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Functional Monomerization of a ClC-Type Fluoride Transporter

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Abstract

Anion channels and antiporters of the ClC superfamily have been found to be exclusively dimeric in nature, even though each individual monomer contains the complete transport pathway. Here, we describe the destabilization through mutagenesis of the dimer interface of a bacterial F^-/H^+ antiporter, ClC^F-eca. Several mutations that produce monomer/dimer equilibrium of the normally dimeric transporter were found, simply by shortening a hydrophobic side chain in some cases. One mutation, L376W, leads to a wholly monomeric variant that shows full activity. Furthermore, we discovered a naturally destabilized homologue, ClC^F-rla, which undergoes partial monomerization in detergent without additional mutations. These results, in combination with the previous functional monomerization of the distant relative ClC-ec1, demonstrate that the monomer alone is the functional unit for several clades of the ClC superfamily.

Keywords

membrane protein; protein oligomerization; antiporter; transmembrane interactions

Introduction

Oligomerization is a recurring motif among membrane transport proteins. Many ion channels require multiple subunits or homologous domains to create an axially symmetric pore, while others have a pore wholly contained within each monomer. Similarly, some solute transporters require oligomeric assembly to support the transport mechanism [1,2], while others operate as functionally independent subunits associated together for no known purpose [3]. ClC family anion channels and transporters are homodimers wherein each subunit contains an independently gated pore for the channels or an independent transport pathway for the antiporters [4]. This picture, inferred from decades of mechanistic analysis [5–8] and sharpened by crystal structures [9,10], was recently buttressed by the engineering of a fully functional monomeric variant of ClC-ec1, a bacterial Cl^-/H^+ antiporter [11]. The two ClC-ec1 subunits were surgically separated by substituting tryptophan residues at structurally known positions in the membrane-embedded dimer interface in order to disrupt surface complementarity and to simultaneously favor interaction with bilayer lipids. In this

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report, we test whether this strategy can also succeed with a phylogenetically distant CIC protein, as a first step toward examining its wider generality. This report examines CIC^F-eca (hereafter denoted Eca), a F⁻/H⁺ antiporter from *Enterococcus casseliflavus*, representative of a clade of bacterial CICs essential for resistance to environmental F⁻ toxicity (CIC^F) [12–14]. Eca is only 21% identical in sequence with CIC-ec1, and it transports a different anion with a different anion/proton stoichiometry (1 F⁻/1 H⁺ versus the 2/1 seen in CIC Cl⁻/H⁺ antiporters). We find that Trp substitutions similar to those established previously can convert Eca from a non-dissociating homodimer in detergent into functionally active monomers that can, for certain mutants, reversibly dimerize on a timescale of several days. Moreover, functional monomers can also be produced by alternative disruptions of the dimer interface.

Results and discussion

We reprised with Eca the previous “warts-and-hooks” approach that produces functionally active CIC-ec1 monomers. This twofold strategy introduces tryptophan residues into the dimer interface near the level of the lipid headgroups in order to disrupt the well-packed dimer surfaces (“warts”) while placing the mutant’s side chain near the lipid–water interface (“hooks”), where amphipathic moieties are energetically favored [15]. Application of this approach to Eca lacks the great advantage of a high-resolution crystal structure that the CIC-ec1 study enjoyed. However, at the sequence level, the four transmembrane helices (H, I, P, and Q) that form the dimerization surface in CIC-ec1 are sufficiently recognizable throughout the superfamily (Fig. 1), so as to provide confidence regarding regions to place mutations.

Wild-type (WT) Eca runs in decylmaltoside micelles as a clean, monodisperse dimer on a size-exclusion column, eluting at 11.3 mL [13] (Fig. 2a), and is confirmed to be dimeric by gel shift upon glutaraldehyde crosslinking (Fig. 2b). This provides a convenient metric for assaying the protein’s oligomeric state, as monomeric Eca is expected to elute ~1 mL later, as with CIC-ec1 [11]. By testing candidate destabilizing mutations in helices H, I, P, and Q, we identified three Trp mutants that produce clear, 1-mL shifts of the elution profile (Fig. 2a): L169W, F361W, and L376W; the first two of these show both dimer and monomer peaks, while the last is fully monomeric.

