Structure/Function Relationships in OxlT, the Oxalate/Formate Antiporter of Oxalobacter formigenes

ASSIGNMENT OF TRANSMEMBRANE HELIX 2 TO THE TRANSLOCATION PATHWAY

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We constructed a single cysteine panel encompassing transmembrane helix two (TM2) of OxlT, the oxalate/formate antiporter of Oxalobacter formigenes. Among the 21 positions targeted, cysteine substitution identified one (phenylalanine 59) as essential to OxlT expression and three (glutamine 56, glutamine 66, and serine 69) as potentially critical to OxlT function. By probing membranes with a bulky hydrophilic probe (Oregon Green maleimide) we also located a central inaccessible core of at least eight residues in length, extending from leucine 61 to glycine 68. Functional assays based on reconstitution of crude detergent extracts showed that of single cysteine mutants within the TM2 core only the Q63C variant was substantially (>95%) inhibited by thiol-specific agents (carboxyethyl methanethiosulfonate and ethylsulfonate methanethiosulfonate). Subsequent analytical work using the purified Q63C protein showed that inhibition by ethylsulfonate methanethiosulfonate was blocked by substrate and that the concentration dependence of such substrate protection occurred with a binding constant of 0.16 mM oxalate, comparable with the Michaelis constant observed for oxalate transport (0.23 mM). These findings lead us to conclude that position 63 lies on the OxlT translocation pathway. Our conclusion is strengthened by the finding that position 63, along with most other positions relevant to TM2 function, is found on a helical face that can be cross-linked to the pathway-facing surface of TM11 (Fu, D., Sarker, R. I., Bolton, E., and Maloney, P. C. (2001) J. Biol. Chem. 276, 8753–8760).

The antiporter OxlT carries out the electrogenic exchange of divalent oxalate with monovalent formate, a reaction that underlies generation of the proton-motive force in the Gram-negative anaerobe Oxalobacter formigenes (1–3). Although this aspect of bacterial cell biology merits further attention, current studies of OxlT are directed to the development of structural models following the success of electron crystallography, which has established a two-dimensional projection map for this protein (4). Such work may have wider significance because OxlT belongs to the major facilitator superfamily (5), the largest group of evolutionarily related antiporters, uniporters, and symporters (6).

The two-dimensional projection map of OxlT reveals a single central cavity representing the substrate translocation pathway (4), but it is not yet possible to recognize the individual helices that border this pathway or to determine which among them contain substrate-binding elements. To address these issues two experimental strategies have been developed. On the one hand, helix proximity is being examined by disulfide trapping in double cysteine variants (7). In addition and as reported here, selected helices are being subjected to biochemical tests to identify a domain(s) that lines the transport pathway (8–10).

Of the twelve OxlT transmembrane helices, TM2 and TM11 are the least hydrophobic (11, 12) and therefore the most likely to specify residues that interact with oxalate (the hydrophilic substrate). In this respect, TM11 has been an attractive candidate for some time because it contains lysine 355, the only charged residue in the OxlT hydrophobic sector and a likely substrate-binding element. Recent work now confirms that TM11 lines the transport pathway and that a positive charge at position 355 is essential to OxlT function (9). By contrast, evidence suggesting that TM2 might line the OxlT pathway has been speculative, deriving largely from its unusually high content of polar residues (Fig. 1) because these may facilitate substrate binding via hydrogen bonding (10, 11).

