Helix Proximity in OxlT, the Oxalate:Formate Antiporter of Oxalobacter formigenes

CROSS-LINKING BETWEEN TM2 AND TM11*

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Experiments were designed to evaluate the proximity of transmembrane helices two (TM2) and eleven (TM11) in the tertiary structure of OxlT, the oxalate:formate exchange transporter of Oxalobacter formigenes. A tandem duplication of the Factor Xa protease cleavage site (IE-GRIEGR) was inserted into the central cytoplasmic loop of an OxlT cysteine-less derivative in which an endogenous cleavage site had been eliminated by mutagenesis (R248Q). Using this host, double cysteine derivatives were constructed so as to pair one of seventeen positions in TM2 with one of four positions in TM11. Following treatment of membrane vesicles with Cu(II)(1,10-phenanthroline), molecular iodine, or N,N'-o-phenylenedimaleimide, samples were exposed to Factor Xa, and disulfide bond formation was assessed after SDS-polyacrylamide gel electrophoresis by staining with antibody directed against the OxlT C terminus. In the absence of disulfide bond formation, exposure to Factor Xa revealed the expected C-terminal 22-kDa fragment, a result unaffected by the presence of reductant. By contrast, after disulfide formation, OxlT mobility remained at 35 kDa, and appearance of the 22-kDa fragment required addition of 200 mM dithiothreitol prior to electrophoresis. The four TM11 positions chosen for cysteine substitution lie on a helical face known to interact with substrate. Similarly, TM2 positions supporting disulfide trapping were also confined to a single helical face. We conclude that TM2 and TM11 are in close juxtaposition to one another in the tertiary structure of OxlT.

Early functional studies showed that OxlT, an oxalate:formate antiporter protein, serves as a virtual proton pump in its host organism, Oxalobacter formigenes, establishing the protonotive force required for survival of this Gram-negative obligate anaerobe (1). Current work (2–5) now focuses on OxlT as a model for understanding structure/function relationships in the Major Facilitator Superfamily (MFS), one of the largest known groups of evolutionarily related transport proteins (6).

The most recent studies of OxlT have addressed the issue of how substrate, divalent oxalate ("OOO-COO"), is bound by the protein. Both the analysis of hydropathy (7) and the determination of topology (5) implicate TM2 and TM11 as likely participants in this event. Moreover, a portion of the translocation pathway through OxlT is lined by TM11 (4), and it appears likely that substrate binding is partly stabilized by electrostatic interaction between an oxalyl carboxylate and Lys-355 (Lys-355), a positively charged residue within the TM11 hydrophobic core (3, 4). Evidence documenting a role of TM2 in substrate binding is less direct, but unpublished work now shows that part of this helix, too, lines the transport pathway.

Unlike TM11, TM2 has no charged residues that might stabilize substrate binding. Rather, it has been suggested (5) that TM2 facilitates ligand binding by hydrogen bonding within a cluster of polar residues (see Fig. 1).

Because TM2 and TM11 are far apart in the linear sequence of OxlT, tertiary structure must bring them into close proximity if they each take part in substrate binding. This idea is supported by the experiments reported here, which document that disulfide bond formation can occur with high efficiency between appropriately placed TM2-TM11 cysteine pairs.

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Expression—Cysteine-less OxlT (3) and its derivatives, each carrying a C-terminal polystyline extension (3), were encoded within a 1.4-kilobase pair Xbal-HindIII fragment in plBluescript II SK+ (Amp+) under plac control (7). This plasmid was the template for site-directed mutagenesis (Camelleo and Stratagen) that removed two potential Factor Xa cleavage sites by substitution of glutamine for arginine (R248Q and R238Q). Using a plasmid specifying cysteine-less OxlT with the R248Q mutation, a silent SmaI site was inserted into the region encoding the OxlT central loop, using the primer: 5′-TTTGCCGCTTGGCTGCCCCTGAAAACCAGATAACG. This construct was restricted with SmaI and Not and then ligated in the presence of two synthetic oligonucleotides: (sense) 5′-GGGCGATT- GAAGTTGCGATGGCAGCCGCAAG and (antisense) 5′-CGCCCTTGCGCGGCTCTGCGTGACCTTAAATCCGCC. The resulting product, termed OxlTFxa, has a tandem duplication of the Factor Xa cleavage site (IEGRIEGR) between Gln-197 and Gln-198 (Fig. 1). The construction of single-cysteine mutants in TM2 and TM11 has been described (3, 5). To obtain the desired double cysteine derivatives, restriction enzymology was used to place either of four single-cysteines (K355C, S359C, G366C, and A370C) into TM11 of OxlTFxa. Similar techniques were then used to introduce the desired cysteine substitutions in TM2. All mutants were confirmed by DNA sequencing.

