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Oligomeric State of the Oxalate Transporter, OxlT

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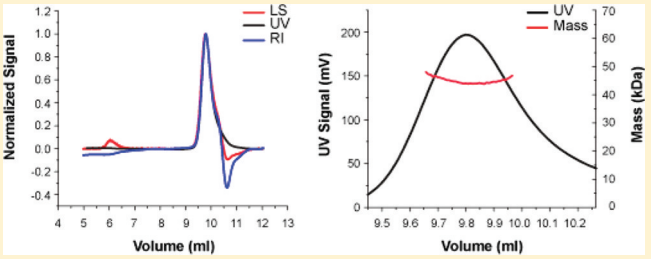
Supporting Information

ABSTRACT: OxlT, the oxalate transporter of *Oxalobacter formigenes*, was studied to determine its oligomeric state in solution and in the membrane. Three independent approaches were used. First, we used triple-detector (SEC-LS) size exclusion chromatography to analyze purified OxlT in detergent/lipid micelles. These measurements evaluate protein mass in a manner independent of contributions from detergent and lipid; such work shows an average OxlT mass near 47 kDa for detergent-solubilized material, consistent with that expected for monomeric OxlT (46 kDa). A disulfide-linked OxlT mutant was used to verify that it was possible detect dimers under these conditions. A second approach used amino-reactive cross-linkers of varying spacer lengths to study OxlT in detergent/lipid micelles and in natural or artificial membranes, followed by analysis via sodium dodecyl sulfate−polyacrylamide gel electrophoresis. These tests, performed under conditions where the presence of dimers can be documented for either of two known dimeric transporters (AdiC or TetL), indicate that OxlT exists as a monomer in the membrane and retains this status upon detergent solubilization. In a final test, we showed that reconstitution of OxlT into lipid vesicles at variable protein/lipid ratios has no effect on the specific activity of subsequent oxalate transport, as the OxlT content varies between 0.027 and 5.4 OxlT monomers/proteoliposome. We conclude that OxlT is a functional monomer in the membrane and in detergent/lipid micelles.



The oxalate/formate antiporter, OxlT, is found in the Gram-negative bacterium *Oxalobacter formigenes*. As a commensal found in the gut of animals and humans, this anaerobic organism plays an important role in clearing dietary oxalate from the intestinal tract.[1] Such clearance occurs via a metabolic cycle in which external oxalate is brought inward via the transporter, OxlT, allowing an intracellular oxalyl decarboxylation system to release both carbon dioxide and formate. The cycle is completed by outward transport of formate, also via OxlT. The overall oxalate^{2-}:formate^{-} exchange process carries negative charge into the cell, at a stoichiometric level with consumption of an intracellular proton by the decarboxylation.[2] As a result, these steps generate an electrochemical proton gradient that can be used to power membrane activities. Indeed, this is the source of energy for ATP synthesis in this cell.[2,3]

OxlT belongs to the major facilitator superfamily (MFS), the largest known transporter superfamily[4,5] (http://www.tcdb.org/), whose members interact with a broad range of substrates, including drugs, neurotransmitters, amino acids, sugars, and inorganic ions. Despite this breadth of substrate selectivity, transporters within the MFS share common structural themes and are therefore believed to utilize similar transport mechanisms.[5,6] Nevertheless, it appears that MFS members can adopt distinct oligomeric organizations. For example, electron crystallography suggests the tetracycline cation/proton antiporter (TetA) from *Escherichia coli* is organized as a trimer,[7] while a biochemical study shows that a related drug antiporter (TetL) functions as a dimer in *Bacillus subtilis*.[8] Analytical ultracentrifugation and freeze-fracture electron microscopy show that the lactose transporter (LacS) from *Streptococcus thermophilus* is also a dimer,[9] yet analytical high-performance liquid chromatography (HPLC) studies along with X-ray crystallography indicate that the glycerol 3-phosphate transporter (GlpT) from *E. coli* is monomeric.[10,11]

The work described here was designed to determine the oligomeric status of OxlT, both in the membrane and in a solubilized state suitable for biochemical studies. Characterization of the oligomeric state, although critical for understanding function and regulation, often proves difficult for membrane proteins.[12] Such proteins may exhibit low levels of protein expression and poor efficiency of purification; membrane proteins also bind variable amounts of detergents and lipids, and this may significantly affect protein stability and physical properties. Further, the molecular organization within the membrane may differ from that of the detergent-solubilized form, either because of the effects of detergents on the protein or because detergent disrupts interactions with lipids.[9] For OxlT, the only currently available information about oligomeric state comes from electron crystallography,[13] in which the limited extent of observed intermolecular contacts suggests that the protein adopts a monomeric state during crystallization. To extend this work, we used three different analytical approaches

Received: July 29, 2011
Revised: August 22, 2011
Published: August 25, 2011

to study the oligomeric status of OxlT both in solution and when embedded in artificial and natural membranes. In each case, our findings are consistent with OxlT functioning as a monomer.

