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Oligomeric State of the Oxalate Transporter, OxIT†

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Abstract

OxIT, 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 OxIT in detergent/lipid micelles. These measurements evaluate protein mass independently of contributions from detergent and lipid; such work shows an average OxIT mass near 47 kDa for detergent-solubilized material, consistent with that expected for monomeric OxIT (46 kDa). A disulfide-linked OxIT mutant was used to verify that it was possible to detect dimers under these conditions. A second approach used amino-reactive cross-linkers of varying spacer lengths to study OxIT in detergent/lipid micelles and in natural or artificial membranes, followed by analysis on SDS-PAGE. 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 OxIT exists as a monomer in the membrane and retains this status on detergent solubilization. In a final test, we showed that reconstitution of OxIT into lipid vesicles at variable protein:lipid ratios has no effect on the specific activity of subsequent oxalate transport, as the OxIT content varies between 0.027 and 5.4 OxIT monomers/proteoliposome. We conclude that OxIT is a functional monomer in the membrane and in detergent/lipid micelles.

The oxalate/formate antiporter, OxIT, 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, OxIT, allowing an intracellular oxalyl decarboxylation system to release both carbon dioxide and formate. The cycle is completed by outward transport of formate, also via OxIT. The overall oxalate²⁻:formate¹⁻ exchange process carries negative charge into the cell, stoichiometric with consumption of an intracellular proton by the

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¹The abbreviations used are: TD-SEC-LS, Triple detector size exclusion chromatography employing light scattering detection; SEC, size exclusion chromatography; DDM, dodecylmaltoside; UDM, undecylmaltoside; DM, decylmaltoside; NM, nonylmaltoside; OG, octylglucoside; DHPC, diheptanoyl-sn-glycerol-3-phosphocholine; Cymal-6, 6-cyclohexyl-1-hexylmaltoside; EGS, ethylene glycolbis[succinimidylsuccinate]; DSP, dithiobis [succinimidyl propionate]; DSG, disuccinimidylglutarate; TCEP, tris[2-carboxyethyl]phosphine

SUPPORTING INFORMATION AVAILABLE

Two figures are included as Supporting Information. Figure S1 describes cases summarized by Table 1, but not shown in Figure 1, in which TD-SEC-LS profiles were obtained for OxIT studied with varying detergents (DM, UDM, DDM), pH (pH 4.3 and pH 7) or substrate (oxalate vs malonate). Figure S2 provides TD-SEC-LS profiles of AdiC and TetL, along with their calculated molecular weights; these data are the positive controls for the experiment described in Figure 2. This information is available free of charge at <http://pubs.acs.org>.

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).

OxIT 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 mechanism(s) (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 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 HPLC studies along with X-ray crystallography indicate that the glycerol-3-phosphate transporter (GlpT) from *Escherichia coli* is monomeric (10, 11).

The work described here was designed to determine the oligomeric status of OxIT, 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, molecular organization in 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 OxIT, the only currently available information about oligomeric state comes from electron crystallography (13), where the limited extent of observed inter-molecular contacts suggest that the protein adopts a monomeric state during crystallization. To extend this work, we used three different analytical approaches to study the oligomeric status of OxIT both in solution and when embedded in artificial and natural membranes. In each case, our findings are consistent with OxIT functioning as a monomer.

EXPERIMENTAL PROCEDURES

Bacterial strain, plasmids and site-directed mutagenesis

In OxIT-Tb-A, the OxIT C-terminal histidine (14) was replaced by a Thrombin recognition sequence (LVPRGS), followed by a His₁₀ tag to facilitate affinity purification. OxIT-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 *Escherichia 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, TX), using as a template a cysteine-less variant (C28G/C271A) with a C-terminal His₉ 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 (8, 16).

