs. 7 and 8) (data not shown). Such findings (Fig. 4) confirm the accessibility of S359C to MTS-linked probes and document that this behavior is described by a simple scheme that assumes access to position 359 is possible only when OxlT is in an unliganded state (see “Experimental Procedures”). We also note that inhibition by MTSCE or MTSES must reflect probe action from the external medium, because an attack from the inner surface (e.g. following any slow inward diffusion of probe) would not take place in the presence of 100 mM internal oxalate. Further, because full inhibition occurs in the absence of substrate, external probe must have access to the entire OxlT population.

**MTSCE and MTSES Approach S359C from Either Surface of OxlT**—The interpretation of such findings depends importantly on whether the orientation of OxlT in proteoliposomes is uniformly right side-out (RSO), as in the intact cell, uniformly inside-out (ISO) or a mixture of RSO and ISO forms. Because UhpT, a related antiporter, is found in both RSO and ISO
orientations after reconstitution (16), a mixed distribution would seem the most likely for OxlT. If so, the full inhibition by external MTSCe and MTSES indicates that the probes gain access to S359C by moving through pathways present in both orientations. By contrast, if OxlT orients as either fully RSO or fully ISO, the approach of such probes to S359C can be confirmed for only one form of the protein. It was essential, therefore, to establish the orientation of reconstituted OxlT.

We performed two experiments to address this issue. In one case, we reconstituted the wild type protein and treated proteoliposomes with excess trypsin, as previously described in "Experimental Procedures." Upper panel, detection of MPB labeling. For each protein, proteoliposomes were divided into four portions of 0.5 ml each, corresponding to the lanes shown: a, solvent control; b, MPB treatment; c, MPB treatment after exposure to MTSET; d, MPB treatment followed by trypsin treatment. Lower panel, detection of OxlT orientation with MPB. The blot used to record MPB labeling (upper panel) was stripped and re-probed with the N-terminal polyclonal antibody. For the two samples treated with trypsin, we assume part of the diminished signals reflect cleavage of the ISO forms present in both A345C and A370C populations.

FIG. 5. Mixed orientation of OxlT in proteoliposomes. A, trypsin susceptibility. After reconstitution of purified wild-type protein, proteoliposomes were placed in Buffer B with (○) or without (△) 1 mg/ml trypsin. At the indicated times, the reaction was quenched by 25-fold dilution into iced Buffer B containing 0.5 mM PMSF. After centrifugation and resuspension in Buffer B, residual OxlT function was determined by a modified filtration assay, using 60-s incubations with 100 µM labeled substrate. Inset, a Western blot showing OxlT present after the 60-min incubation in the presence (+) or absence (−) of trypsin, using a polyclonal antibody reactivity with the OxlT N terminus (3). B, thiol labeling of the ends of TM11. The A345C and A370C proteins were separately purified and reconstituted, after which thiol labeling and trypsin treatment were performed as noted in A and under "Experimental Procedures." Upper panel, detection of MPB labeling. For each protein, proteoliposomes were divided into four portions of 0.5 ml each, corresponding to the lanes shown: a, solvent control; b, MPB treatment; c, MPB treatment after exposure to MTSET; d, MPB treatment followed by trypsin treatment. Lower panel, detection of OxlT orientation with MPB. The blot used to record MPB labeling (upper panel) was stripped and re-probed with the N-terminal polyclonal antibody. For the two samples treated with trypsin, we assume part of the diminished signals reflect cleavage of the ISO forms present in both A345C and A370C populations.

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We performed two experiments to address this issue. In one case, we reconstituted the wild type protein and treated proteoliposomes with excess trypsin, as previously described in studies with UhpT (16). Because of the distribution of positively charged residues in transporters within the major facilitator superfamily (4, 16), we anticipated that trypsin would cleave at the OxlT cytoplasmic surface, but not at its extracellular face. Indeed, that oxalate transport was reduced by nearly 50% (Fig. 5A), in parallel with an equivalent loss of immunoreactive material (Fig. 5A, inset), consistent with this view, provided RSO and ISO forms have comparable kinetic properties and are present in about equal proportion, as is true for UhpT (16).

In a second kind of experiment, we monitored the accessibility of cysteines placed at either end of TM11, using MPB and MTSET, two water-soluble, thiol-reactive probes of low membrane permeability (16–18). As targets, we selected the A345C and A370C single-cysteine derivatives, because in these variants cysteine is placed at the cytoplasmic or extracellular face of TM11, respectively (10)2 (see Fig. 2), and because cysteine at either of these locations reacts with both maleimide- and MTSET-linked probes (102) (Fig. 3). If reconstitution of OxlT yields a uniformly RSO population, only the A370C derivative should be labeled, while a fully ISO population would allow labeling of only the A345C protein. On the other hand, if reconstitution gives a population containing both RSO and ISO forms, the cysteine on either derivative will be available to impermeant, external probes. Results obtained in this kind of experiment clearly favor the last scenario, since both mutant proteins can be labeled by external MPB (Fig. 5B). Moreover, in each case, MPB modification was blocked by a prior exposure of proteoliposomes to external MTSET, indicating that the reaction with MPB occurs from the external medium. As well, in this same experiment, MPB-labeled proteoliposomes were treated with trypsin to test the idea (Fig. 5A) that only one surface of OxlT contains an accessible trypsin cleavage site(s). Exposure to trypsin led to disappearance of the label on A345C, whereas signal derived from A370C was retained. This verifies the inference that the cytoplasmic, but not the periplasmic, face of OxlT contains a trypsic cleavage site(s) and further strengthens the conclusion that MPB labeling is confined to residues facing the external medium.

