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

TOPOLOGICAL FEATURES OF TRANSMEMBRANE HELIX 11 AS VISUALIZED BY SITE-DIRECTED FLUORESCENT LABELING*

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Analysis of hydrophathy suggests that in OxlT, the oxalate/formate antiporter of Oxalobacter formigenes, lysine 355 is within transmembrane helix no. 11. To test this idea, we used single-cysteine, histidine-tagged OxlT variants to study the organization of a 30-residue segment (residues 344–373) containing this region. Topology was examined by probing the A345C and A370C proteins with Oregon Green maleimide carboxylic acid, an impermeant and fluorescent thiol-reactive agent. Examination of purified protein showed that only A370C was fluorescent after treating intact cells with the probe, while both proteins were modified in tests with isolated membrane ghosts. In addition, labeling of A370C, but not A345C, was blocked when external cysteines were protected with the impermanent and nonfluorescent agent, methanethiosulfonate ethyltrimethylammonium. These findings confirm that A345 faces the cytoplasm, while A370C faces the periplasm. A similar study focused on 13 single-cysteine variants positioned throughout the target segment. That work revealed a striking discontinuity in reactivity toward Oregon Green maleimide; cysteines within a 10-residue central core (residues 351–360) were not labeled when membranes were probed, but were readily modified after protein denaturation. We suggest this core resides within the lipid bilayer, unavailable to an impermanent reporter. Since this region includes position 355, we also suggest that lysine 355 lies within the OxlT hydrophobic sector, where it may facilitate the binding and translocation of the anionic substrates, oxalate and formate. Combination with consumption of a single internal proton during oxalate decarboxylation, functions as a virtual proton pump, enabling this cell to generate ATP by decarboxylative phosphorylation (3, 8, 9). Theoretical considerations (5, 8, 9), and the finding of OxlT variants during genomic sequencing of Archaeoglobus fulgidis (10), suggest that added biochemical study of OxlT can be of value to the ongoing investigation of membrane transport.

Hydropathy analysis of the OxlT amino acid sequence suggests the presence of 12 hydrophobic segments (11), and circular dichroism spectroscopy of purified, solubilized OxlT supports the idea that these segments are structured as membrane-spanning α-helices (6). A closer examination of the putative OxlT topology reveals an unexpected feature, however; the hydrophobic sector appears to contain a single charged residue, a lysine (Lys355) positioned near the center of transmembrane helix 11 (TM11)1 (11). This is counterintuitive, since placement of an uncompensated charge within a hydrophobic environment can significantly destabilize a membrane protein (12, 13). On the other hand, the location of Lys355 might be rationalized if such an energetic disadvantage contributed an electrostatic component utilized in the process of transport. This idea is all the more attractive because OxlT must capture and translocate anions (oxalate and formate), implying direct interaction between the negatively charged substrate and unidentified positive element(s) within a translocation pathway. In this latter circumstance, Lys355 becomes a likely candidate.

Such an argument depends heavily on one interpretation of hydrophathy, and it is essential to have positive experimental evidence that confirms or rejects this view before embarking on any extended study. The work reported here was designed to address this specific issue by answering two questions. (i) Does the segment containing Lys355 actually span the plasma membrane? (ii) Where within this interval does Lys355 reside, relative to the internal and external aqueous phases? As a tool to answer these questions, we generated a panel of single-cysteine mutants spanning the entirety of TM11, so that the environment at each position could be probed with a membrane-impermeable fluorescent reporter, OGM. Use of this approach allowed us to confirm the postulated topology in this region and to identify a set of 10 contiguous residues (residues 351–360) that form an inaccessible core within TM11, thereby defining the location of Lys355 as at the center of the OxlT hydrophobic sector.