Protein expression and purification

A single colony of XL3 harboring a plasmid encoding OxIT or one of its variants was placed in 50 ml LB medium, and after overnight growth at 37°C, this pre-culture 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. OxIT-Tb-A and OxIT 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 harvesting 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 phenylmethane-sulfonylfluoride (from a 0.25M stock in dimethylsulfoxide). Cells were disrupted by sonication on ice in a cold room for 10 min (5 sec on, and 5 sec off), at power level 5, using an Ultrasonic XL2020 sonicator (Misonix, NY). Membrane proteins were solubilized by addition of 1% (w/v) DDM¹ (Anatrace, OH) at 4°C for 1 h. After removing insoluble material by ultracentrifugation (324,000g for 30 min), the supernatant was incubated with 4 ml Ni-NTA resin (Qiagen, CA) 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. Protein concentration in the eluate was measured as described (14, 15); the yield of purified OxIT was 0.8-1.2 mg/Liter of LB.

Size exclusion chromatography

After affinity purification of OxIT, 100 μ g of protein were loaded onto a preparative size-exclusion Superdex 200 (10/300 GL) column for FPLC (AKTA PrimePlus, GE Healthcare) at 23°C, at the 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 absorbance at 280 nm.

Molecular weight determination 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 μ g 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 of known extinction coefficients (ϵ_{280}) and molecular weights (29 kDa to 161 kDa), as described previously (17). For purposes of calculation, a value of 1.7 was used for the ϵ_{280} of OxIT-Tb-A and its derivatives based on 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 software (Precision Detectors, Inc.), and the molecular mass of OxIT was calculated as described (17, 18), using the instrument calibration constant (see above) and the integrated area under the OxIT elution peak of the signals from the three detectors. The ratio of detergent plus 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 OxIT and AdiC were diluted to 0.05 mg/ml with buffer containing 20% glycerol, 20 mM potassium phosphate, 100 mM potassium oxalate, and 0.1 % DDM (pH 7). Aliquots of 50 μ l were incubated at room temperature for 30 min with the indicated concentrations of EGS, DSP, DSG (Pierce, IL) 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 SDS-PAGE, followed by silver staining. Masses were estimated relative to the mobilities of standard proteins (Bio Rad, CA) of known mass run on the same gel.

For cross-linking in native membranes, OxIT and AdiC cell pellets were lysed by sonication, followed by low speed centrifugation (23,000g $\times$ 15 min) to remove unbroken cells. Membranes were collected by ultracentrifugation (180,000g $\times$ 90 min), and the pellets were suspended at pH 7 in 20% glycerol, 20 mM KPi, 100 mM potassium oxalate. Subsequent cross-linking reactions were carried out as described above for analysis of solubilized protein. Samples were analyzed with SDS-PAGE followed by immunoblotting.

Reconstitution and OxIT functional assay

Solubilized protein was reconstituted by detergent dilution as described (14, 15, 20), to generate proteoliposomes loaded with 100 mM potassium oxalate and 50 mM potassium phosphate (pH 7). OxIT function was measured by gently pipetting proteoliposomes onto a 0.22 μ m pore size Milipore filter, rinsing twice with 5 ml assay buffer (100 mM potassium sulfate, 50 mM MOPS, pH 7), and then overlaying the trapped proteoliposomes with assay buffer containing 100 μ M [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 iced assay buffer.

Other assays

For maltosides and glucosides, detergent levels were determined by assays of reducing sugar content (21); phospholipid bound to OxIT was estimated by its phosphorous content, using reactions with ferrothiocyanate (22) or with 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. Factor Xa protease was from New England Biolabs. *E. coli* phospholipid was from Avanti Polar Lipids.

RESULTS

Molecular weight determination by TD-SEC-LS

The presence of associated detergents and lipids makes it difficult to use traditional chromatographic methods to estimate 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 independently of bound, non-UV absorbing materials (18, 19, 24). As a test of this approach for OxIT, we determined the mass of the purified protein under several different conditions of solubilization. In these trials, we focused on use of maltoside-based detergents (DDM, UDM and DM), because of their minimal destabilizing effects on wild-type OxIT, and on use of two different substrates (oxalate, malonate) and 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 OxIT solubilized in 0.1% DDM (Fig. 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, as well as 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 suggest this region has no significant protein or detergent components (17).