EXPERIMENTAL PROCEDURES

Bacterial Strain, Plasmids, and Site-Directed Mutagenesis. In OxlT-Tb-A, the OxlT C-terminal histidine[14] was replaced with a thrombin recognition sequence (LVPRGS), followed by a His_{10} tag to facilitate affinity purification. OxlT-Tb-A was encoded within a 1.4 kb XbaI−HindIII fragment in pBluescript II SK^+ (Amp^r) under *plac* control. To suppress protein expression prior to induction, plasmids were housed in *E. coli* strain XL3, which is strain XL1 carrying plasmid pMS421 ($Spec^r$, $LacI^q$).[14] Single- and double-cysteine variants (F18C, A342C, A307C, F18C/A342C, F18C/A307C, and H413C) were generated by QuikChange site-directed mutagenesis (Stratagene), using as a template a cysteine-less variant (C28G/C271A) with a C-terminal His_9 tag but without the thrombin cleavage site.[15] As control proteins, we used the transporters AdiC (arginine/agmatine antiporter) and TetL (drug/cation antiporter), which were expressed and purified as described previously.[8,16]

Protein Expression and Purification. A single colony of XL3 harboring a plasmid encoding OxlT or one of its variants was placed in 50 mL of LB medium, and after being grown overnight at 37 °C, this preculture was diluted 100-fold into four 1.2 L batches of fresh LB medium for further growth at 35 °C until A_{650} reached 0.1. OxlT-Tb-A and OxlT mutant expression was then induced by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, and growth was continued for an additional 3 h before the cells were harvested by centrifugation. Cell pellets were combined and suspended in 100 mL of buffer (pH 7.5) containing 20% (v/v) glycerol, 200 mM potassium oxalate, 50 mM Tris-HCl, and 1 mM phenylmethanesulfonyl fluoride (from a 0.25 M stock in dimethyl sulfoxide). Cells were disrupted by sonication on ice in a cold room for 10 min (5 s on and 5 s off), at power level 5, using an Ultrasonic XL2020 sonicator (Misonix). Membrane proteins were solubilized by addition of 1% (w/v) DDM (Anatrace) at 4 °C for 1 h. After insoluble material had been removed by ultracentrifugation (324000*g* for 30 min), the supernatant was incubated with 4 mL of Ni-NTA resin (Qiagen) at 4 °C for 4 h, followed by washing with 100 bed volumes of buffer (pH 7.5) containing 20% glycerol, 200 mM potassium oxalate, 20 mM Tris-HCl, 40 mM imidazole, and the indicated detergent (0.1% DDM, 0.1% UDM, 0.2% DM, 0.6% NM, 1.2% OG, 0.1% Cymal-6, 0.1% DHPC, or 1% CHAPS). Protein was then eluted at low pH (pH 4.3), using a buffer containing 20% glycerol, 200 mM potassium oxalate, 50 mM acetic acid, and the desired detergents. The protein concentration in the eluate was measured as described previously;[14,15] the yield of purified OxlT was 0.8−1.2 mg/L of LB.

Size Exclusion Chromatography. After affinity purification of OxlT, 100 μg of protein were loaded onto a preparative size exclusion Superdex 200 (10/300 GL) column for fast protein liquid chromatography (AKTA PrimePlus, GE Healthcare) at 23 °C, at a flow rate of 0.6 mL/min, using a buffer (pH 7) containing 5% glycerol, 20 mM Tris-HCl, 100 mM NaCl, 100 mM potassium oxalate, and detergents at the concentrations indicated above. Protein elution was monitored by the absorbance at 280 nm.

Determination of Molecular Masses by TD-SEC-LS. Analytical size exclusion chromatography was conducted using a Postnova Analytics PN1122 HPLC pump in conjunction with a Shodex KW-G guard column in line with a Shodex KW-803 size exclusion column. A total of 50−100 μg protein was injected for each run. The eluate from the size exclusion column flowed first through a Postnova PN3210 UV−vis detector, followed by a Precision Enterprise^{MDP} system containing static light scattering and refractive index detectors (Precision Detectors, Inc.). The system (column and detector flow cells) was equilibrated overnight with buffer (pH 4.3) containing 5% glycerol and 50 mM acetic acid or a similar buffer (pH 7) containing 50 mM potassium MOPS instead of acetic acid; these buffers also contained 100 mM potassium oxalate or potassium malonate, as indicated, along with the specified detergents. The system was maintained at room temperature, as lower temperatures resulted in unsteady baseline from the refractive index detector. For each set of trials, an instrument calibration constant was obtained with a set of six soluble proteins with known extinction coefficients (ε_{280}) and molecular masses (from 29 to 161 kDa), as described previously.[17] For the calculation, a value of 1.7 was used for the ε_{280} of OxlT-Tb-A and its derivatives on the basis of the known amino acid sequences (http://ca.expasy.org/cgi-bin/protparam).

The tracings of A_{280} (UV), light scattering (LS), and refractive index (RI) were aligned using Discover 32 (Precision Detectors, Inc.), and the molecular mass of OxlT was calculated as described previously,[17,18] using the instrument calibration constant (see above) and the integrated area under the OxlT elution peak of the signals from the three detectors. The ratio of detergent and lipid to protein was also derived from the three detector signals,[17] using a value of 0.187 mL/g for the increment in refractive index with concentration (dn/dc) for protein, as commonly used for soluble proteins,[18,19] and the dn/dc values for detergents obtained from the supplier.

EGS-, DSP-, DSG-, and Glutaraldehyde-Mediated Cross-Linking. For cross-linking in detergent micelles, purified OxlT and AdiC were diluted to a concentration of 0.05 mg/mL with buffer containing 20% glycerol, 20 mM postassim phosphate, 100 mM potassium oxalate, and 0.1% DDM (pH 7). Aliquots of 50 μL were incubated at room temperature for 30 min with the indicated concentrations of EGS, DSP, DSG (Pierce), or glutaraldehyde (Sigma-Aldrich), in the presence or absence of 0.5% SDS. Reactions were quenched by dilution with 50 mM Tris-HCl (pH 7.5). Samples were then processed for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), followed by silver staining. Masses were estimated relative to the mobilities of standard proteins (Bio-Rad) of known mass run on the same gel.