The experiments summarized here were designed to address the specific question of whether one or more residues on TM2 lies on the translocation pathway. To explore the issue, we used cysteine-scanning mutagenesis together with application of hydrophilic and impermeant thiol-specific probes. Our findings provide direct evidence supporting the idea that TM2 lies on the OxlT substrate translocation pathway and that this domain contributes residues critical to OxlT function.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Protein Expression—Mutations in the 21-residue stretch representing TM2 (Fig. 1) (11) were generated by a double-stranded protocol (Chameleon, Stratagene) using as host a parental plasmid (pOxlTHis) that specifies OxlT lacking its two normal cysteines (C28G and C271A) and containing a C-terminal polyhistidine extension to enable metal chelate affinity chromatography (13). All mutants were confirmed by DNA sequencing. Cysteine-less OxlTHis and its variants were carried in Escherichia coli strain XL3, which harbors plasmid pMS421 (SpecrLacIq) to limit basal expression (12). A few colonies from a fresh transformation were grown overnight at 37 °C with shaking in Luria-Bertani medium containing ampicillin (100 μg/ml) and spectinomycin (50 μg/ml). Overnight cultures were diluted 20-fold into 40 ml of Luria-Bertani medium with antibiotics and grown for 1 h before OxlT expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside; cells were harvested by centrifugation after an additional 2.5-h growth.
Functional Reconstitution and Assays of Oxalate Transport—Harvested cells were suspended in 5 ml of a lysis solution (300 μg/ml lysozyme, 40 μg/ml DNase, 10 mM Tris-HCl, 5 mM EDTA, 0.75 mM phenylmethylsulfonyl fluoride, pH 7.5) and incubated at 37 °C for 15 min. To prepare membranes, cells were disrupted by 10-fold dilution into 45 ml of iced distilled water after which released cytoplasmic proteins were removed by two cycles of centrifugation and washing with iced distilled water (1). The membrane pellet, which contained a mixture of unsealed sheets and vesicles of normal and everted polarity, was taken up in 2 ml of a solubilization buffer (20 mM MOPS/KOH, 10 mM potassium oxalate, 0.75 mM phenylmethylsulfonyl fluoride, 20% (v/v) glycerol, 1.5% (v/v) octyl-β-D-glucopyranoside, 0.5% (v/v) E. coli phospholipid, pH 7) and shaken at 4 °C for 30 min. The crude extract was clarified by centrifugation (15,000 g for 15 min). To prepare membranes, cells were disrupted by 10-fold dilution into 45 ml of iced distilled water after which released cytoplasmic proteins were removed by two cycles of centrifugation and washing with iced distilled water (1). The membrane pellet, which contained a mixture of unsealed sheets and vesicles of normal and everted polarity, was taken up in 2 ml of a solubilization buffer (20 mM MOPS/KOH, 10 mM potassium oxalate, 0.75 mM phenylmethylsulfonyl fluoride, 20% (v/v) glycerol, 1.5% (v/v) octyl-β-D-glucopyranoside, 0.5% (v/v) E. coli phospholipid, pH 7) and shaken at 4 °C for 30 min. The crude extract was clarified by centrifugation (15,000 g for 30 min) at 4 °C in an Eppendorf refrigerated microfuge and then was stored at −80 °C until use.

OxlT function was assessed after reconstitution of the crude extract into proteoliposomes loaded with 100 mM potassium oxalate as described (1). Unless otherwise noted, initial rates of [14C]oxalate entry into proteoliposomes were measured in duplicate at 4 °C by a filtration assay (11). Proteoliposomes were applied directly to the center of a 0.22-μm pore size GST Millipore filter and washed twice with 5-ml volumes of chilled assay buffer (100 mM potassium sulfate, 50 mM MOPS/KOH, pH 7). On release of the vacuum, proteoliposomes were covered with chilled assay buffer containing 0.1 mM [14C]oxalate, and the reaction was terminated after 40 s by filtration and washing. OxlT function is usually reported as OxlT function as determined by immunoblot analysis (described below).

Immunoblot Analysis—After SDS-PAGE protein was transferred to nitrocellulose and probed with a mouse monoclonal antibody directed against tetrahistidine (Qiagen). Antibody binding was detected by chemiluminescence and quantitated using a Fuji LAS 1000 gel documentation system; the expression of single cysteine mutants was evaluated with relative specific activity by normalization of observed rates to levels of OxlT expression as determined by immunoblot analysis (described below).

