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 66, glutamine 69, and serine 72) 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 (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 C37A) 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 (Spec’ Lac+) 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-ß-D-galactopyranoside; cells were harvested by centrifugation after an additional 2.5-h growth.

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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% (w/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 were measured in duplicate at 4 °C by a filtration assay (1). Proteoliposomes were applied directly to the center of a 0.22-μm Millipore filter and washed twice with 5-ml volumes of chilled assay buffer (100 mM potassium sulfate, 50 mM MOPS/KOH, pH 7) and incubated at 37 °C for 40 s by filtration and washing. OxalT function is usually reported as the relative specific activity by normalization of observed rates to levels of OxlT expression 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 with chemiluminescence and quantitated using a Fuji LAS 1000 gel documentation system; the expression of single cysteine mutants was evaluated with reference to that of the cysteine-less parent, processed in parallel on the same gel.

Site-directed Fluorescence Labeling— Exposure of TM2 positions to the aqueous medium was assessed in single cysteine variants whose expression levels and specific activities were ≥20% of the cysteine-less parent. Membrane pellets (prepared as above) were resuspended in 20 mM potassium phosphate (pH 8) and incubated for 10 min at 23 °C with 40 μM OGM, an impermeable thiol-active agent (see Ref. 11). After a quench by addition of 6 mM β-mercaptoethanol and three cycles of washing with distilled water, the labeled membranes were solubilized (as above), and OxlT was purified by metal chelate affinity chromatography as described (8, 11). After SDS-PAGE of the purified protein a fluorescence profile was recorded using the Fuji LAS 1000 gel documentation system; the protein content of the same gel was then assessed by staining with Coomassie Brilliant Blue. To verify the presence of a reactive cysteine, control samples were incubated in SDS-PAGE sample buffer containing 40 μM OGM just prior to electrophoresis.

Modification by MTS-linked Probes—To characterize the inhibition of single cysteine variants by MTS-linked probes, proteoliposomes trapped on a Millipore GSTF filter (see above) were washed twice with assay buffer and then overlaid in quadruplicate for 7 min at room temperature with 0.5 ml of assay buffer containing 2 mM MTSCe, MTSES, or MTSET. The probe was removed by rinsing with two 5-ml volumes of assay buffer after which OxlT function was measured by a second overlay with assay buffer containing labeled substrate. When protection by substrate was monitored, incubation with probe was carried out in the presence of increasing concentrations of potassium oxalate (0–2 mM) together with compensatory decreases in potassium sulfate (100–98 mM). In a few experiments, the kinetic behavior of the Q63C variant and its inhibition by MTSES were examined in detail. For that work, both the Q63C protein and the cysteine-less parental protein were purified using standard methods (8). Analysis of MTSES inhibition was evaluated as described (9) using a kinetic model assuming that unliganded OxlT reacts with either substrate or the probe to generate either liganded OxlT or an irreversibly inhibited complex. If only liganded OxlT is modiﬁed by the probe, F, the fraction of the OxlT population that remains unmodiﬁed, is given by: ln(F) = −kπ(t/1 + S/Kp), where S and P represent substrate (oxalate) and probe (MTSES) concentrations, respectively, t is time, k is the rate constant governing probe modiﬁcation of unliganded OxlT, and Kp is the dissociation constant for the substrate-liganded complex. A linear transform of this relationship is used to extract the value of Kp.

Chemicals—Puriﬁed E. coli phospholipid was obtained as a lyophilized powder from Avanti Polar Lipids. MTSCe, MTSES, and MTSET were from Toronto Research Chemicals, and OGM was from Molecular Probes. Roche-Calbiochem provided octyl-β-D-glucopyranoside, whereas [13C]oxalate was from PerkinElmer Life Sciences.

RESULTS

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 Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I). Severe defects found in the Q63C, Q66C, and S69C mutants (2) ranged from 2 to 120% of the parental level (Table I).
tutions displayed significantly reduced function with specific activities of less than 10% of the parent. Of those remaining, the Q56N variant was the most active (41%), followed by the Q56T and Q56S derivatives (31% and 16%, respectively). This preliminary analysis supports the general idea that these two activities of less than 10% of the parent. Of those remaining, the Q56N variant was the most active (41%), followed by the Q56T and Q56S derivatives (31% and 16%, respectively). This preliminary analysis supports the general idea that these two positions may be important to OxlT function.