For cross-linking in native membranes, OxlT and AdiC cell pellets were lysed by sonication, followed by low-speed centrifugation (23000*g* for 15 min) to remove unbroken cells. Membranes were collected by ultracentrifugation (180000*g* for 90 min), and the pellets were suspended at pH 7 in 20% glycerol, 20 mM KP_i, and 100 mM potassium oxalate. Subsequent cross-linking reactions were conducted as described above for analysis of solubilized protein. Samples were analyzed via SDS−PAGE followed by immunoblotting.

Reconstitution and OxlT Functional Assay. Solubilized protein was reconstituted by detergent dilution as described previously,[14,15,20] to generate proteoliposomes loaded with 100 mM potassium oxalate and 50 mM potassium phosphate (pH 7). We measured OxlT function by gently pipetting

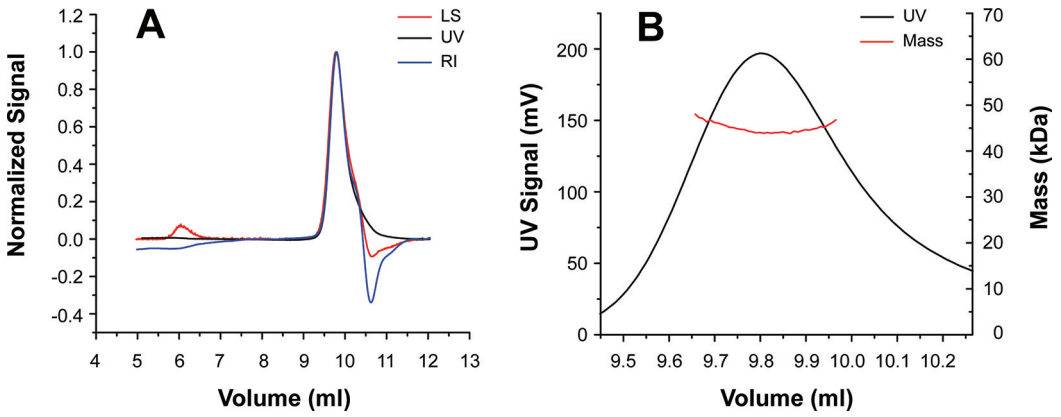


Figure 1. Determination OxlT molecular masses using TD-SEC-LS. (A) OxlT (50−100 μg) was loaded onto a Shodex kw-803 HPLC column in the presence of 5% glycerol, 50 mM potassium MOPS, 100 mM potassium oxalate, and 0.1% DDM (pH 7). The tracings show normalized outputs of detectors for measurement of A_{280} (UV, black), light scattering (LS, red), and refractive index (RI, blue). In a parallel trial in the same experiment with the same buffer conditions, the instrument calibration constant K_1 was obtained using a reference set of six protein standards with masses between 29 and 141 kDa (see Experimental Procedures). (B) For illustrative purposes, using data from panel A, the OxlT mass (red) was calculated for the region across the A_{280} trace at ~75% peak height. For this experiment and other experiments, the mass values reported in Tables 1 and 2 were calculated at the peak of the A_{280} trace.

Table 1. TD-SEC-LS Measurements of OxlT Molecular Masses in Detergent Micelles

buffer condition	elution volume (mL)	apparent molecular mass (kDa)[a]	average K_1[b]	TD-SEC-LS calcd molecular mass (kDa)[c]	detergent/protein (g/g)[d]
0.1% DDM, 100 mM oxalate, pH 7[e]	9.65	160	23700	44.6	2.58
0.1% DDM, 100 mM oxalate, pH 4.3[f]	10.3	157	23100	46.9	2.34
0.1% DDM, 200 mM malonate, pH 7[f]	9.66	153	25500	48.7	2.15
0.1% UDM, 100 mM oxalate, pH 7[f]	10.0	131	25700	46.4	1.83
0.2% DM, 100 mM oxalate, pH 7[f]	10.1	120	25600	48.7	1.47

[a]Apparent molecular mass derived from elution time in comparison to protein standards. [b]Derived from the elution profiles of protein standards (see Experimental Procedures). [c]Calculated for the polypeptide component, without contributions from bound detergent, as described in Experimental Procedures. [d]Detergent/protein ratios were estimated as described in refs 17 and 18 (see Experimental Procedures). [e]See Figure 1. [f]See Figure S1 of the Supporting Information.

proteoliposomes onto a 0.22 μm pore size Millipore filter, rinsing twice with 5 mL of assay buffer [100 mM potassium sulfate and 50 mM MOPS (pH 7)], and then overlaying the trapped proteoliposomes with assay buffer containing 100 μM [^{14}C]oxalate (5 mCi/mmol, American Radiolabeled Chemicals, Inc.).[20] After an incubation period of 1 min, the reaction was terminated by washing with 5 mL of iced assay buffer.

Other Assays. For maltosides and glucosides, detergent levels were determined by assays of reducing sugar content;[21] the amount of phospholipid bound to OxlT was estimated by its phosphorus content, using reactions with ferrothiocyanate[22] or Malachite Green.[23]

Chemicals. All detergents were purchased from Affymetrix-Anatrace Inc. The detergents were of Anagrade quality for TD-SEC-LS experiments; Sol-Grade was used in other work. Thrombin protease was obtained from GE Healthcare.

RESULTS

Determination of Molecular Masses by TD-SEC-LS. The presence of associated detergents and lipids makes it difficult to use traditional chromatographic methods to estimate the molecular size of membrane proteins. By contrast, TD-SEC-LS uses the well-established relationship between protein molecular mass and UV absorbance (UV), light scattering (LS) and refractive index (RI) to monitor protein mass in a manner independent of bound, non-UV-absorbing materials.[18,19,24] As a test of this approach for OxlT, we determined the mass of the purified protein under several different solubilization conditions. In these trials, we focused on use of maltoside-based detergents (DDM, UDM, and DM), because of their minimal destabilizing effects on wild-type OxlT, and on use of two different substrates (oxalate and malonate) at the pH extremes (pH 4.3 and pH 7) normally encountered during biochemical analysis of this transporter.