Functional Impact of TM2 Single Cysteine Substitutions—Single cysteine mutants were individually engineered into a 21-residue stretch (serine 51 to proline 71) known from previous work (11) to encompass TM2 (Fig. 1). Analysis of this panel showed that with one exception (F59C) such mutagenesis had little effect on OxlT expression (Table I). By contrast, these variants showed considerable variation of specific activity, ranging from 2 to 120% of the parental level (Table I). Severe defects found in the Q56C, Q66C, and S69C mutants (2–5% residual function) point to three TM2 polar residues (glutamine 56, glutamine 66, and serine 69) as potentially essential to OxlT function. Among the latter, further mutagenesis focused on glutamine 56 and glutamine 66, whose cysteine derivatives had especially low specific activities. In these cases, we introduced one of five residues (Ser, Thr, Asn, Lys, Arg) that might function as alternative proton donors as well as one residue (Leu) of a non-polar character. Most of these additional substi-

RESULTS

Fig. 1. Topology of OxlT. A, superimposed on the topology of OxlT (11), the 21 residues comprising TM2 are shown using the single letter code, with polar residues indicated by white letters on a black background. Residues shown on TM11 are on the helical face that abuts TM2, as shown by disulfide trapping (7) (see also Fig. 4). Open circles indicate modifications introduced to generate the cysteine-less, polyhistidine-tagged protein used here. B, TM2 using an expanded scale to indicate residue numbers.
TM2 Lies on the Pathway through OxlT

During the course of a single turnover (10). Such residues would include a variety of residues including (but not restricted to) those directly involved in substrate recognition. Two types of practical tests establish the experimental criteria that identify this collection of conformationally active positions (9, 10, 15, 16). The first test asks if a target

Table I
Levels of expression and specific activities of TM2 single-cysteine variants are relative to those of the cysteine-less parent

<table>
<thead>
<tr>
<th>Variant</th>
<th>Expression</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S51C</td>
<td>57</td>
<td>138</td>
</tr>
<tr>
<td>L52C</td>
<td>145</td>
<td>16</td>
</tr>
<tr>
<td>A53C</td>
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<td>67</td>
</tr>
<tr>
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<td>15</td>
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<td>101</td>
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<td>T57C</td>
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<td>57</td>
</tr>
<tr>
<td>A58C</td>
<td>76</td>
<td>23</td>
</tr>
<tr>
<td>F59C</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>T60C</td>
<td>70</td>
<td>16</td>
</tr>
<tr>
<td>L61C</td>
<td>108</td>
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<tr>
<td>S62C</td>
<td>98</td>
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</tr>
<tr>
<td>Q63C</td>
<td>103</td>
<td>24</td>
</tr>
<tr>
<td>V64C</td>
<td>128</td>
<td>15</td>
</tr>
<tr>
<td>I65C</td>
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<td>61</td>
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</tr>
<tr>
<td>P71C</td>
<td>123</td>
<td>21</td>
</tr>
</tbody>
</table>

* Not determined.

Definition of the TM2 Inaccessible Core Region—Earlier work shows that cysteine residues exposed to the aqueous phase can be identified by their accessibility to the hydrophilic fluorescent probe OGM (8, 11), which is known to be membrane-impermeant under the conditions used here (11). The TM2 single cysteine panel was used to generate membranes of mixed orientation for use in tests of OGM reactivity. In such tests there was a notable discontinuity of response (Fig. 2). Control experiments using OGM to treat denatured protein showed that each example contained a cysteine that could be modified by the fluorophore (not shown; see Ref. 8). Yet when intact membranes containing OxlT were examined, significant labeling was observed only for cysteines at three positions near the periplasmic end of TM2 (A53C, V55C, and T57C) and two cysteines at the TM2 cytoplasmic surface (Q70C and P71C). A negative response was found for each of six cysteines within an eight-residue stretch at the TM2 center (L61C, S62C, Q63C, I65C, A67C, and G68C). We interpret these in situ findings as identifying a centrally placed TM2 core, minimally of eight residues in length, that is inaccessible to OGM because of a more rigid helix packing and lowered mobility that restricts access of the bulky probe (463 daltons) to targets deep within the hydrophobic sector (8, 11). Of the four residues identified earlier as important to TM2 function, at least two (phenylalanine 59 and glutamine 66) lie within this core. A third (serine 69) may also lie within this core (the low specific activity of the S69C variant precluded tests of OGM accessibility), whereas the fourth (glutamine 66) lies at the periplasmic border of the core domain.