Plasmids were carried in Escherichia coli strain XL-1, together with plasmid pMS421 (Spe′, LacI′) to limit inappropriate basal expression (7). A few colonies of freshly transformed cells were placed in LB broth containing antibiotics and grown overnight at 37°C. After overnight growth, cells were diluted 100-fold into fresh medium and grown to A600 of 0.09, at which point OxlT expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested 3.5 h thereafter, washed twice in 20 mM Tris/HCl (pH 7.5), and resuspended in the same buffer for high-pressure lysis (18,000 p.s.i.) in a French press. After low speed centrifugation to remove debris and unbroken amide gel electrophoresis; octylglucoside, octyl-β-D-glucopyranoside; DTT, dithiothreitol.

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The abbreviations used are: TM, transmembrane; PAGE, polyacrylamide gel electrophoresis; octylglucoside, octyl-β-D-glucopyranoside; DTT, dithiothreitol.

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cells, membrane vesicles were pelleted (150,000 g × 1 h) and stored at −80 °C at 10–20 mg protein/ml in 20 mM Tris/HC1 containing 20% (v/v) glycerol and 0.25 mM 4(2-aminoethyl)benzenesulfonfonyl fluoride.

Measurement of OxlT Specific Activity—An aliquot of membrane vesicles was processed for SDS-PAGE, and OxlT expression was evaluated by chemiluminescence using a mouse monoclonal antibody (Qiagen) directed against tetra-histidine (4). Signals collected by an automated documentation system (Fuji LAS 1000) were quantitated using FujiFilm Image Gauge v3.3. Expression of each mutant is reported relative to that of its OxlTfxa parent, processed in parallel on the same gel.

To assess OxlT function, 10 mg of membrane vesicles were suspended for 1 h at 4 °C in 4 ml of a solubilization buffer containing 50 mM Tris/HC1, 20% (v/v) glycerol, 10 mM potassium oxalate, 1.5% (w/v) octylglucoside, and 0.5% (w/v) E. coli phospholipid (pH 7.5). After clarification by centrifugation at 4 °C (150,000g × 30 min), 200 μl of the crude detergent extract was placed with 1.75 mg bath-sonicated liposomes and 1.5% (w/v) octylglucoside for 20 min on ice. Proteoliposomes were formed at room temperature by dilution of the mixture with 5 ml of a loading buffer containing 100 mM potassium oxalate and 50 mM Tris/HC1 (pH 7.5). After 20 min, unincorporated debris was removed by low speed centrifugation (10,000 g × 15 min) and the proteoliposome suspension was stored on ice until assay. In most cases, the initial rate of [14C]oxalate transport was measured at room temperature by rapid filtration (8). In brief, duplicate 100–200 μl aliquots of the proteoliposome suspension were placed at the center of Millipore GSTF filters (0.22 μm pore size) and washed twice with 100 mM potassium sulfate and 50 mM Tris/HC1 (pH 7.5) to remove external (unlabelled) oxalate, and after interrupting the vacuum, proteoliposomes trapped on the filter were overlaid with 300 μl of the same buffer containing 0.1 mM [14C]oxalate. After 1 min, the reaction was terminated by filtration, followed by two washes using 5 ml icid reaction buffer lacking oxalate. When an extended time course of transport was studied, proteoliposomes were pelleted (300,000 g × 15 min), washed, and resuspended as a concentrated stock using 100 mM potassium sulfate and 50 mM Tris/HC1 (pH 7.5). Stock proteoliposomes were then diluted 10-fold into this buffer, and 5 min later, 0.1 mM [14C]oxalate was added. Samples were taken for filtration and washing at the indicated times.