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, G65C, Q66C, V69C). We interpret these in situ findings as identifying a centrally placed TM2 core, minimal 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 Q63C variant as a suitable candidate for quantitative tests that might identify TM2 as lining the OxlT translocation pathway.

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 recon-

TABLE I

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

| Variant | Expression | Specific Activity |
|---------|------------|------------------|
| Parent  | 100        | 100              |
| S51C    | 57         | 138              |
| L52C    | 145        | 16               |
| A53C    | 167        | 67               |
| A54C    | 75         | 28               |
| V55C    | 96         | 115              |
| Q56C    | 101        | 1.6              |
| T57C    | 105        | 57               |
| A58C    | 76         | 23               |
| F59C    | 0          | —                |
| T60C    | 70         | 16               |
| L61C    | 108        | 55               |
| S62C    | 98         | 57               |
| Q63C    | 103        | 24               |
| V64C    | 128        | 15               |
| I65C    | 105        | 61               |
| Q66C    | 105        | 1.9              |
| A67C    | 102        | 28               |
| G68C    | 122        | 59               |
| S69C    | 126        | 4.6              |
| Q70C    | 118        | 35               |
| P71C    | 123        | 21               |

* Not determined.

The translocation pathway is defined as the set of residues whose modification of crude extracts. We chose agents whose bulk (182–242 daltons) was significantly smaller than OGM, and we used an extended exposure to excess probe (9, 11) to maximize the chances of finding residues whose modification might have functional impact. OxlT function was for the most part unaffected by such maneuvers (Fig. 3), and only two variants (V55C and Q63C) showed significant responses. One of the two susceptible targets (V55C) was affected by all three probes, but in no case did inhibition exceed 50%. Substantial inhibition (80–95%) was recorded for only a single target (Q63C) and then only with the negatively charged MTSCE and MTSES. Other work has shown that OxlT is found in both right side-out and inside-out orientations after reconstitution (9); each orientation is present in about equal amount, and each is of equivalent activity. With this in mind, and assuming the observed inhibition-reflected action of excess probe, it seems likely that the cysteine present in the V55C protein is accessible in only one of these conformations, whereas the cysteine in the Q63C variant is accessible in both (9, 14).

These findings (Fig. 3) were integrated with information derived from mutagenesis (Table I and accompanying text) and studies of disulfide trapping (7) to develop a working model of TM2. When such data are displayed as a helix wheel (Fig. 4), there is a clear asymmetry to the attributes of TM2 residues (Fig. 4A). Thus, one helical face is enriched for residues whose substitution by cysteine is usually without marked functional impact (excepting only S69C), with a mean residual activity of 66%. By contrast, at the other helical face cysteine-scanning mutagenesis has far more substantial impact, yielding a mean residual activity of 17%; note that this latter surface includes the three instances in which residual activity is near zero (Q66C, F59C, and Q63C) as well as the single position that shows high sensitivity to MTS-linked probes (Q63C).

Q63C Lies on the Substrate Translocation Pathway—Glutamine 63, although not essential to OxlT function, is found in the TM2 core on the helical face enriched for residues of functional significance (Fig. 4). For this reason, it seemed likely that further work targeting this residue could be informative, especially in regard to tests exploiting its sensitivity to MTS-linked probes (Fig. 3).

Kinetic study of the purified Q63C protein showed that its diminished function reflects modest changes in both the Michaelis constant for oxalate (0.23 versus 0.13 mM for the Q63C variant and its cysteine-less parent, respectively) and the maximal velocity for oxalate transport (32 and 73 μmol/mg per min, respectively). Under standard assay conditions, one therefore finds reduced function on reconstitution of either crude extract (24% residual activity, Table I) or purified material (31% residual activity). These data confirm that glutamine 63 plays no critical role in substrate transport, an idea further supported by the finding that the Q63A Q63S, Q63T, and Q63N derivatives have essentially normal function with relative specific activities 70–130% of the parental protein (not shown; tested by reconstitution of crude extracts). These findings, coupled with observations noted earlier (Fig. 3), point to the Q63C variant as a suitable candidate for quantitative tests that might identify TM2 as lining the OxlT translocation pathway.

The translocation pathway is defined as the set of residues that becomes exposed to solvent (and substrate) at either side of the membrane as the substrate binding site is alternately exposed to either surface 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
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
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 $K_p$ 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). In UhpT, for example, it is clear that TM11 contains residues that 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 MelB, the Na⁺/melibi-
Osm 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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