Modification by MTS-linked Probes—Cysteine-scanning mutagenesis (Table I) highlights four TM2 residues as relevant to OxlT expression or function (glutamine 56, phenylalanine 59, glutamine 66, and serine 69). In an attempt to identify additional residues of interest, we selected other targets in our single cysteine panel and exposed proteoliposomes containing each variant to each of three MTS-linked probes after reconstruc-
residue can be approached from both cytoplasmic (internal) and periplasmic (external) surfaces by a suitable probe; if so, the second test asks if such access is prevented by the presence of substrate. With respect to OxlT TM2, the first of these questions is answered affirmatively by the finding (Fig. 3) that both MTSES and MTSEC give nearly complete inhibition of Q63C variant. Thus, because OxlT is present in both orientations (inside-out and right side-out) after reconstitution (9), full

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**Fig. 2. In situ labeling of OxlT with OGM.** Membranes derived from cells expressing the indicated TM2 single cysteine variants were exposed to 40 μM OGM at pH 8 to label cysteines exposed to the aqueous medium. The membranes were solubilized, OxlT was purified, and the purified material was subjected to SDS-PAGE. After recording the fluorescence profile (bottom), the same gel was stained with Coomassie Brilliant Blue to reveal protein content (top). Positions of the OxlT monomers, dimers, and n-mers are indicated.

**Fig. 3. Inhibition of TM2 single cysteine variants by MTS-linked agents.** Membranes containing the indicated TM2 single cysteine variants were solubilized, and proteoliposomes were prepared by reconstitution of the crude detergent extracts. Each preparation was separately exposed to 2 mM MTSES, MTSET, and MTSEC, and residual activity was compared with that measured in the absence of inhibitor. The disulfide-linked modifications introduced by the MTS-linked agents are indicated (top), with RS representing the targeted cysteine.
inhibition requires that the external probe must approach its single target (Q63C) from both the inner (cytoplasmic) and outer (periplasmic) surfaces of the protein. We have now strengthened this conclusion by quantitative tests using MTSES as the probe of purified and reconstituted material. In these additional experiments (Fig. 5) we established that for the usual conditions of treatment (Figs. 3 and 5) a 50% inhibition is given by about 0.25 mM MTSES and that this inhibition follows approximate first-order kinetics with respect to probe concentration (Fig. 5A). For the same conditions we then exposed the purified and reconstituted Q63C protein to excess (2 mM) external MTSES in the presence of increasing concentrations of oxalate. Without substrate, nearly complete inhibition was recorded (6% residual activity), whereas nearly complete protection (80%) was afforded in the presence of 2 mM oxalate. From the concentration dependence of this substrate protection (Fig. 5B, inset) one may derive (see "Experimental Procedures") an effective $K_D$ of 0.16 mM oxalate, a value that compares favorably with the measured $K_m$ (0.23 mM) for oxalate transport (see above). As noted earlier (15), such protection may arise as a result of steric blockage of the substrate binding site or following conformational changes that prevent exposure of Q63C to the external phase, but it is not possible to distinguish the relative contributions of each factor. We note also that the charged character of MTSES (Fig. 3) suggests that it exerts its inhibitory effect by interactions at the external surface. This supposition is strengthened by the finding of substrate protection because the presence of excess (100 mM) internal oxalate would have blocked any attack from the proteoliposomal interior. That substrate protects Q63C against modification by MTSES fulfills the second criterion noted above, and together with the observation that MTSES approaches Q63C from either surface of the protein (Figs. 3 and 5), leads us to conclude that position 63 (and by extension TM2) lies on the OxlT substrate translocation pathway.