Taken together, these two different experimental approaches (Fig. 5) show that OxlT reconstitutes in mixed orientation, as does UhpT (16), and that RSO and ISO forms of equivalent kinetic properties are present in about equal proportion. This conclusion, coupled with the fact that external MTSCe or MTSES yields full inhibition of S359C (Fig. 4), shows that these probes are able to approach S359C by entering from either surface of the protein.

Discussion

Solute transporters such as OxlT have a network of key residues whose role is to facilitate substrate movement into and out of an appropriate binding site, thereby defining a translocation pathway through the protein (19, 20). One also presumes that, for transporters of polar molecules such as oxalate, this pathway will be enriched for residues of a more hydrophilic character than found elsewhere in the protein (20–24). For these reasons, Lys-355 of OxlT merits close attention. Not only is Lys-355 the only charged residue in the OxlT hydrophobic sector (3), it is strategically placed at the center of TM11 (10), where it might take part in binding of the anionic substrate, oxalate. If so, one may expect that residues on the same helical face as Lys-355 might also contribute to the substrate translocation pathway. Mutagenesis of Lys-355, along with thiol-specific modification of single-cysteine variants throughout TM11, has now given strong positive support to these ideas.

Lys-355 Is Essential—The results of directed mutagenesis indicate that Lys-355 is critical to OxlT function, for among the substitutions examined (Lys-355 → Cys, Gly, Gln, Arg, or Thr), only the K355R variant retains any vestige of function (Fig. 1). In that case, however, catalytic efficiency is markedly reduced, reflecting reductions in both the affinity for and velocity of oxalate transport. The phenotype of such mutants leads us to conclude that anion transport by OxlT requires a positive charge, preferably lysine, at position 355. The kinetic changes found in the K355R mutant also provide direct evidence supporting the proposed role of Lys-355 in substrate binding.

Assignment of TM11 to the Translocation Pathway—A cysteine scan of TM11 strengthens the idea that Lys-355 lies in a region of significance, since the helical face containing this residue is highly susceptible to mutational perturbation (Fig. 2). Further work with this single-cysteine panel led to identification of a variant (S359C) that is reversibly modified by MTSS-linked agents (Fig. 3). Study of S359C became especially revealing when it was shown that OxlT reconstitutes as a mixed population (Fig. 5), as does UhpT (16); in both cases, about half the molecules orient as in the intact cell (RSO), and half with the reverse polarity (ISO) (see Ref. 25 for recent discussion). This observation is significant, because in oxalate-loaded proteoliposomes, these two orientations should also correspond to the two main conformations in the OxlT catalytic
cycle (Fig. 6) (26). Thus, in the absence of external substrate, efflux of internal oxalate through RSO molecules should freeze OxlT in the open conformation normally used to receive external substrate at the beginning of the first half-turnover. By contrast, efflux through ISO molecules would leave the pathway trapped in the form used normally to accept internal substrate as the concluding half-turnover is initiated (Fig. 6).

As a result, we are able to ask if a single residue, S359C, is accessible to a hydrophilic probe (MTSCE, MTSES) when OxlT adopts the conformation used to initiate influx (RSO) as well as the conformation used to initiate efflux (ISO). This is an important question, since a defining criterion of residues on the translocation pathway is that within a single catalytic cycle they are alternately exposed to the cis and trans environments (via cis- and trans-facing conformations) (19, 20). That OxlT is fully inhibited by external MTSCNE or MTSES (Fig. 4) indicates that S359C is, in fact, available in both conformations, fulfilling the requirement needed to assign position 359 to the translocation pathway. Equally significant, the quantitative analysis of substrate protection suggests that approach of such probes to their target is blocked by the conformational changes associated with substrate binding and/or transport (e.g., Fig. 4). This is taken as strong additional support for the idea that position 359 lies on the pathway taken by substrate itself.