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1 The abbreviations used are: TM, transmembrane helix; OGM, Oregon Green 488 maleimide carboxylic acid; MTSET, methanethiosulfonate ethyltrimethylammonium; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholino propane sulfonic acid.
EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Expression—OxlT, encoded within pBlue-script II SK+ (Amp'), carried a C-terminal extension of nine tandem histidine residues to facilitate protein purification (6). Oligonucleotide-directed mutagenesis (Chameleon14, Stratagene) was used to replace the two endogenous cysteines, and the C28G/C271A, cysteine-less variant was used as parent to 30 single-cysteine derivatives constructed at positions 344 through 373. All mutants were confirmed by DNA sequencing (14).

To suppress uninduced protein expression, plasmids specifying OxlT were carried together with pMS421 (Spe', Lac') (11). After co-transformation of Escherichia coli XL-1 cells, one or a few colonies were inoculated into 1 ml of LB broth (plus antibiotics) and grown at 37 °C overnight with vigorous shaking. These cells were subcultured in 50 ml of LB broth (plus antibiotics) and grown for 1–2 h until the exponential phase (A600, of 0.2), at which point isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM. Growth continued for an additional 4 h before cells were harvested.

Thiol-specific Fluorescent Labeling—Site-specific fluorescent labeling was designed to selectively attack either cysteines exposed to the extracellular medium or cysteines exposed at the intracellular surface. We also performed labeling to identify cysteines exposed to an aqueous phase, without regard for their normal disposition.

To label cysteines exposed at the extracellular surface, intact cells were harvested by centrifugation (4,000 × g × 2 min) and resuspended at moderate density (A600 of 1) in Buffer A (100 mM potassium sulfate, 50 mM potassium phosphate, pH 8.0) to which was added 40 µM freshly prepared OGM. After a 20-min incubation at 23 °C, the labeling reaction was quenched by addition of 2 mM 3-mercaptoethanol, followed immediately by three cycles of centrifugation and washing using Buffer A alone. Labeled cells were then resuspended in 20 ml of lysis solution (300 µg/ml lysozyme, 40 µg/ml DNase, 5 mM EDTA, and 10 mM Tris/ HCl, pH 7.5), and incubated at room temperature for 10–15 min to initiate cell rupture (15). Membrane ghosts were obtained by 10-fold dilution in distilled water, and cytoplasmic proteins were removed by three cycles of centrifugation (6,000 × g × 10 min) and resuspension in distilled water (15). These membranes served as the source of OxlT for solubilization and purification (below).

Cysteines exposed at the intracellular surface were identified by a two-step protocol. External cysteines were blocked by an initial 5-min preincubation of cells in Buffer A containing (freshly dissolved) 200 µM MTSET, a nonfluorescent, membrane-impermeable thiol-specific reagent. MTSET was removed by three cycles of centrifugation and washing using Buffer B (100 mM potassium sulfate, 50 mM MOPS/K, pH 7), without mercaptoethanol, and preblocked cells were used for preparation of membrane ghosts, as above. This exposed unmodified cysteines which had faced the cytoplasm, and these were labeled by incubating membrane ghosts for 20 min in Buffer C (20 mM potassium phosphate, pH 8) containing 40 µM OGM; the reaction was quenched with 2 mM 3-mercaptoethanol and followed immediately by three cycles of centrifugation and resuspension, using distilled water, and by protein solubilization and OxlT purification (below).

To simultaneously label cysteines exposed at both surfaces, membrane ghosts were prepared directly, without blocking external cysteines, and incubated for 20 min in Buffer C with 40 µM OGM before quenching the reaction as above.