In TD-SEC-LS, the chromatographic profile of OxlT solubilized in 0.1% DDM (Figure 1A) is indicative of a monodisperse preparation with an aggregate mass (based on elution time) of 160 kDa (see Table 1). We noted that following elution of the protein peak, the RI and LS traces may also display an additional positive or negative deflection. These late-eluting features, which are of varying amplitudes in different trials, appear to arise because of different concentrations of detergent and glycerol in the injected samples relative to the elution buffer, varying concentrations of detergent and other reagents in the injected samples, and possible sequestration of detergent by injected material. In some samples, a minor peak in the LS signal also appears at an elution time corresponding to the void volume of the column. In these cases, however, the absence of UV and RI signals at this position suggests this region has no significant protein or detergent components.[17]

Table 1 lists the masses of OxlT as determined by TD-SEC-LS for five different conditions (and see Figure S1 of the Supporting Information). In each case, calculation of the

dx.doi.org/10.1021/bi201175y | Biochemistry 2011, 50, 8445−8453

molecular mass was based on values for UV, LS, and RI signals that were averaged over a small area centered on the protein peak. For illustrative purposes, a point-by-point molecular mass profile was also calculated for each elution time corresponding to a UV absorbance greater than 75% of the maximal peak absorbance, as shown in Figure 1B. Throughout this work (Table 1), the measured mass of OxlT [mean of 47.1 ± 0.9 kDa (standard error of the mean) for all trials] agrees well with its predicted mass of 46 kDa.

The apparent hydrodynamic radius of OxlT varied considerably in the presence of different detergents; the predicted mass of the complex, as calculated from the elution times, ranged from 120 to 160 kDa, and the corresponding detergent lipid/protein ratio calculated by comparison of detector signals in the TD-SEC-LS system varied from 1.47 to 2.34 g/g (Table 1). Because most lipid is expected to be removed during purification of the solubilized protein (ref 25 and unpublished measurements of bound lipid), it is likely that this associated material is predominantly detergent. For this reason, it is reasonable that the hydrodynamic radius of the complex increases as the detergent acyl chain length increases. Similar relationships between detergent chain length and complex size have been observed in studies of solubilized forms of the yeast mitochondrial ADP/ATP exchange carrier[26] and the bacterial zinc transporter (YiiP) from *E. coli*.[27]

Size exclusion chromatography was also conducted on OxlT in a variety of additional detergents, including DHPC, NM, DM, UM, DDM, and Cymal-6, ranging in acyl chain length from 6 to 12 carbons and containing glucosyl, maltosyl, or phosphocholine headgroups. In each case, OxlT exhibited predominantly monodisperse elution profiles (not shown). Retention of monodisperse profiles in the additional detergents suggests that OxlT is monomeric under these conditions as well.

Cysteine Cross-Linking. Because disulfide cross-linking has been used previously to determine intramolecular distances in OxlT,[28] we investigated whether this methodology could provide an alternative approach to determining the oligomeric state of detergent-solubilized OxlT. For these purposes, because the two endogenous cysteines (C28 and C271) are buried,[29] we tested for formation of disulfide cross-links between OxlT monomers engineered to contain one or two accessible cysteine residues (see Experimental Procedures). In an attempt to facilitate formation of intermolecular cross-links, we constructed seven cysteine substitution mutants, using the OxlT homology model[30] to select positions at exposed cytoplasmic or periplasmic surfaces. For six of these mutants, TD-SEC-LS of DDM-solubilized material indicated a monomer as the sole species present [mean of 47 ± 0.9 kDa (standard error of the mean)] (Table 2). The seventh mutant contained a substitution of Cys for His413, located immediately prior to the C-terminal His_9 tag. In this case, TD-SEC-LS performed in the absence of reducing agents yielded two forms of OxlT (Figure 2A). The larger of these had a calculated mass of 103 kDa, while the smaller species exhibited a calculated mass of 56.4 kDa (legend of Figure 2). Moreover, the 103 kDa form was not observed when the elution buffer contained the reducing agent TCEP (10 mM) (Figure 2B), suggesting that this species is a disulfide-linked dimer. To assess the sensitivity of this assay for detecting an OxlT dimer, we also studied the arginine/agmatine antiporter (AdiC) and the drug/cation antiporter (TetL). These proteins have a monomer size (47 and 50 kDa, respectively) comparable to that of OxlT, but both AdiC and TetL are found as dimers.[8,16,31] Consistent with those published findings, TD-SEC-LS of AdiC yielded a single dominant peak with a molecular mass

Table 2. TD-SEC-LS Measurements of Molecular Masses of OxlT Cysteine Substitution Mutants[a]

mutant	TD-SEC-LS calcd molecular mass (kDa)[b]
C27G/C271A (cysteine-less parent)	44.2
F18C	49.4
A342C	46.9
A307C	48.2
F18C/A342C	45.1
F18C/A307C	48.2

[a]Single- or double-cysteine substitution mutants were constructed in the background of the OxlT cysteine-less variant. TD-SEC was performed in 0.1% DDM in the presence of 100 mM potassium oxalate (pH 7). [b]Calculated as described in Table 1 for the polypeptide, without contributions from bound detergent.