Cross-linking—In most experiments, cross-linking was initiated by exposing membrane vesicles (5 μg in 100 μl) for 10 min at 37 °C to 0.5 mM freshly prepared CuII (I,10-phenanthroline), followed by a wash with 10 mM DTT and 10 mM EDTA (pH 7) (9, 10). In a few experiments, cross-linking was achieved at room temperature by addition of either freshly prepared 0.05 mM iodoine (11) followed after 5 min by a quench with 10 mM n-ethylmaleimide or by addition of 0.5 mM N,N'-o-phenylendimaleimide (11) quenched 30 min later by 10 mM DTT. In all cases, cross-linked samples and uncontrolled reactions were washed twice by centrifugation (300,000 g × 10 min) with 1 ml of 20 mM Tris/HC1 (pH 7.5) and resuspended in 100 μl of the same buffer containing 100 mM NaCl, 2 mM CaCl2 (or MgSO4), and 2% (w/v) octylglucoside. Iodoines were incubated overnight at 4 °C with or without Factor Xa protease (1 μg of protease/10 μg of sample) as indicated. Samples were then processed for SDS-PAGE, with or without a 30 min preincubation with 200 mM DTT at room temperature, as noted. After transfer to nitrocellulose, OxlT mobility was assessed using antibody directed against the C-terminal polyhistidine extension (above); in a few experiments, we also used a rabbit polyclonal antibody raised against the OxlT N terminus (12).

Chemicals—Copper sulfate, 1,10-phenanthroline, molecular iodine, and N,N'-o-phenylendimaleimide were from Sigma. Octylglucoside was obtained from Boehringer-Calbiochem Corp., and E. coli phospholipid from Avanti Polar Lipids, Inc. [14C]Oxalate was from PerkinElmer Life Sciences and Factor Xa protease was from New England Biolabs; all other reagents were of reagent grade.

RESULTS

Designing OxlT for Cross-linking Studies—TM2 and TM11 have been implicated as the most likely of the 12 OxlT transmembrane helices to take part in substrate binding (3–5, 7) (Fig. 1). To test the idea that these helices lie sufficiently close to each other to share in construction of a ligand-binding site, we modified OxlT to facilitate detection of disulfide trapping between selected positions on these two domains. For this purpose, we introduced a Factor Xa protease cleavage site midway between the target helices (Fig. 1) so that disulfide formation could be assessed by the effect of reductant on SDS-PAGE mobility of protease-treated samples. The Factor Xa protease was chosen because its preferred cleavage site (IEGR) does not occur in OxlT, and because insertion of this cleavage site has proven feasible for other membrane proteins including LacY (11, 13) and the muscarinic (M3) acetylcholine receptor (14). In preliminary trials, we found partial cleavage of OxlT after exposure to Factor Xa (Fig. 2), even without insertion of the preferred IEGR site. To evaluate the possibility that the protease might act with low efficiency at one or more secondary sites, we modified the two GR sequences of OxlT, hoping to limit such a background response. Indeed, background proteolysis was negligible in a cysteine-less variant carrying the R248Q modification although not the R284Q mutation (Fig. 2). Further trials showed that insertion of a Factor Xa cleavage site (IEGR) into the OxlT central loop was well tolerated but that efficient proteolysis required insertion of the cleavage site in tandem duplication (not shown; Ref. 11)3. Accordingly, OxlTfxa, the derivative used to house double cysteine mutants, carried both the R248Q mutation and an IEGRIEGR insertion (Fig. 1). Functional analysis of this host indicated that it catalyzed oxalate self-exchange at the same rate as the cysteine-less parental protein (not shown).

Intramolecular Cross-linking in OxlT—In our initial studies we sought to maximize the likelihood of disulfide trapping by first studying a variant carrying cysteines known to react with hydrophilic sulfhydryl probes in a substrate-protectable fashion because of their location on the substrate translocation pathway (3). The selected variant (OxlTfxa/63–359) harbored cysteines at positions 63 (TM2) and 359 (TM11). When analyzed separately, these mutations had each been associated with a functional deficit, the Q63C mutation lowering maximal specific activity would have been expected, if these deficiencies been fully expressed in the double mutant, a 20-fold reduced specific activity would have been expected, rather than the observed 5-fold reduction (Table I).

Early trials (not given) established that OxlTfxa was effi-

3 We thank Dr. H. R. Kaback, Jr. for this suggestion.
FIG. 2  Latent Factor Xa cleavage site. Membrane vesicles containing the indicated OxlT variants were placed overnight at 4 °C with and without Factor Xa (see “Experimental Procedures”), after which samples were taken for SDS-PAGE and immunoblotting using antibody reactive to the polyhistidine C terminus. From the left, lanes show profiles for cysteine-less OxlT, cysteine-less OxlT with an R248Q mutation, cysteine-less OxlT with both R248Q and R284Q mutations. The positions of OxlT and its derivative, OxlT-frag, are indicated.