OxlT Purification—Protein was solubilized by resuspending membrane ghosts in 5 ml of solubilization buffer (20 mM MOPS/K, 20% (v/v) glycerol, 0.42% (v/v) E. coli phospholipid, 6 mM β-mercaptoethanol, 10 mM potassium oxalate, and 1.5% (v/v) octyl-β-D-glucopyranoside). After incubation at 4 °C for 1–2 h on a rotary platform, insoluble debris was removed by centrifugation in the cold (6,000 × g × 30 min), and OxlT was purified in an one-step affinity procedure (6). In brief, the polyhistidine fraction was applied to a Ni-NTA resin by an overnight batch incubation in the cold on a rotary platform shaker. The resin, with bound OxlT, was then packed into a Micro Bio-Spin chromatography column (Bio-Rad) and washed on ice with a total of 3 ml of wash buffer (solubilization buffer supplemented with 200 mM sodium fluoride and 50 mM imidazole). Elution of OxlT was achieved by centrifugation in the cold (100,000 × g) of 50 mM Tris/HCl, 2% SDS, 10% glycerol, 0.5 mM imidazole, pH 7.

SDS-PAGE—Protein (1–2 µg/lane) was subjected to SDS-PAGE using a 12% polyacrylamide gel matrix. After electrophoresis, the gel was rinsed briefly with a destaining solution (10% glacial acetic acid, 15% methanol), and fluorescent profiles were recorded with a Molecular Dynamics STORM fluorescence imaging system (blue fluorescent-chemiluminescence mode, excitation wavelength of 450 ± 30 nm, photomultiplier voltage of 900 mV, pixel size of 100 µm). After recording a fluorescence profile, the protein content of each lane was evaluated by staining the same gel with Coomassie Brilliant Blue. The Coomassie-stained profile was recorded on film and digitized using a UMax PowerLook scanner.

Immunoblot Analysis—Detergent extracts of membrane ghosts (0.35 mg of protein/ml) were analyzed by SDS-PAGE. Each of three gels contained samples (1.75 µg of protein/lane) of 10 single-cysteine mutants and the cysteine-less parent. After electrophoresis, proteins were transferred to nitrocellulose and probed with a rabbit polyclonal antibody directed against the OxlT N terminus (11). OxlT expression was assayed by chemiluminescence, and signals were quantitated using NIH Image v1.59. Expression of each OxlT variant was normalized to that of the cysteine-less parental control found on the same gel.

Assay of Oxalate Transport—To determine the specific activity of single-cysteine OxlT mutants, a sample (14 µg of protein) of the detergent extract (above) was used for reconstitution into proteoliposomes loaded with 100 mM potassium oxalate, as described previously (6, 11). [14C]Oxalate transport was measured by an abbreviated assay (10, 12) that was terminated after steady state was reached (10 min) (11). Oxalate transport by each mutant was normalized to that of the cysteine-less parental protein, and specific activity relative to the parent was calculated for protein expression as assessed by immunoblot analysis of the same sample (above).

RESULTS

Characterization of OxlT Single-cysteine Variants—To construct single-cysteine OxlT variants, we first removed the two endogenous cysteine residues by site-directed mutagenesis. In work using another bacterial transporter, UhpT, substitution of cysteine with serine generated a cysteine-less variant whose activity was reduced by 60% relative to the wild type (17). For this reason, we replaced OxlT cysteines with residues more commonly found in membrane proteins, so that cysteine at position 28 was changed to glycine, while cysteine at position 271 was replaced by alanine. This gave a cysteine-less (C28G/C271A) derivative whose expression and activity levels were nearly identical (90%) to that of wild type OxlT. We next used this derivative as a template for construction of 30 new variants, each containing a single cysteine, throughout the region (residues 344–373) expected to contain transmembrane helix 11.

Immunoblot analysis of this panel (Fig. 1, left) indicated that with the exception of the N347C, A368C, and I369C derivatives, for which no detectable protein was found, most variants were expressed in membranes at levels comparable to that of the cysteine-less parent. Each of three independent experiments gave similar estimates of protein expression, with individual variants scored at levels ranging from 30 to 230% of the cysteine-less parent (Fig. 1, right). Since the parental (and wild type) proteins are present at 3–5% of total membrane protein in both O. formigenes (5) and E. coli (11) we expected that most of the single-cysteine mutants could be purified in microgram quantities from a few milliliters of culture, a yield sufficient for the anticipated fluorescent labeling experiments (below).