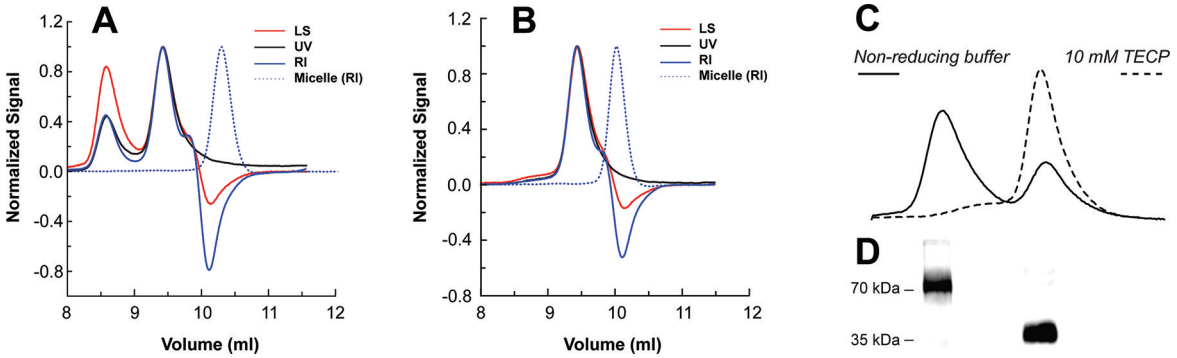


Figure 2. Cross-linking of a cysteine substitution mutant. The OxlT H413C variant was subjected to TD-SEC-LS or SEC after solubilization and purification. (A) Two forms of OxlT were detected by TD-SEC-LS in 0.1% DDM at pH 7. On the basis of TD-SEC-LS measurement, the calculated molecular masses of these forms are 103 and 56 kDa, corresponding approximately to dimer and monomer forms of OxlT, respectively. (B) The dimer form was not found after addition of a reducing agent (10 mM TCEP). In both panels A and B, parallel chromatographic runs without protein were used to identify the elution time of the DDM micelle, as reflected by its RI trace (dotted lines). (C) SEC using Superdex 200 was performed after overnight incubation at 23 °C and pH 8.5, to facilitate disulfide bond formation. A sample of 100 μg protein was loaded onto the column in elution buffer (pH 7.5) containing 5% glycerol, 20 mM Tris-HCl, 100 mM NaCl, 100 mM potassium oxalate, and 0.1% DDM, with (---) or without (—) 10 mM TCEP. (D) Nonreducing SDS–PAGE of samples taken from the apparent dimer and monomer peaks showed the presence of OxlT species corresponding to ~70 and ~35 kDa, respectively.

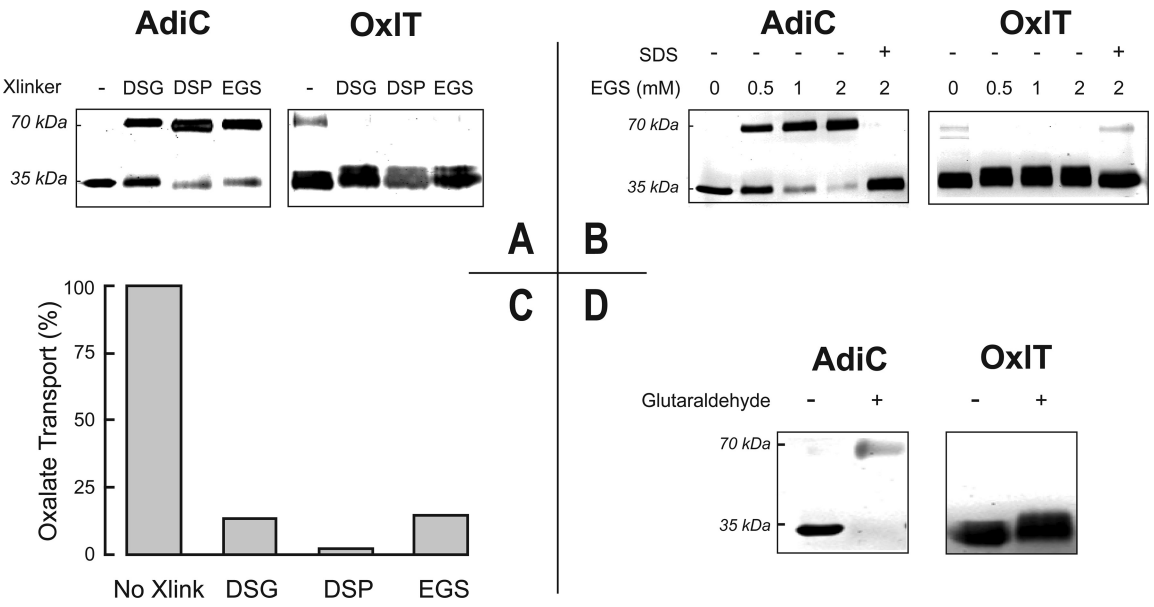


Figure 3. Amino-directed cross-linking of solubilized OxlT. After purification, AdiC and OxlT were each suspended (50 μg/mL) in buffer (pH 7.5) with 100 mM potassium oxalate, 20 mM potassium phosphate, 20% (w/v) glycerol, and 0.1% DDM and exposed to the indicated cross-linking agent for 30 min at 23 °C. The reaction was quenched by addition of Tris-HCl (pH 7.5) to a final concentration of 50 mM, and samples were processed for SDS–PAGE. Protein was detected using Silver Stain Plus (Bio-Rad); standard markers on the same gels were used to extrapolate the indicated mass values. (A) Samples were exposed to 2 mM DSG, DSP, or EGS. (B) Samples were exposed to increasing concentrations of EGS (0 to 2 mM), in the absence or presence of 0.5% SDS, as indicated. (C) Samples were collected before and after addition of DSG, DSP, or EGS, as in panel A, to evaluate residual function by reconstitution. The observed initial rates of [^{14}C]oxalate transport are reported relative to that found for the untreated sample. (D) Samples were treated with 100 mM glutaraldehyde.

of 107 kDa, while analysis of TetL showed a single species of 122 kDa (see Figure S2 of the Supporting Information). In further tests, we also confirmed the presence of dimers and monomers of the H413C variant of OxlT by nonreducing SDS–PAGE of fractions from traditional size exclusion chromatography (Figure 2C,D). Finally, we noted that disulfide-mediated dimerization of H413C was more prominent at higher protein concentrations (results not shown), suggesting that such dimers formed spontaneously as a result of random collision of separated monomers after detergent solubilization.