TABLE I  Double cysteine derivatives surveyed for disulfide trapping

| Mutant | Expression Specific activity | Mutant | Expression Specific activity |
|--------|-----------------------------|--------|-----------------------------|
| G49C-K355C | 70 ±5 | G49C-G366C | 30 ±5 |
| V50C-K355C | 57 ±5 | V50C-G366C | 84 ±5 |
| A53C-K355C | 30 ±5 | S51C-G366C | 28 ±12 |
| Q56C-K355C | 14 ±5 | A53C-G366C | 29 ±5 |
| T57C-K355C | 30 ±5 | A54C-G366C | 61 ±60 |
| V63C-K355C | 60 ±5 | V55C-G366C | 44 ±5 |
| V64C-K355C | 60 ±5 | Q56C-G366C | 51 ±5 |
| L61C-K355C | 68 ±17 | T57C-G366C | 38 ±30 |
| Q66C-K355C | 37 ±5 | T60C-G366C | 81 ±5 |
| A67C-K355C | 82 ±5 | Q63C-G366C | 29 ±42 |
| G68C-K355C | 86 ±5 | V64C-G366C | 96 ±30 |

In principle, cross-linking could reflect an intramolecular and/or intermolecular reaction. Results given here strongly favor the first possibility because no significant accumulation of higher molecular weight aggregates (dimers, etc.) was found after cross-linking but before protease treatment (Fig. 3). In addition, for the experiment shown (Fig. 3), we separately probed duplicate blots with antibodies to the OxlT N and C termini because the intra- and intermolecular reactions yield different predictions regarding fragments arising after Factor Xa cleavage but before exposure to DTT. Specifically, the intramolecular reaction yields a single 35-kDa species in the absence of reductant. By contrast, all intermolecular reactions (TM2-TM2', TM11-TM11', TM2-TM11', etc) give either a small fragment (18 or 22 kDa corresponding to the N-terminal or C-terminal portions of OxlT, respectively), or a mixture of the large (35 kDa) and small (18 kDa or 22 kDa) forms. Because both antibodies detected only the 35-kDa species after cleavage of the oxidized sample (Fig. 3), we conclude that the predominant cross-linking reaction is within a single OxlT monomer. 

FIG. 3  Cross-linking between positions 63 (TM2) and 359 (TM11). Membrane vesicles of a TM2/TM11 double cysteine variant (OxlTFxa/63–359) were exposed to 0.5 mM Cu(II)(1, 10-phenanthroline), or an equivalent volume of its suspension buffer for 10 min at 37 °C. After quenching (see “Experimental Procedures”), vesicles were incubated overnight at 4 °C with and without Factor Xa; a portion of the cross-linked sample was given 200 mM DTT for 30 min at room temperature before SDS-PAGE. (Left panel) After SDS-PAGE (0.25 μg/lane) OxlT and its C-terminal fragment were detected by immunoblotting with antibody directed against the polyhistidine C terminus. (Right panel) After SDS-PAGE (2 μg/lane) OxlT and its N-terminal fragment were detected by immunoblotting with a polyclonal antibody directed against the OxlT N terminus. For untreated controls, the dimer/monomer ratio was 7–9% (samples at 0.25 μg/lane) or 21–26% (samples at 2 μg/lane); these ratios were unchanged by exposing vesicles to oxidizing conditions. The higher dimer/monomer ratio (right panel) is presumed to reflect the known tendency of OxlT to aggregate at higher protein concentrations (2, 3).

In our work, we found little or no evidence of spontaneous disulfide formation because protease treatment of the unoxidized sample always led to efficient (usually 90%–95%) conversion of the 35-kDa form to the 22-kDa product (and see below).

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Cross-linking between TM2 and TM11—Based on the results of these initial experiments (Fig. 3), we conducted a broader search for pairs of positions on TM2 and TM11 sufficient to enable disulfide bond formation. To do this, we selected seventeen positions on TM2 that (with the exception of Q56C and Q66C) yield functional products as single-cysteine variants (typically, 15–115% of the parental specific activity).2 A subset of this cohort was then paired with one of four cysteines on the polar face of TM11 lining the substrate translocation pathway (3). Because the polar face of TM11 is highly sensitive to perturbation by mutagenesis (3), this experimental design at times led to analysis of non-functional products (see Table I).
For example, the TM11 K355C mutant displays undetectable levels of oxalate transport (2, 3). It was therefore expected that double cysteine variants incorporating this mutation would be of low specific activity, as observed (Table I). A significant loss of function is also associated with the TM11 G366C mutation, whose specific activity is 5% of its cysteine-less parent (3). In this case, however, we note that several double cysteine derivatives incorporating G366C have a substantially increased specific activity, suggesting a form of internal complementation and phenotypic suppression. Prior study of the two other TM11 partners (S359C and A370C) revealed derivatives of moderate specific activity (33 and 45%, respectively, of the cysteine-less parent), and a corresponding level of residual function is recorded in most double cysteine variants based on these mutations (Table I).