The functional analysis of these single-cysteine variants used measurement of the oxalate self-exchange reaction (8) in oxalate-loaded proteoliposomes. These assays indicated a broad range of capacities (Fig. 2); almost two-thirds of the mutants (21/30) had a specific activity comparable to that of the cysteine-less parent (≥30%); a minority (5/30) had reduced activity relative to the parent (2–20%), while in four cases there was no detectable oxalate transport. This last group included the N347C, A368C, and I369C variants, as expected from their reduced levels of expression (Fig. 1). We noted that the K355C
variant, despite its normal level of expression (Fig. 1), failed to exhibit oxalate transport, suggesting a significant functional role for this charged residue.

**Orientation of TM11—Hydropathy analysis of the OxlT amino acid sequence suggests the presence of 12 transmembrane segments (TM1–TM12) and that Lys355 may lie within TM11 (11). The peak hydropathy in the area surrounding TM11 only marginally exceeds the usual criterion for a transmembrane structure (12, 13), so our initial experiments were designed to establish whether this region actually traverses the membrane. This was accomplished by examining the accessibility to a membrane-impermeable, thiol-specific fluorescent probe (OGM) of single-cysteine targets predicted to be at either the extracellular or intracellular surface. Labeling of cysteine exposed to the external phase was achieved by direct exposure of intact cells to the probe, while labeling of cysteine exposed at the internal surface was performed by a general attack using isolated membranes, after external targets were protected by a nonfluorescent blocking agent (MTSET).

These initial experiments utilized two single-cysteine variants, A345C and A370C, whose high levels of expression (Fig. 1) and activity (Fig. 2) ensured an overall structure closely resembling authentic OxlT. In preliminary work, we considered both tetramethylmaleimiderhodamine and OGM as candidate probes. The latter proved far superior, and only OGM showed a side-specific reactivity consistent with an impermeant character. We attribute this differential behavior to either or both of two factors. First, the OGM carboxyl and hydroxyl groups have \( pK_a \) values, while the single carboxyl of tetramethylmaleimiderhodamine has a \( pK_a \) near neutral pH (\( pK_a \approx 6.8 \)); thus, only OGM was fully deprotonated under our conditions of labeling (pH 8). Second, it seemed feasible that the rhodamine-linked probe could be rendered permeable by formation of an internal salt bridge between its anionic and cationic groups.

This preliminary work indicated OGM was suitable for our purposes, a conclusion validated by the topological mapping of cysteines at positions 345 and 370. Thus, after intact cells were directly exposed to OGM, both the A345C and A370C proteins could be readily purified, but only the latter contained the fluorescent label (Fig. 3, experiment 1). In contrast, when extracellular cysteines were first blocked by the nonfluorescent MTSET, subsequent exposure of isolated membranes to OGM yielded fluorescent labeling of only the A345C protein (Fig. 3,
Fig. 3. Positions 345 and 370 lie on opposite membrane surfaces. Bottom drawings indicate the predicted OGM alternating patterns (top right), while OxlT content was revealed by staining the same gel with Coomassie Blue (top left). In experiment 1, intact cells were exposed to OGM before a quench by β-mercaptoethanol, cell lysis, and preparation of membrane ghosts. In experiment 2, external cysteines were blocked by preincubation with MTSET, which was removed prior to preparation of membrane ghosts and OGM labeling of the newly exposed internal cysteines. In experiment 3, direct labeling of membrane ghosts with OGM allowed detection of cysteines exposed to both internal and external phases.

experiment 2). Moreover, under these latter conditions we could show equally efficient labeling of the external cysteine, provided the preblock with MTSET was omitted (Fig. 3, experiment 3). We also labeled membranes in the presence of 100 mM potassium oxalate, but use of excess substrate (and high ionic strength) had no effect on labeling of the A345C and A370C proteins (not shown; other conditions as in Fig. 3, experiment 3). Two conclusions follow from these findings. First, it is clear that positions 345 and 370 are on opposite membrane surfaces, with the former facing the cytoplasm and the latter exposed to the external medium. This confirms the general topology derived from the original analysis of hydropathy in this region (11) and verifies the existence of TM11. Second, perhaps more important, these experiments may form the basis of a general method for determining topology by site-directed fluorescent labeling.