Lysine-Mediated Cross-Linking. The inability to detect disulfide-linked dimers of most of the cysteine substitution mutants tested could reflect the restricted number of locations tested as sites of cysteine substitution, because such sites would have to be located at a dimer interface to form cross-linked dimers. Thus, we also examined cross-linking agents that would be expected to react with multiple sites in OxlT. In particular, we used three membrane-permeable, homobifunctional cross-linkers, DSG, DSP, and EGS, agents that react with primary amino groups to generate cross-links with well-defined maximal distances of 7.7, 12, and 16 Å, respectively.[32,33] We also used glutaraldehyde, which also targets primary amines (and occasionally arginine[34]) but has the further advantage of potentially linking widely separated sites, because it is present largely as polymers of variable size in aqueous solution.[35,36]

To test the effects of these bifunctional probes, we examined their abilities to induce cross-links in OxlT as well as AdiC, a known dimer (see above). When AdiC was subjected to cross-linking with DSG, DSP, or EGS (each used at a concentration of 2 mM) and then subjected to SDS–PAGE, the mobility of a significant fraction (≥40%) of the protein shifted from a position corresponding to the monomeric species (35 kDa) to a position corresponding to the dimer (70 kDa) (Figure 3A). In contrast, OxlT remained as a monomer when treated with these agents; however, treatment with cross-linkers did lead to broadening of the OxlT band, presumably reflecting intramolecular cross-linking,[16] as found for some other membrane proteins.[37] Formation of the higher-molecular mass species of AdiC increased as the cross-linker (EGS) concentration increased (Figure 3B), while OxlT migrated as monomer, even at the highest EGS concentration. Notably, when AdiC (or OxlT) was pretreated with 0.5% SDS before addition of EGS, cross-linking was not found. This negative finding is consistent with the argument that subunits within a larger structure are often close enough to be linked by bifunctional agents, but if the target is monomeric or consists of denatured and dissociated subunits, individual monomers will generally be too distant (in solution) to support cross-linking. We also confirmed that cross-linkers were actually reacting with OxlT under these conditions by examining samples from such experiments in functional assays. As shown in Figure 3C, the capacity of OxlT to conduct [^{14}C]oxalate transport was significantly limited by exposure to DSG, DSP, or EGS, suggesting that OxlT becomes locked or is more restricted in its conformational flexibility, as might be expected from intramolecular linkage(s).

To reduce the likelihood that the failure to cross-link OxlT (Figure 3) reflected insufficient length of the cross-linkers' spacer arms to bridge between two monomers, we conducted a similar analysis using glutaraldehyde, which can form cross-links of varying lengths.[35,36] Whereas AdiC was readily shifted to a higher-molecular mass species upon treatment with 100 mM glutaraldehyde, OxlT remained essentially unshifted (Figure 3D).

Cross-Linking in the Membrane. We also used cross-linking to analyze the oligomeric status of OxlT in its functional state, embedded in native or artificial membranes. Crude membrane suspensions derived from OxlT-expressing or AdiC-expressing *E. coli* were treated with increasing concentrations

dx.doi.org/10.1021/bi201175y | *Biochemistry* 2011, 50, 8445–8453

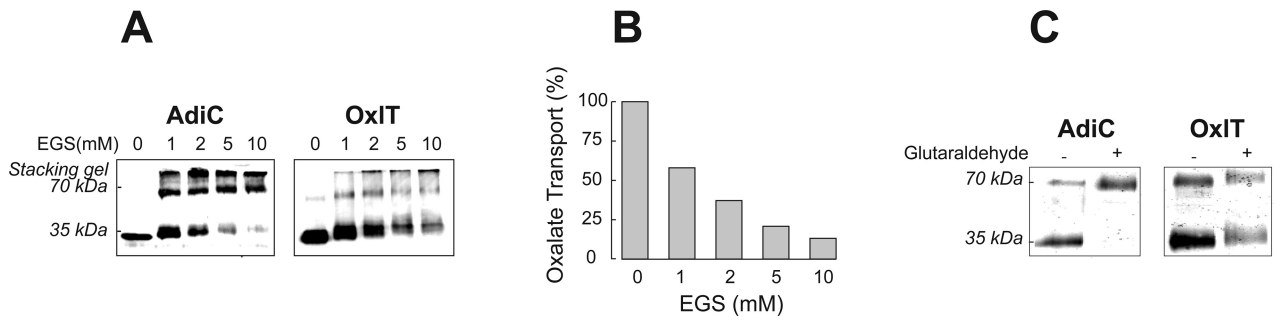


Figure 4. Amino-directed cross-linking of membrane-embedded OxlT. (A) Washed membranes from cells expressing either AdiC or OxlT were suspended in buffer (pH 7.5) containing 100 mM potassium oxalate and 50 mM potassium phosphate and then treated with 0−10 mM EGS for 30 min at 23 °C. After the reaction was quenched, samples were taken for SDS−PAGE (see the legend of Figure 3). (B) In a parallel experiment using the same conditions, samples were taken for reconstitution to evaluate residual OxlT function, as described in the legend of Figure 3C. (C) Purified OxlT or AdiC was reconstituted into proteoliposomes prepared using at 50/50 mixture of palmitoyloleoylphosphatidylcholine and palmitoyloleoylphosphatidylglycerol. Cross-linking was assessed by treatment with 100 mM glutaraldehyde, using AdiC as the positive control.