Assignment of S359C to the translocation pathway does not require that cysteine (in S359C) or serine (in wild type OxlT) take part in substrate binding, although this may occur. Instead, this interpretation specifies only that position 359 be part of the surface enclosing substrate as it passes through the protein, across the membrane (19, 20). Similar arguments were presented when assigning positions on TM7 to the translocation pathway of UhpT (19, 20), but in that case the experimental system was complex, requiring comparisons of probe accessibility in the intact cell and everted membrane vesicles. We believe the present work is far more convincing in that it exploits a reconstituted preparation with purified protein under conditions that allow one to monitor the response of both conformations of the target transporter.

Comparisons with Other Transporters—UhpT offers instructive parallels to the work presented here. Thus, in TM11 of UhpT one finds an intrahelical salt bridge involving Lys-388 and Asp-391 (27). Neither of these charged residues is required for the transport of glucose 6-phosphate, since normal function is present in mutants lacking both residues (the K388C/D391S variant) (27). On the other hand, when TM11 of UhpT contains a lone positive charge (the D391C mutant), substrate selectivity is biased to favor molecules such as PEP, which carry an additional negative charge into the active site (27). At least, such findings suggest that portions of TM11 lie on the translocation pathway through UhpT and that they can do so without at the same time directly influencing substrate specificity, much as we suggest for position 359 in OxlT. Work with UhpT makes it equally clear, however, that residues lining the pathway may exert a dominant influence on substrate preference if there is a required electrostatic interaction between substrate and protein, as we suggest is normal for Lys-355 in OxlT.

The correspondence between the behavior of OxlT, UhpT, and their mutants, is in accord with their common membership in the MFS (9). In this regard, one might also note that within this superfamily of mostly 12-helix transporters, TM11 is the least hydrophobic transmembrane helix (21). This, considered along with our findings in OxlT and UhpT (26), leads us to suggest that TM11 will be found to line the transport pathway in all other examples within the MFS.

Relevance to Mechanism—It is commonly accepted that, for energetic reasons, charged residues are frequently found in transmembrane a-helices, an expectation generally borne out in surveys of transmembrane helices known at atomic resolution (see Ref. 10). The same appears true if less direct criteria are used, as in UhpT or LacY, where one finds that such charged residues may be organized by intra- or interhelical salt bridges (11, 26, 28, 29). Accordingly, the presence of uncharged residues in the hydrophobic sector of such membrane proteins is viewed as introducing inappropriate destabilizing influences. For proteins involved in electron transport, where a fixed geometry between prosthetic groups may be of paramount importance, this argument would appear justified. However, just this kind of structural flexibility could be of advantage to solute carriers and facilitators, which adopt multiple conformations as they enclose and transport their substrates. Indeed, demonstration that TM11 lies on the OxlT transport pathway leads us to suggest that such cost-benefit considerations may be essential to this antiporter. Thus, we imagine the oxalate-binding site comprising liganding groups arising from several transmembrane helices. One of these groups is Lys-355, whose presence should, for thermodynamic reasons, promote a conformation that is “open” to the external hydrophilic environment and at the same time establish the electrostatic driving force that attracts negatively charged substrate, oxalate. Once within the transporter, the formation of an ion pair between Lys-355 and a substrate carboxyl would enable closure of the translocation pathway, which may then reopen spontaneously to face either membrane surface. If substrate dissociates when the pathway faces the original (cis) surface, no net substrate movement occurs; but if the pathway is facing the opposite (trans) surface when substrate leaves, net transport will occur. This mechanistic view is not only suggested by our findings, it may also rationalize the elevated $K_d$ and $K_m$ found for the S359C variant. Thus, the proximity of Lys-355 may lower the pK$_a$ of the thiol at position 359, placing the anionic sulfide group (-S$^-$) in the pathway and reducing fractional occupancy of the pathway by the anionic substrate. Placement of a deprotonatable group in the pathway may also have consequences for the electrogenic character of the heterologous oxalate:formate exchange, a question to be explored in later work.

We emphasize that Lys-355 is only one of several residues that may be involved in construction of the substrate-binding site. Judging from the OxlT amino acid sequence, and with the assumption that this binding site lies in the hydrophobic sector (see Refs. 22 and 27), we would expect TM2 to be of equal importance, since it is the only other OxlT transmembrane helix with significant polarity. The experimental determination of OxlT topology indicates that, of the 20 residues that can now be assigned to TM2 (Asn-47 through Gln-66), there are 8...
potential hydrogen bond donors (Asn-47, Ser-51, Gln-56, Thr-57, Thr-60, Ser-62, Gln-63, Gln-66). We suggest, therefore, that binding of divalent oxalate is accommodated by two distinct mechanisms: (a) by the electrostatic interaction between one carboxyl group and Lys-355 on TM11, and (b) by interaction of the second carboxyl group with the cluster of hydrogen bond donors on TM2. It also seems feasible that, in the absence of substrate, the net charge on Lys-355 is partly stabilized by the cluster of electronegative centers (O, N) in TM2. For these reasons, we consider it significant that TM2 and TM11 are nearest neighbors in the general structural model for proteins within MFS (21) and in the specific model generated by biochemical studies of LacY (22).

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