Altogether, 46 double cysteine pairs were tested (Table I), among which 26 proved positive and 20 negative for disulfide trapping (summarized in Fig. 4). We believe these results support three broad conclusions. First, it is apparent that TM2 and TM11 are sufficiently close in the OxlT tertiary structure to support significant disulfide trapping of appropriately placed cysteine pairs. In turn, this suggests these helices are sufficiently close to cooperate in substrate binding. Second, it also appears that TM2 and TM11 are juxtaposed along their full-lengths because the most distant TM11 partners (K355C and A370C) each cross-links to a similarly placed group on TM2. Thus, K355C, which lies in the cytoplasmic half of TM11 (2), links with positions in a comparable region of TM2 but not to positions near the TM2 periplasmic pole. In the same way, A370C, on the outside edge of TM11, links with a similarly placed group on TM2 but not to positions at the TM2 center. Third, when seen in helical representation (Fig. 4E), the pattern of positive and negative responses indicates that a single helical face of TM2 is juxtaposed to the TM11 helical face containing positions 355, 359, 366, and 370. We note as well that TM2 positions that fail to cross-link lie on a helical face opposite to that supporting productive interaction with TM11. Notably, such patterns hold whether or not the targeted variant displays function, a finding consistent with observations in other membrane proteins analyzed in this way (11, 15, 16).

Cross-linking Blocks OxlT Function—It was also of interest to determine whether a covalent link between TM2 and TM11 affects OxlT function. To address this question, we conducted cross-linking of the OxlTFxa/63–359 double cysteine mutant by the usual methods, and then solubilized both treated vesicles and untreated controls to prepare oxalate-loaded proteoliposomes. These proteoliposomes were then used for both SDS-PAGE, to evaluate the extent of TM2-TM11 cross-linking, and

Fig. 4. Cross-linking between TM11 and TM2. Panels A–D summarize experiments evaluating disulfide trapping between K355C, S359C, G366C, or A370C in TM11 and the specified targets in TM2. In each panel, representative immunoblots (as in Fig. 3) illustrate one negative and two positive responses as indicated by SDS-PAGE of untreated control vesicles (lanes 1), untreated control vesicles exposed to Factor Xa protease (lanes 2), vesicles treated with Cu(II)(1,10-phenanthrline), and exposed to Factor Xa protease (lanes 3), and vesicles treated with oxidizing agent and Factor Xa protease and then DTT prior to electrophoresis (lanes 4). Cartoons give helical representations of TM2 (left) and TM11 (right) with colored circles indicating those positions examined; green indicates the finding of a cross-link; red indicates a negative response. Panel E gives TM2 and TM11 helical wheels with residues participating in cross-links (panels A–D) shown in green and those for which cross-links were not found in red; a gray shading indicates lack of expression of the cysteine derivative.
for assays of oxalate self-exchange, to assess OxlT function (Fig. 5). Immunoblot analysis (Fig. 5, inset) showed that proteoliposomes from control and cross-linked samples contained similar levels of protein and that after proteolysis the untreated control yielded the expected 22-kDa fragment without regard to the presence of DTT. By contrast, the cross-linked sample showed no change of OxlT mobility unless DTT was present, indicating that in proteoliposomes prepared from the oxidized sample most of the OxlT population (≥ 80%) was in a cross-linked configuration. Parallel study of [14C]oxalate transport showed that such cross-linking was correlated with a significant decrease in OxlT function (Fig. 5). Equally important, exposure of proteoliposomes to reductant (100 mM DTT) just before assay led to a 3- to 4-fold increase in the rate and extent of [14C]oxalate transport, restoring it to the level found in control preparations. Such findings suggest that cross-linking inhibits OxlT function and that this inhibited state should be attributed to the covalent link between TM2 and TM11 rather than to an irreversibly altered OxlT structure.