of EGS (0−10 mM). Under these conditions, AdiC gradually shifted from a predominantly monomeric band to one migrating at the position expected for a dimer (Figure 4A). We also noted that aggregates appeared at the interface between the stacking and resolving gels, presumably reflecting the cross-linking of AdiC to other, unidentified membrane proteins. By contrast, although EGS treatment of OxlT also yielded large aggregates in the stacking gel, protein that entered the gel remained largely confined to the monomer band; no major dimer or higher-order oligomeric species was observed, aside from a commonly observed minor SDS-induced oligomeric band[13,38] (see also Figure 3B) that did not increase in magnitude with increasing cross-linker concentrations. As described above (Figure 3C), we concluded that EGS does react with OxlT under these conditions, because protein extracted and reconstituted from EGS-treated membranes showed weakened function as the EGS concentration was increased (Figure 4B). These findings (Figure 4A) suggest that OxlT is predominantly monomeric in the membrane.

A difficulty in the analysis of OxlT (and AdiC) in native membranes is the nonspecific cross-linking of the target protein with other proteins or, possibly, amino-containing lipid (e.g., phosphatidylethanolamine), which may account for the generation of high-molecular mass forms trapped in the stacking gel (Figure 4A,B). In an attempt to circumvent this limitation, we also conducted cross-linking of purified OxlT reconstituted into artificial membranes containing only phosphatidylcholine and phosphatidylglycerol. [In control experiments (not shown), we confirmed that OxlT retains function in this phospholipid environment.] Reconstituted AdiC treated with glutaraldehyde was efficiently shifted from monomer to the higher-molecular mass species, yet the cross-linker had no effect on the ratio of low- to high-molecular mass species of OxlT (Figure 4C). This finding reinforces the conclusion that OxlT exists as a monomer in the membrane environment.

Reconstitution of OxlT at Low Protein/Lipid Ratios. As an additional test of the functional oligomeric state of OxlT in membranes, we measured the transport activity of OxlT as a function of lipid/protein ratio in the membrane, extending the experiment to conditions where the protein is present, on average, at less than one OxlT monomer per proteoliposome. In this instance, we followed the protocol established for UhpT, which functions as a monomer both in solution and in the membrane.[20] Assuming the presence of approximately 2×10^{12} proteoliposomes/mg of phospholipid after reconstitution,[20] we adjusted conditions to reconstitute approximately 0.03−6 OxlT monomers/proteoliposome (Figure 5). Throughout this range,

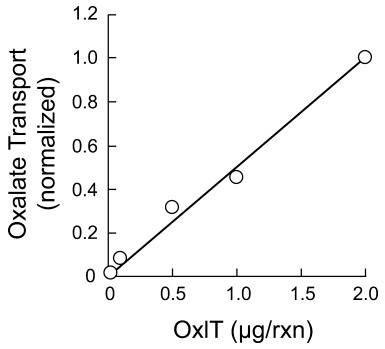


Figure 5. Reconstitution of OxlT at low protein/lipid ratios. Initial rates of [^{14}C]oxalate transport were measured after reconstitution of OxlT at variable protein/lipid ratios. Each reconstitution mixture contained 2.7 mg of phospholipid together with 0.01−2 μg of OxlT, achieving a nominal monomer/proteoliposome ratio between ~0.03 and 6. The mean values of duplicate measurements are given, normalized to the value obtained at the highest dose of OxlT. The line drawn reflects the theoretical expectation if OxlT specific activity is constant with OxlT content.

the total transport activity of OxlT was proportional to the protein concentration, indicating that the specific activity of the protein remained constant. This indicates that OxlT does not reversibly oligomerize and that the functional unit remains constant down to very low concentrations. The most likely explanation of this is that OxlT functions as a monomer, because a protein that exists as a monomer in the solubilized state would not be expected to efficiently oligomerize during reconstitution at very low average numbers of proteins per reconstituted vesicle.

DISCUSSION

Understanding the oligomeric status of a transporter such as OxlT is an essential part of its biochemical description and is critical for understanding its mechanism of action. Unfortunately, the approaches commonly used for determining the oligomeric state of a soluble protein can easily be confounded by the presence of the lipid and detergent required for biochemical study of a membrane protein. In the work reported here, we circumvented these problems by exploiting the ability of

TD-SEC-LS to evaluate the molecular mass of detergent-solubilized purified OxlT, in a manner independent of the hydrodynamic properties of the complex and the contributions of bound lipid and detergent. The unambiguous result was that OxlT exists as a monomer in the presence of a variety of detergents and substrates (Figure 1 and Tables 1 and 2). The utility of this approach to discriminating between monomers and oligomers was reinforced by successful detection of dimeric species of two known dimeric proteins, AdiC and TetL (Figure S2 of the Supporting Information). Moreover, at high concentrations, the H413C OxlT variant exhibited a subpopulation of faster migrating species that exhibited a molecular mass corresponding to dimer, as determined by TD-SEC-LS (Figure 2). This species was likely the result of disulfide cross-linking of randomly colliding monomers, as its formation was concentration-dependent and was not observed in the presence of reducing agents.