DISCUSSION

Two-dimensional crystallography of OxlT (4) has generated a projection structure that provides an initial glimpse at architectural features likely to characterize members of the major facilitator superfamily. An immediate goal, therefore, is to integrate the emerging structural information with ongoing functional studies. For this purpose we have focused on identification of helices likely to line the OxlT substrate translocation pathway, using criteria developed in earlier studies of UhpT, the sugar phosphate carrier of E. coli (10, 14, 15). Such tests require documentation to prove that at least one position on the targeted helix is accessible to a suitable probe from both surfaces of the protein (that is, from the cytoplasmic (internal) as well as the periplasmic (external) surface) and that such access is blocked by the presence of substrate(s). These experimental criteria were developed initially using cells and vesicles of known orientation (10, 15), but the analysis was subsequently extended to the use of purified material when it became clear that reconstitution by detergent dilution (as used here) yields a population in which half of the molecules orient as in the cell (RSO) while the other half orients in the opposite configuration (ISO), each configuration showing equivalent kinetic behavior.
during the self-exchange reaction (9, 14). After reconstitution, then, the presence of the two different populations indicates that both cytoplasmic and periplasmic surfaces of the protein are accessible (in different molecules) to probes added in the external medium. Moreover, the presence of internal substrate ensures that both populations are in a physiologically relevant state. That is, in RSO molecules, efflux of internal substrate leads to a transport pathway poised to initiate influx, whereas ISO molecules rest in a configuration normally associated with efflux (9). Proteoliposomes therefore comprise a convenient experimental system for the analysis of the sidedness with which a probe gains access to its target and for asking whether a target is on the translocation pathway. For example, in the case of the V55C mutant the finding of a 50% inhibition by MTSES (Figs. 3 and 5) must reflect that the external probe can reach only if the probe(s) travels inward from one surface of the protein. Judging from the position of valine 55 (Figs. 1 and 4), we presume that this is the periplasmic surface. If so, position 55 in TM2 resembles position 370 in TM11 as well as a number of residues at the periplasmic surface of TM7 in the sugar phosphate transporter UhpT, where a similarly restricted accessibility is found (8, 9, 15). On the other hand, the complete inhibition of the Q63C variant by either MTSCE or MTSES (Figs. 3 and 5) must reflect that the external probe can move along TM2 toward its single target from either the cytoplasmic or periplasmic surfaces. The simplest interpretation of this observation, when considered together with the fact that such modification is substrate protectable, leads to the conclusion that Q63C lies on the substrate translocation pathway.

When these same criteria were applied earlier to OxlT TM11, we were able to assign S359C to the translocation pathway (9). This and other findings supported the proposal that lysine 355, which lies on the same helical face as serine 359, engages in an electrostatic interaction with one of the substrate carboxylates (8, 9, 13). But that model leaves open the question of how the second anionic group on oxalate (‘OOC–COO’) might be accommodated. With its collection of polar residues (Fig. 1) that might facilitate substrate stabilization via hydrogen bonds, TM2 is the most likely of the remaining OxlT helices to take part in substrate binding along with TM11. Moreover, disulfide cross-linking shows that TM11 is close to TM2 (7), further implicating TM2 as a key player in OxlT function. To sustain this working hypothesis in the long term, however, one would have to show that at least some part of TM2 borders the translocation pathway, a requirement now fulfilled by our analysis of the Q63C mutant. We note that the TM11 and TM2 residues known to be on the pathway (serine 359 and glutamine 63) also fall on TM11 and TM2 faces known to cross-link with each other (7) (Fig. 4A). We conclude that both functional tests (as reported here and in Ref. 9) and structural (7) information support the idea that TM2 and TM11 form an integral part of the substrate transport pathway and that they contain residues likely to contribute to the substrate binding site.

Information gathered from other model systems within the major facilitator superfamily is also consistent with our assignment of TM2 and TM11 to the translocation pathway and with the idea that they may each contribute to a ligand binding site. In UhpT, for example, it is clear that TM11 contains residues which influence substrate specificity (16, 18), whereas in LacY, the H+–lactose symporter, TM2/TM11 proximity has been documented by extensive trials of cross-linking (21). Because each of these three examples (LacY, OxlT, and UhpT) is found in a distinct family within the major facilitator superfamily, it is plausible that TM2 and TM11 play equivalent roles throughout the entire superfamily. Such conclusions may also extend to other 12-helix transporters, because in MeIB, the Na+/melibi-
ose symporter, determinants of Na\(^+\) selectivity have been mapped to TM2 (19), and the identification of an interhelical salt bridge suggests TM2 and TM11 are in close proximity (20).

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**REFERENCES**

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