**DISCUSSION**

The availability of several well established techniques has greatly facilitated an understanding of the two-dimensional topology of membrane proteins (4, 17). On the other hand, the three-dimensional arrangement of such proteins is poorly documented, largely because application of the most reliable methods for this purpose, two- and three-dimensional crystallography, has met with limited success. In this setting, use of cross-linking agents, particularly those based on thiol reactivity, has become an attractive alternative tool with which to assess proximity relationships. In a few notable cases, such as the bacterial chemotaxis receptors (18) and LacY, the lactose transporter of *E. coli* (19), the use of disulfide trapping and/or thiol-based cross-linking agents has led to robust models of tertiary structure in the absence of crystallographic evidence.

In applying this approach to the antiporter, OxlT, we elected to first study the proximity of TM2 and TM11, two helices likely involved in substrate binding (Fig. 1 and accompanying text). Because the substrate of OxlT, oxalate ("OOC-COO"), is small (about 6 Å in length) ligand-binding atoms in TM2 and TM11 could not be separated by much more than this dimension if they were each to contribute to substrate binding. And because disulfide bond formation does not occur efficiently if cysteinyl sulfur atoms are much more than 8 Å apart (9), demonstration of disulfide bond formation between TM2 and TM11 could strengthen the idea that these helices cooperate together in substrate binding. Indeed, less direct support for this hypothesis came from an unanticipated source. Several TM2 mutants, when combined with a TM11 variant of low specific activity, suppressed the TM11 phenotype. The Q63C/S359C pairing was noted earlier, but the most striking example is the G49C/G366C doublet, in which the low activity of the G366C variant (~5%) (3) is fully suppressed in the context of the double mutant (Table 1). Had such complementation arisen during a search for second-site suppressors, it would have presented genetic evidence favoring interaction between TM2 and TM11. The extent to which this suppression reflects true helix-helix interaction must now await crystallographic reconstruction.

Trials with OxlT double cysteine variants yield a pattern of cross-linking that clearly suggests a single helical face of TM2 abuts the polar surface of TM11 that lines the translocation pathway (Fig. 4). It is also worth comment that in OxlT the linear dimension along TM2 defined by a capacity to cross-link corresponds to about ± two turns of an α helix (see, for example, the behavior of G366C), or about ± 11 Å, consistent with the observations of Careaga and Falke (9). Equally relevant, it is clear that cross-linking itself need not significantly perturb the native OxlT structure because in at least one case the functional deficit associated with disulfide trapping is fully reversed under reducing conditions (Fig. 5). Finally, we note that our experiments have been done in the absence of substrate, and we do not know if a substrate-bound conformation of OxlT will yield similar findings. In fact, we have observed inhibition of cross-linking by oxalate,4 but this phenomenon is not interpreted easily because oxalate itself may restrict availability of the catalytic metal required for disulfide formation. It may be possible to pursue this issue in later work, now it is clear that N,N′-o-phenylenedimaleimide also mediates TM2-TM11 cross-linking (see above).

Our work follows an extensive series of cross-linking experiments in LacY (19), another member of the Major Facilitator Superfamily. In those studies, cross-linking was most often achieved by using bifunctional maleimides to connect separately expressed N- and C-terminal portions of the protein. Such experiments indicate that in LacY, as in OxlT, TM2 and TM11 are neighbors in the native structure of the transporter. Nevertheless, significant differences are apparent in the two model systems. In LacY, the effectiveness of cross-linking between TM2 and TM11 does not generally rise above about 30% (11), a level reached only for cysteine pairs placed near the center of the hydrophobic core. Further, in LacY cross-linking between TM2 and TM11 diminishes in efficiency as more external positions are examined, leading to the suggestion that these helices diverge at their periplasmic ends (20). In studies with OxlT, cross-linking efficiency is ~80% or better at all

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4 Y.-M. Kim, L. Ye, and P. C. Maloney, unpublished material.
positions tested (Fig. 4). Although a kinetic study of cross-linking along the TM2/TM11 interface (15) may reveal a similar divergence in OxlT, the present data fit best with the idea that in TM2 and TM11 are closely juxtaposed along their full-lengths. It is feasible that differences in the efficiency of cross-linking LacY and OxlT arise because the latter is a more compact and rigid structure as implied by the tight helix packing found by two-dimensional crystallography (21).

Cross-linking studies of the sort summarized here have value beyond supporting the idea that TM2 and TM11 might jointly contribute to construction of a substrate-binding site. The determination of an OxlT projection structure (21) offers a general view of helix packing in the Major Facilitator Superfamily, but of itself this information cannot match specific helices to the observed electron density profiles. That correlation will require added biochemical study, including the analysis of helix proximity as documented here.

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