Definition of a TM11 Hydrophobic Core Surrounding Lys355.—The main goal of these experiments was to test the idea that Lys355 is located within the OxlT hydrophobic core. If so, it seemed unlikely that hydrophilic probes such as OGM and MTSET could be used to label the single-cysteine K355C variant. If, on the other hand, such reactivity were found, the null phenotype of this variant (Fig. 2) would not support any simple interpretation. To avoid such ambiguity, we chose to evaluate the topology of TM11 by studying single-cysteine variants of normal specific activity (≥30% parental level). In this way, the location of Lys355 could be inferred from the locations of its neighbors.

With this strategy in mind, we selected 13 single-cysteine variants, each of robust activity, as representative of TM11. For each protein, the accessibility of its cysteine to OGM was determined by the generalized labeling of membrane ghosts obtained after cell lysis, using conditions which had enabled labeling of both external and internal positions (Fig. 3, experiment 3). In principle, because such labeling took place with OxlT embedded within the membrane, this allowed identification of cysteines normally exposed to the internal or external aqueous phases, but not of cysteines at positions within the lipid bilayer. Indeed, an examination of the fluorescence profile obtained in such an experiment (Fig. 4, bottom panel) revealed a discrete pattern of reactivity to OGM according to the location of cysteine within the target interval. Significant fluorescent signals were found at positions 345, 346, 348, and 350; little or no labeling could be detected at positions 351, 353, 356, 357, and 360; while fluorescent signals reappeared at the more distal positions, 361, 364, 367, and 370. This differential response was not attributable to differences in protein content, since Coomassie staining of the same gel showed comparable levels of protein in all lanes (Fig. 4, top panel). Nor did this pattern reflect variability in the cysteine content of these variants, since each could be labeled in the parallel control when OGM addition was delayed until protein had been denatured by SDS (Fig. 4, middle panel). In that case, only the cysteineless parent failed to take up the fluorescent probe. Such controls indicate that any differential labeling by OGM reflects the relative accessibility of the individual cysteines, and since this occurred only when the proteins were labeled in situ, it seemed most likely that the unreactive positions (residues 351–360) lie within the hydrophobic core of OxlT, at the center of TM11.

DISCUSSION

The work described here has focused on a 30-residue segment (residues 344–373) in the anion exchange transporter, OxlT. In particular, we sought to confirm or reject the idea of a transmembrane segment in this region and to determine whether a basic residue in the center of this segment (Lys355) is within the OxlT hydrophobic sector, where the presence of a resident positive charge might influence substrate binding and translocation. To address these issues, we exploited affinity purification of histidine-tagged OxlT (6) along with cysteine scanning mutagenesis and use of impermeant and fluorescent, thiol-directed probes. Together, these tools allowed us to docu-
It is also evident that the entire population of OxlT is of periplasm, as predicted by the initial analysis of hydropathy faces, with A345C facing the cytoplasm and A370C the sions. Clearly, these positions lie at opposite membrane sur-

mean probes (OGM, MTSET) (Fig. 3) supports several conclu-

ctions for such findings. One interpretation is that the region of low reactivity (positions 351–360) is within the lipid bilayer and hence unavailable to the impermeant probe, and a semi-quantitative argument is consistent with this suggestion. Thus, on modification of its target, the OGM carbohydrate lies about 8–10 Å from the cysteiny1 sulfur. Accordingly, one ex-