These three destabilized variants demonstrate that a range of intermediate dimer affinities can be attained through different mutations. Furthermore, the case of L376W shows that dramatic destabilization can be seen via only a single Trp substitution. We studied this construct in more depth in order to confirm its monomeric nature and to assess its proper folding. A mild glutaraldehyde treatment of WT Eca in detergent completely crosslinks the subunits, as is evident by a shift to the dimeric position on SDS-PAGE (Fig. 2b). In contrast, the L376W mutant fails to crosslink after 30 min of glutaraldehyde treatment, thus confirming it to be monomeric in detergent. To determine whether L376W remains monomeric in a lipid environment, we used Eca variants carrying a cysteine residue at a position (F177C) that leads to subunit crosslinking in a mildly oxidizing environment established with a glutathione redox pair [16]. The F177C mutant was selected after screening several Eca cysteine mutants for robust crosslinking in a region aligning well with

an extended loop in the CIC-ec1 structure and that was positioned close to the dimeric 2-fold axis. These variants were incorporated into liposomes, treated with the redox pair, and examined on SDS-PAGE (Fig. 3a). A substantial fraction of the cysteine-bearing “WT” Eca molecules crosslink under these conditions, while the L376W substitution shows no crosslinking and is fully monomeric in the lipid bilayer and in detergent.

We gauged the L376W mutant’s functional activity to determine whether this construct is stable and well folded in its monomeric state. Transport was assayed in “F⁻ dump” experiments, where F⁻ efflux from liposomes pre-loaded with a high concentration of KF is measured using a light-scattering technique [13]. WT Eca and the monomeric mutant show essentially identical F⁻ transport rates, with efflux half-times in the range 25–30 s (Fig. 3b). The functionality of the L376W mutant, a compelling surrogate for proper folding, demonstrates that the protein’s monomeric character does not reflect structural disruption of the monomer arising from either the mutation or the loss of its partner subunit.

We wondered about the moderately destabilizing mutations described above, which show both dimer and monomer peaks on gel-filtration profiles. Might these profiles reflect thermodynamically reversible monomer/dimer equilibrium? This question was examined with the F361W mutant to see if the protein would redistribute toward monomer upon dilution. Membrane protein “concentration” in a micellar solution is properly quantified by the molar ratio of protein to micellar detergent, P:D [17], rather than the absolute concentration of protein in solution (the protein:water ratio). We can thus manipulate the protein chemical potential by either decreasing the amount of protein at fixed detergent concentration or increasing the amount of detergent at fixed protein.

The kinetics of monomer/dimer redistribution after dilution was followed in order to know if thermodynamically valid dimerization equilibrium could be achieved on an experimentally tractable timescale. Upon initial purification, F361W is at a high protein:detergent ratio and is accordingly mostly dimeric (Fig. 4a). After >40-fold dilution to P:D = 5.0×10^{-5} (unitless mole fraction, 1.35 μ M Eca, 27 mM micellar detergent), the monomer/dimer distribution was followed at a series of timepoints by gel filtration. Over the course of roughly a week at 22 °C, the oligomeric distribution re-equilibrates, with a half-time of ~40 h, such that monomer becomes the dominant species at equilibrium.

Despite the slow dimerization kinetics, this approach allows an estimate of the mutant’s dimerization constant in decylmaltoside detergent. The protein was diluted to varying concentrations and was assessed for monomer/dimer distribution after 7 days. The dimer fraction as a function of total protein concentration (Fig. 4b) fulfills the quantitative expectation for a monomer/dimer equilibrium, with the most concentrated sample (P:D = 1.7×10^{-4}) primarily dimeric and the most dilute (P:D = 2.1×10^{-5}) almost entirely monomeric. These points were fit according to a dimerization equilibrium isotherm:

$$\text{fraction dimer} = \frac{1 + 4K [\text{P:D}] - \sqrt{1 + 8K [\text{P:D}]}}{4K [\text{P:D}]} \quad (1)$$

where K is the dimerization constant. The mutant Eca thus engages in a reversible dimerization equilibrium with estimated dimerization constant $K = 8.7 \times 10^3$, equivalent to

$G_{\text{dim}}^0 = -5.4$ kcal/mol (mole fraction standard state). This value of dimerization free energy also allows an estimate of subunit interface destabilization produced by the Phe-to-Trp substitution on “fully dimeric” WT protein; at the lowest protein-to-detergent molar ratio, a lower limit of detection of WT monomer was by conservative estimate <20%, implying a destabilization of at least 2.3 kcal/mol by the mutation. Similarly, even the “fully monomerized” L376W mutant is seen to dimerize slightly when pushed to very high concentration (Fig. S1), leading to a rough estimate of $G_{\text{dim}}^0 = -2$ kcal/mol for this more destabilizing Trp substitution, with the caveat that this small amount of dimerization might be partly influenced by the relatively high protein concentration with a limiting amount of free detergent micelles.