Table 1 lists the mass of OxIT as determined by TD-SEC-LS for five different conditions (and see Fig. S1). In each case, calculation of 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 purposes of illustration, a point-by-point molecular weight profile was also calculated for each elution time corresponding to a UV absorbance greater than 75% of the maximal peak absorbance, as shown in Fig. 1B. Throughout this work (Table 1), the measured mass of OxIT (mean of 47.1 ± 0.9 kDa [$\pm$ SEM] all trials) agrees well with its predicted mass of 46 kDa.

The apparent hydrodynamic radius of OxIT varied considerably in the presence of different detergents, such that the predicted mass of the complex, as calculated from the elution times, ranged from 120-160 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). Since most lipid is expected to be removed during purification of the solubilized protein (25; 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 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 OxIT 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 head groups. In each case, OxIT displayed predominantly monodisperse elution profiles (not shown). Retention of monodisperse profiles in the additional detergents suggests that OxIT is monomeric under these conditions as well.

Cysteine cross-linking

Because disulfide crosslinking has been used previously to determine intramolecular distances in OxIT (28), we investigated whether this methodology could provide an alternative approach to determining the oligomeric state of detergent-solubilized OxIT. For these purposes, since the two endogenous cysteines (C28, C271) are buried (29), we tested for formation of disulfide crosslinks between OxIT monomers engineered so as to contain one or two accessible cysteine residues (see *Experimental Procedures*). In an attempt to facilitate formation of inter-molecular crosslinks, we constructed seven cysteine substitution mutants, using the OxIT 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 [$\pm$ SEM]) (Table 2). The seventh mutant contained a substitution of Cys for His413, located immediately prior to the C-terminal His₉ tag. In this case, TD-SEC-LS performed in the absence of reducing

agents yielded two forms of OxIT (Fig. 2A). The larger of these had a calculated mass of 103 kDa, while the smaller species showed a calculated mass of 56.4 kDa (legend to Fig. 2). Moreover, the 103 kDa form was not observed when the elution buffer contained the reducing agent TCEP (10 mM) (Fig. 2B), suggesting that this species is a disulfide-linked dimer. To assess the sensitivity of this assay for detecting an OxIT 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 OxIT, 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 of 107 kDa, while analysis of TetL showed a single species of 122 kDa (see Fig. S2). In further tests, we also confirmed the presence of dimers and monomers of the H413C variant of OxIT by non-reducing SDS-PAGE of fractions from traditional size exclusion chromatography (Fig. 2C, 2D). Finally, we noted that disulfide-mediated dimerization of H413C was more prominent at higher protein concentration (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

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, since such sites would have to be located at a dimer interface in order to form cross-linked dimers. Thus, we also examined cross-linking agents that would be expected to react with multiple sites in OxIT. 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, since 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 OxIT as well as AdiC, a known dimer (see above). When AdiC was subjected to cross-linking with DSG, DSP or EGS (each used at 2 mM concentration), and then subjected to SDS-PAGE, the mobility of a significant fraction ($\geq 40\%$) of the protein shifted from a position corresponding to the monomeric species (35 kDa) to a position corresponding to the dimer (70 kDa) (Fig. 3A). In contrast, OxIT remained as a monomer when treated these agents; however, treatment with cross-linkers did lead to broadening of the OxIT band, presumably reflecting intramolecular cross-linking (16), as found for some other membrane proteins (37). Formation of the higher molecular weight species of AdiC increased as cross-linker (EGS) concentration increased (Fig. 3B), while OxIT migrated as monomer, even at the highest EGS concentration. Notably, when AdiC (or OxIT) was pre-treated 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 OxIT under these conditions by examining samples from such experiments in functional assays. As shown in Figure 3C, the capacity of OxIT to carry out [^{14}C]oxalate transport was significantly limited by exposure to DSG, DSP or EGS, suggesting that OxIT becomes locked or is more restricted in its conformational flexibility, as might be expected from intramolecular linkage(s).