The oligomeric state of OxlT was also probed using chemical cross-linking of both solubilized and membrane-embedded protein. Except as noted above, no significant population of cross-linked OxlT species was generated by disulfide linkage between cysteine-containing OxlT variants, nor was it possible to generate OxlT oligomers using lysine-specific cross-linkers with spacer lengths ranging from 7.7 Å (DSG) to ≥16 Å (EGS and glutaraldehyde).[35,36] This negative finding is significant for several reasons. (1) OxlT contains 14 lysine residues, of which 13 are expected to be accessible at either the cytoplasmic or periplasmic surface.[14,15] (2) The ability of lysine-reactive agents to modify OxlT was confirmed by loss of OxlT transport function by protein treated with cross-linkers (Figure 3) and by the heterogeneous mobility of cross-linker-treated OxlT on SDS−PAGE (Figure 3). (3) Identical treatment with cross-linkers yielded efficient formation of covalent dimers of AdiC, a known dimeric transporter of similar size and net charge.[16,31,39] Similar negative (for OxlT) and positive (for AdiC) results were obtained for cross-linking conducted in natural or artificial membranes (Figure 4 and text), although cross-linking in native membranes led to formation of higher-molecular mass adducts, presumably reflecting linkage of OxlT or AdiC to other membrane proteins or phospholipids.

The functional oligomeric state of OxlT in membranes was also examined by assays of transport function following reconstitution of the protein into lipid vesicles at protein/lipid ratios designed to yield, on average, an OxlT content equivalent to 0.03−6 monomers/liposome. As argued in previous work with UhpT[20] and AdiC,[16] retention of a constant specific activity of reconstituted protein over such a wide range of lipid/protein ratios indicates that the minimal functional unit is no larger than the oligomeric state found in the solubilized material, because individual units are not expected to associate at reconstitution levels averaging much fewer than one protein per vesicle. Because we show that solubilized OxlT is a monomer (Figures 1−3), the observed linear dependence of activity on protein/lipid ratio implies that the functional state of the transporter is also a monomer. We conclude, therefore, that unlike some proteins that appear to exist in different oligomeric states when solubilized as compared to when they are in membranes,[9,40−42] OxlT is a monomer both in detergent and in situ.

In membrane transport proteins, dimeric, trimeric, or higher-order oligomeric states are in many cases directly related to transport function. This is exemplified by K^+ and Na^+ channels, where structural analyses indicate that the translocation pathway is formed by apposition of individual subunits,[43,44] in agreement with early suggestions that substrates moving through membrane proteins would travel at the interfaces of interacting subunits.[45,46] Similar arguments have been made with regard to members of the MFS, because it is likely that the earliest form of these transporters operated as homodimers of six-helix subunits,[47] with the transport pathway formed at the subunit interface.[6] Contemporary members of the MFS are assumed to have arisen by ancestral gene duplication−fusion events, allowing each six-helix unit to evolve independently while keeping the same overall fold, with the large central loop between TM6 (transmembrane 6) and TM7 providing the connection between the ancestral subunits. Viewed in this context, the finding that OxlT (and other bacterial members of the MFS) functions as a monomer is consistent with the existence of their pseudodimeric internal structures, because the translocation pathway lies at the interface of the TM1−6 and TM7−12 helix bundles corresponding to the separate ancestral subunits.[11,48,49]

The ability of OxlT to function as a monomer, together with experimental evidence of monomeric states of other bacterial MFS proteins, including the proton/lactose cotransporter, LacY,[49] the sugar-phosphate/phosphate antiporter (UhpT),[20] and the glycerol 3-phosphate/phosphate antiporter (GlpT),[10,11] suggests that transport functions of these proteins may not, in general, require oligomerization. This leads to the prediction that, in instances in which MFS proteins appear in higher-order structures,[50] the individual monomers will be functional. This appears to be the case for certain non-MFS transporters, such as the aquaglyceroporins,[51] the amino acid transporter, LeuT,[52] the Na/H antiporter, NhaA,[42,53] where dimerization appears to enhance stability,[53] and the bacterial ClC proton/chloride antiporter, where it is possible to disrupt the dimer interface without compromising monomer function.[54] If oligomerization is not required for transport function, oligomerization of transporters may have initially resulted from a chance formation of dimers without a functional consequence that was fixed by subsequent evolution,[55] perhaps providing for mechanisms of regulation of transport activity, such as allosteric interactions, that may be more difficult to achieve in the monomeric state.[50]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Cases summarized by Table 1, but not shown in Figure 1, in which TD-SEC-LS profiles were obtained for OxlT studied with varying detergents (DM, UDM, and DDM), pH values (4.3 and pH 7), and substrates (oxalate and malonate) (Figure S1) and TD-SEC-LS profiles of AdiC and TetL, along with their calculated molecular masses (Figure S2), which are the positive controls for the experiment described in Figure 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

Supported by Grants GM24195 (P.C.M.), DK27495 (M.E.D.), and GM94611 (M.E.D.) from the National Institutes of Health.

■ ACKNOWLEDGMENTS

We thank Dr. Yiling Fang (Brandeis University, Waltham, MA) and Bryan Czyzewski (New York University, New York, NY) for generously providing the AdiC and TetL protein samples, respectively. We also thank Dr. Kathleen M. Clark for help with the TD-SEC-LS setup and for useful discussion.

■ ABBREVIATIONS

TD-SEC-LS, triple-detector size exclusion chromatography employing light scattering detection; SEC, size exclusion chromatography; DDM, dodecyl maltoside; UDM, undecyl maltoside; DM, decyl maltoside; NM, nonyl maltoside; OG, octyl glucoside; DHPC, diheptanoyl-*sn*-glycero-3-phosphocholine; Cymal-6, 6-cyclohexyl-1-hexyl maltoside; EGS, ethylene glycol bis(succinimidylsuccinate); DSP, dithiobis(succinimidyl propionate); DSG, disuccinimidyl glutarate; TCEP, tris(2-carboxyethyl)phosphine.

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