pects cysteines that react with OGM to be within 8–10 Å of the external or internal aqueous phases. And if TM11 is a trans-

membrane α-helix, of the 20–25 residues that span the 30–40 Å bilayer, one expects a central core of some 10 residues (e.g. residues 351–360) to be inaccessible to OGM for purely geometric reasons. A biotin-linked maleimide of similar dimensions defines a comparable set of core residues along TM7 of UhpT, the E. coli sugar phosphate transporter (20). A second line of reasoning suggests that cysteines in this core are unreactive for reasons of chemistry, in that they display the high pK_a (pK_a = 14) characteristic of cysteine in a nonpolar (i.e. lipid) environ-

ment. In this second case, the reactive sulfide (–S^−) would not be at sufficiently high concentration to support modifica-

tion. One presumes this principle underlies the low N-ethylmaleimide reactivity of single-cysteine derivatives of TM9 in the tetracycline antiporter, TetA (21). We caution, however, that such alternative explanations are not mutually exclusive. Rather, a transmembrane helix may have positions facing other helices or the hydrophilic translocation pathway (cf. Goswitz and Brooker (22)); these positions may be inaccessible, but possibly chemically reactive. Or, there may be positions facing membrane lipid, and while these may be accessible, they are most likely chemically unreactive. In either case, the finding that residues 351–360 form the core of TM11 effectively posi-

tions Lys^{355} at the center of the OxlT hydrophobic sector.

While the conclusion that TM11 has an inaccessible core of about 10 residues is not surprising, it is unusual that this core includes a basic residue, Lys^{355}. As judged from examples known at a crystallographic level, transmembrane helices do not typically contain charged residues (23–28), and if they are present, it is almost always in combination with a second residue of opposite charge (24–26), a conclusion also reached during genetic and biochemical studies of the LacY symporter (29–31). Hydropathy analysis of the OxlT sequence does not suggest an anionic partner which might pair with Lys^{355} (11), and for this reason one should now consider the alternative hypothesis that this basic residue, by virtue of its location at

2 T. Jung and P. C. Maloney, unpublished results.
the center of TM11, is strategically placed to interact directly with OxIT substrates as they enter the hydrophilic translocation pathway, much as anionic residues in the Na\(^+\)/melibiose MelB symporter are thought to interact directly with the co-substrate, Na\(^+\) (32, 33). This last possibility has further merit in that it may help explain the marked effect of oxalate on OxIT stability. Thus, substrate binding energy dramatically increases the stability of the purified protein in lipid/detergent micelles (6, 34). An attractive explanation for this phenomenon is that the anionic substrate donates a negative charge that removes an unavoidable destabilizing element contributed by the uncompensated, resident cation, Lys\(^{355}\).

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REFERENCES
1. Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A., and Henderson, P. J. F. (1992) Curr. Opin. Cell Biol. 4, 684–695
2. Marger, S. D., and Saier, M. H., Jr. (1993) Trends Biochem. Sci. 18, 13–20
3. Maloney, P. C. (1994) Curr. Opin. Cell Biol. 6, 571–582
4. Clayton, R. A., White, O., Ketchum, K. A., and Venter, J. C. (1997) Nature 387, 459–4602
5. Ruan, Z.-S., Anantharam, V., Crawford, I. T., Ambudkar, S. V., Rhee, S. Y., Allison, M. J., and Maloney, P. C. (1992) J. Biol. Chem. 267, 10537–10543
6. Fu, D., and Maloney, P. C. (1997) J. Biol. Chem. 272, 2129–2135
7. Pao, S. S., Paulsen, I. T., and Saier, M. H., Jr. (1998) Microbiol. Mol. Biol. Rev. 62, 1–34
8. Anantharam, V., Allison, M. J., and Maloney, P. C. (1989) J. Biol. Chem. 264, 7244–7250
9. Harold, F. M., and Maloney, P. C. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R., Ingraham, J. L., Low, E. C. C., Ma...