To reduce the possibility that the failure to cross-link OxIT (Fig. 3) reflected insufficient length of the cross-linkers' spacer arms to bridge between two monomers, we conducted

similar analysis using glutaraldehyde, which can form crosslinks of varying lengths (35, 36). Whereas AdiC was readily shifted to a higher molecular weight species upon treatment with 100 mM glutaraldehyde, OxIT remained essentially unshifted (Fig. 3D).

Cross-linking in the membrane

We also used crosslinking to analyze the oligomeric status of OxIT in its functional state, embedded in native or artificial membranes. Crude membrane suspensions derived from OxIT-expressing or AdiC-expressing *E. coli* were treated with increasing concentrations of EGS (0 -10 mM). Under these conditions, AdiC gradually shifted from a predominantly monomer band to one migrating at the position expected for dimer (Fig. 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 OxIT also yielded large aggregates in the stacking gel, protein that entered the gel remained largely confined to the monomer band; no major dimer nor higher order oligomeric species was observed, aside from a commonly-observed minor SDS-induced oligomeric band (13, 38; see also Fig. 3B) that did not increase with increasing cross-linker concentrations. As before (Fig. 3C), we concluded that EGS does react with OxIT under these conditions, since protein extracted and reconstituted from EGS-treated membranes showed reduced function as EGS concentration was increased (Fig. 4B). These findings (Fig. 4A) suggest that OxIT is predominantly monomeric in the membrane.

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

Reconstitution of OxIT at low protein:lipid ratios

As an additional test of the functional oligomeric state of OxIT in membranes, we measured the transport activity of OxIT 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 OxIT monomer per proteoliposome. In this instance, we followed the protocol established for UhpT, which functions as a monomer in both solution and in the membrane (20). Assuming the presence of approximately 2×10^{12} proteoliposomes/mg of phospholipid after reconstitution (20), we adjusted conditions so as to reconstitute approximately 0.03-6 OxIT monomers/proteoliposome (Fig. 5). Throughout this range, the total transport activity of OxIT was proportional to the protein concentration, indicating that the specific activity of the protein remained constant. This indicates that OxIT does not reversibly oligomerize and that the functional unit remains constant down to very low concentrations. The most likely explanation of this is that OxIT functions as a monomer, since 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 OxIT 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 OxIT, independent of the hydrodynamic properties of the complex and the contributions of bound lipid and detergent. The unambiguous result was that OxIT exists as a monomer in the presence of a variety of detergents and substrates (Fig. 1, 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 (Fig. S2). Moreover, at high concentrations, the H413C OxIT variant exhibited a sub-population of faster migrating species that exhibited a molecular mass corresponding to dimer, as determined by TD-SEC-LS (Fig 2). This species was likely the result of disulfide crosslinking of randomly colliding monomers, as its formation was concentration dependent and was not observed in the presence of reducing agents.

The oligomeric state of OxIT was also probed using chemical cross-linking of both solubilized and membrane-embedded protein. Except as noted above, no significant population of cross-linked OxIT species was generated by disulfide linkage between cysteine-containing OxIT variants, nor was it possible to generate OxIT 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) OxIT 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 OxIT was confirmed by loss of OxIT transport function by protein treated with cross-linkers (Fig. 3) and by the heterogeneous mobility of cross-linker-treated OxIT on SDS -PAGE (Fig. 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 OxIT) and positive (for AdiC) results were obtained for cross-linking conducted in natural or artificial membranes (Fig. 4 and text), although cross-linking in native membranes led to formation of higher molecular weight adducts, presumably reflecting linkage of OxIT or AdiC to other membrane proteins or phospholipid.