We wondered whether maneuvers less dramatic than warts-and-hooks Trp substitution could also destabilize the dimer interface. In particular, we tested removal of a large, hydrophobic side chain to see if this could also destabilize the dimer by weakening a presumed steric complementarity. The I364A substitution clearly leads to the formation of a monomer peak (Fig. 5), thus showing that the warts-and-hooks approach is not the only way to destabilize the dimer interface.

All previous reports of CIC proteins have viewed them exclusively as irreversibly assembled dimers. However, now with two CIC homologues, Eca and CIC-ec1, showing monomeric forms achieved by single point mutations, we also sought naturally occurring examples of CICs with moderately destabilized dimer interfaces. A screen of bacterial CIC^F homologues identified CIC^F-rla from *Ruminococcus lactaris* (denoted here “Rla”) that is partially monomeric in detergent solution (Fig. 6a). Rla, as expected from its sequence [14], ~50% identical with Eca, is a functional F⁻ transporter (Fig. 6b). As with Eca and CIC-ec1, the Rla dimer affinity in detergent can be further weakened through the introduction of mutations without impairing its transport function (Fig. 6a and b). The behavior of native Rla suggests a diversity of dimerization free energies among the CIC superfamily. We emphasize that the monomer form of WT Rla may be observable only in detergent and may not represent its state in its biological membrane, as with the dimeric lactose transporter LacS [18]. However, considering that the detergent-micelle behaviors of Eca and CIC-ec1 mirror their inherent dimer stability in the lipid membrane, it is perhaps not surprising that a dimerically less stable CIC would be found in nature.

Ever since the realization over two decades ago that CIC proteins are independently functioning homodimers [6], the evolutionary reasons for this architecture have been a mystery. We are loath to speculate too freely as to the purpose of CIC dimeric structure, but it is striking that dimeric architecture is maintained across the wide evolutionary distance separating Eca and CIC-ec1 despite both transporters remaining functional upon monomerization. Perhaps dimerization eliminates stability problems that would arise from hydrophobic mismatch between the monomer and the lipid bilayer [19]. At the end of the day, however, we can only assert with confidence that nature has seen fit to maintain this arrangement for some still unknown reason.

Materials and methods

Biochemical

Decylmaltoside was purchased from Anatrace. *Escherichia coli* polar lipids were obtained from Avanti Polar Lipids. All other chemicals purchased from Sigma-Aldrich.

Eca (GenBank EEV37149.1) and Rla (EDY33609.1) were expressed and purified from a pASK vector with a C-terminal hexahistidine tag as previously described [13]. The two changes from that protocol were that the cobalt affinity wash solution contained 20 mM imidazole and that the final gel-filtration buffer was 10 mM *N*-(2-acetamido)iminodiacetic acid, 100 mM NaF, and 5 mM decylmaltoside (pH 6.5). Both monomeric and dimeric peaks were collected and pooled when present.

Proteoliposome reconstitution

Purified protein in detergent was mixed with 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid solubilized *E. coli* polar lipids to a final ratio of 1 μ g protein per milligram of lipid and was dialyzed for 36 h against 25 mM Hepes and 300 mM NaF (pH 7) for crosslinking or against 25 mM Hepes and 300 mM KF (pH 7) for functional assays.

Analytical gel filtration

Purified protein was diluted to the reported P:D ratios in 10 mM *N*-(2-acetamido)iminodiacetic acid and 100 mM NaF (pH 6.5) with final total decylmaltoside concentrations ranging from 5 mM to 33.4 mM. All reported P:D ratios use the micellar detergent concentration, which is determined by subtracting the decylmaltoside CMC (1.8 mM) from the total detergent concentration. Unless otherwise stated, samples were incubated for 7 days at 22 °C. Analytical gel filtration was performed over a Superdex 200 increase column, equilibrated to the sample buffer, unless otherwise stated. Care was taken to have similar detergent concentration in the gel-filtration buffer as in the sample, and samples were injected on the gel-filtration column with no further dilution.

Subunit crosslinking

Crosslinking in detergent was performed by adding 0.125% glutaraldehyde to Eca at P:D ratio of 1.7×10^{-3} , and it was quenched through the addition of 90 mM Tris (pH 8.0). Samples were analyzed by SDS-PAGE stained with Coomassie Blue. Crosslinking of the F177C Eca in liposomes (oxidized samples) was performed by adding 0.075 mM oxidized glutathione and 1.4 mM reduced glutathione to the proteoliposomes. Samples were freeze-thawed 3 $\times$ and were incubated at 22 °C for 3 days. The reaction was quenched with 17 mM *N*-ethylmaleimide prior to SDS-PAGE analysis. Reduced samples were treated with 50 mM tris(2-carboxyethyl)phosphine prior to analysis. SDS-PAGE loading buffer contained no reducing agent, with a final SDS concentration of 2%. Gel was stained with SYPRO Ruby (Invitrogen) and was visualized via Typhoon fluorescence imager (GE Healthcare).

F⁻ efflux assay

Liposomes reconstituted with CIC protein were loaded with 300 mM KF and diluted ~300-fold into 300 mM K-isethionate, in a 3-mL fluorimeter cuvette. KF efflux, initiated by addition of valinomycin (0.9 μ M), was followed by 90° light scattering as previously described [13].

Data analysis

Multiple sequence alignment was performed using Clustal Omega. Protein monomer and dimer species were quantified by fitting gel-filtration profiles with two Gaussians with a shared standard deviation in MatLab and integrating. Any errors describe the standard error of the mean of at least 3 independent measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

WT wild type

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Fig. 1.

Multiple sequence alignment of the dimer interface region of CIC family proteins. The four transmembrane helices that form the membrane-embedded dimer interface (H, I, P, and Q) are shown. Regions of high homology are shown in yellow. Known destabilizing tryptophan mutants in CIC-ec1 [11] and positions mutated here are highlighted in blue and red, respectively. Crystal structures of CIC-sy1 (PDB ID 3NDO), CIC-st1 (1KPL), and CIC-ec1 (1OTS) allow clear placement of the dimerization interface in the alignment. Additional sequences shown are CIC-sy1 (*Synechocystis* sp.), CIC-st1 (*Salmonella typhimurium*), CIC-ec2 (*E. coli*), CIC^F-rla (*R. lactaris*), CIC^F-eve (*Eubacterium ventriosum*), and CIC^F-psy (*Pseudomonas syringae*).

Fig. 2.

Disruption of the Eca dimer interface in detergent. (a) Gel-filtration profiles of Eca mutants. Broken lines indicate elution volumes for dimer (11.3 mL) and monomer (12.3 mL). Samples were pre-incubated at a protein:detergent ratio of 5.0×10^{-4} (WT, L169W, L376W) or 1.7×10^{-4} (F361W). (b) SDS-PAGE of WT or L376W Eca. Samples were treated with 0.125% glutaraldehyde (“+ glut”) or without (“C”) for 1 or 30 min. No higher-order oligomers were observed.

Fig. 3. Oligomeric state and function of Eca in phospholipid membranes. (a) SDS-PAGE of F177C or F177C/L376W Eca incorporated into *E. coli* polar lipids. Samples treated with a reducing agent (Red) or incubated with a glutathione redox pair (Ox). (b) F^- efflux from liposomes reconstituted with WT or L376W Eca. Flux was initiated at time 0 by addition of valinomycin, and F^- transport was followed by 90° light scattering. Traces are normalized to the final level. Half-time of transport is 26 ± 3 s for WT and is 29 ± 4 s for L376W.

Fig. 4. Monomer/dimer equilibrium of F361W Eca in detergent. (a) Timecourse of equilibration upon dilution of concentrated F361W Eca to a final P:D ratio of 5.0×10^{-5} . Samples taken at 0 day (blue), 1 day (purple), 3 days (green), 5 days (orange), and 7 days (red) were analyzed by gel-filtration elution profiles shown. Inset: timecourse of the dimeric fraction following dilution. Line represents the single exponential fit. (b) Final F361W Eca oligomeric distribution. P:D ratios are 1.7×10^{-4} (blue), 8.5×10^{-5} (purple), 5.0×10^{-5} (green), and 2.1×10^{-5} (red). Inset: dimer fraction at 7 days as a function of total protein

concentration. Continuous curve represents the predicted fraction dimer for $K = 8.7 \times 10^3$, according to Eq. (1), equivalent to -5.4 kcal/mol, with a run-to-run variation of ~ 0.4 kcal/mol.

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Fig. 5.
Gel-filtration profile of I364A Eca. Sample was incubated at a P:D ratio of 2.5×10^{-4} . WT Eca trace from Fig. 1a is overlaid (broken line).

Fig. 6.

Rla dimer destabilization and transport activity. (a) Gel-filtration profiles of WT and the destabilized L383W and L356W A371W mutants of the Rla homologue. WT and L383W samples incubated at a P:D ratio of 5×10^{-4} . L356W A371W trace is from the initial purification on a Superdex 200 column. (b) F^- efflux from liposomes reconstituted with WT or L356W A371W Rla. Flux was initiated at time 0 by addition of valinomycin, and F^- transport was followed by 90° light scattering. Traces are normalized to the final level.