The functional oligomeric state of OxIT 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 OxIT content equivalent to between 0.03 and 6 monomers/liposomes. 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, since individual units are not expected to associate at reconstitution levels averaging much less than one protein per vesicle. Since we show that solubilized OxIT is a monomer (Figs. 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), OxIT 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 in regard to members of the MFS, since 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 and TM7 providing the connection between the ancestral subunits. Viewed in this context, the finding that OxIT (and other bacterial members of the MFS) function as monomers is consistent with the existence of their pseudodimeric internal structures, since 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 OxIT to function as a monomer, together with experimental evidence for 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 where 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 CIC 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 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. OxIT molecular weight determination using TD-SEC-LS

(A) 50-100 μ g of OxIT 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 and using the same buffer conditions, the instrument calibration constant K_1 was obtained using a reference set of six protein standards of mass between 29 kDa and 141 kDa (see *Experimental Procedures*). (B) For illustrative purposes, using data from Part A, OxIT mass (red trace) was calculated for the region across the A_{280} trace at ca. 75% peak height. For this and other experiments, the mass values reported in Tables 1 and 2 were calculated at the peak of the A_{280} trace.

Figure 2. Cross-linking of a cysteine substitution mutant

The OxIT H413C variant was subjected to TD-SEC-LS or SEC after solubilization and purification. (A) Two forms of OxIT were detected by TD-SEC-LS in 0.1% DDM at pH 7. Based on TD-SEC-LS measurement, the calculated molecular masses of these forms are 103 kDa and 56 kDa, corresponding approximately to dimer or monomer forms of OxIT. (B) The dimer form was not found after addition of reducing agent (10 mM TCEP). In both parts 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 at pH 8.5, so as 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 (dotted trace) or without (solid trace) 10 mM TCEP. (D) Non-reducing SDS-PAGE of samples taken from the apparent dimer and monomer peaks showed the presence of OxIT species corresponding to ca. 70 kDa and 35 kDa, respectively.

Figure 3. Amino-directed cross-linking of solubilized OxIT

After purification, AdiC and OxIT were each suspended (50 $\mu\text{g/ml}$) in buffer (pH 7.5) with 100 mM potassium oxalate, 20 mM potassium phosphate, 20% (w/v) glycerol, 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/Cl (pH 7.5) to 50 mM, and samples were processed for SDS-PAGE. Protein was detected using Silver Stain Plus (BioRad); standard markers on the same gels were used to extrapolate the mass values indicated. (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 Part 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.

Figure 4. Amino-directed cross-linking of membrane-embedded OxIT

(A) Washed membranes from cells expressing either AdiC or OxIT were suspended in buffer (pH 7.5) containing 100 mM potassium oxalate, 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 legend to Fig. 3). (B) In a parallel experiment using the same conditions, samples were taken for reconstitution to evaluate residual OxIT function, as described in the legend to Figure 3C. (C) Purified OxIT or AdiC was reconstituted into proteoliposomes prepared using a 50/50 mixture of palmitoyl-oleoylphosphatidyl choline and palmitoyl-oleoylphosphatidyl glycerol. Cross-linking was assessed by treatment with 100 mM glutaraldehyde, using AdiC as the positive control.

Figure 5. Reconstitution of OxIT at low protein-to-lipid ratios

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

Table 1

TD-SEC-LS measurement of OxIT molecular weight in detergent micelles

Buffer condition	Elution volume (ml)	Apparent MW (kDa) ^a	Average K _I ^b	TD-SEC-LS Calculated MW (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 weight 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 (17, 18; see *Experimental Procedures*).

^e See Figure 1

^f See Figure S1

Table 2TD-SEC-LS measurements of molecular weight of OxIT cysteine substitution mutants^a

Mutant	TD-SEC-LS Calculated Molecular Weight (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 OxIT cysteine-less variant. TD-SEC was performed in 0.1% DDM in the presence of 100 mM potassium oxalate, at pH 7.

^b Calculated as described in Table 1 for the polypeptide, without contributions from bound detergent.