Solution structure and elevator mechanism of the membrane electron transporter CcdA

Yunpeng Zhou and John H. Bushweller

Membrane oxidoreductase CcdA plays a central role in supplying reducing equivalents from the bacterial cytoplasm to the envelope. It transports electrons across the membrane using a single pair of cysteines by a mechanism that has not yet been elucidated. Here we report an NMR structure of the Thermus thermophilus CcdA (TtCcdA) in an oxidized and outward-facing state. CcdA consists of two inverted structural repeats of three transmembrane helices (2 × 3-TM). We computationally modeled and experimentally validated an inward-facing state, which suggests that CcdA uses an elevator-type movement to shuttle the reactive cysteines across the membrane. CcdA belongs to the LysE superfamily, and thus its structure may be relevant to other LysE clan transporters. Structure comparisons of CcdA, semiSWEET, Pnu, and major facilitator superfamily (MFS) transporters provide insights into membrane transporter architecture and mechanism.

Results

Sample conditions and NMR structure determination. We screened multiple CcdA proteins from bacteria and archaea for the feasibility of solution NMR studies. Oxidized TtCcdA was stable, monodisperse, and showed promising initial NMR spectra (Fig. 1b and Supplementary Fig. 1b). An E35A mutation was introduced to suppress truncation during heterogeneous expression. The protein was partially oxidized during expression and became fully oxidized after purification. Purified TtCcdA is redox active with its native Trx substrate (Supplementary Fig. 1c). The structure represents an oxidized conformation in which the redox-active disulfide bond is formed. Reduced TtCcdA showed conformational heterogeneity and increased dynamics; thus, a structure determination was not feasible (Fig. 1c). For oxidized TtCcdA, 86% of the backbone resonances and 58% of the side chain β-carbon resonances have been assigned. The long-range restraints used to determine the global fold of TtCcdA are derived from eight paramagnetic relaxation enhancement (PRE) samples and residual dipolar couplings (RDC) measured in two alignment conditions (Table 1 and Supplementary Fig. 2a–d). These restraints were measured using sensitive 2D 15N-1H TROSY-based experiments and rely on the readily obtained protein backbone amide resonance assignments.

The structure of oxidized TtCcdA was validated in two ways. First, we calculated a series of ensemble structures using partial long-range RDC and PRE restraints (Supplementary Fig. 2e). These structures were examined using the withheld RDC restraints. The low Qrms factors are indicative of the quality of these structures. The reported structure, which is calculated with all restraints, is similar to these partially restrained structures. Second, solvent-accessibility data were obtained using the water-soluble paramagnetic reagent Gd-DTPA (Supplementary Fig. 3a,b). Residues accessible to the solvent and the paramagnetic reagent exhibited a marked reduction of NMR signal intensity. These residues are in the periplasmic and cytoplasmic loops, whereas residues with relatively small signal reduction are in the central regions of the TM helices. The solvent-accessibility data are in good agreement with the structure of TtCcdA.
Structure of oxidized TtCcdA in an outward-facing state. The most prominent structural feature of TtCcdA is the symmetry of the 3-TM repeats (Fig. 2a,b). For convenience of discussion, we named the three helices of the 3-TM repeats A-helix, B-helix, and C-helix. The A-helix is long and bends ~60° at the central PCxxP motif. Its N- and C-terminal helical segments are labeled a and b, respectively (TM1a, TM1b, TM4a, TM4b). The B- and C-helices together form a U shape. A characteristic bend in the C-terminal one-third of the B-helix forms the bottom of the U. The two 3-TM repeats are antiparallel to one another. Each inserts its A-helix into the U of the opposite one. The A-helices TM1 and TM4 are central transport helices and are connected by the active-site disulfide bond. Outside, C-helices TM3 and TM6 pack tightly against each other via a pair of conserved Gx6G motifs (Supplementary Fig. 3c). B-helices TM2 and TM5 form similar packing. The two inverted U shapes stack together, forming an “O scaffold” domain. On the basis of the membrane orientation prediction using the PPM server, the O scaffold is highly tilted, with its long axis passing through residues 69 and 176 forming a 38° angle with the membrane norm, which results in a resemblance of the interior portion of the O to a channel. The loops connecting the two central A-helices and the O scaffold (L1, L3, and L4) are flexible (Supplementary Fig. 3d). The overall architecture of TtCcdA could be described as two kinked transport helices inserted into an O-scaffold channel with flexible loops connecting them (Fig. 2c).

The NMR structure of oxidized TtCcdA represents an outward-facing state. This results from TM1 and TM4 adopting different orientations relative to the O scaffold (Fig. 2b). Viewed from the periplasmic side, the disulfide bond is at the bottom of a shallow pocket surrounded by the N terminus of TM3, TM4b, the C terminus of TM6, and helix H1 (Fig. 2c). From the cytoplasmic side, TM1b and TM4a block access to the disulfide bond. The N\textsubscript{out}–C\textsubscript{out} membrane topology was inferred using the ‘positive inside’ rule and is consistent with previous studies\textsuperscript{11,12} (Supplementary Fig. 3e).

Comparison to Archaeoglobus fulgidus CcdA. We have compared our structure with the published NMR structure of a mutant Archaeoglobus fulgidus CcdA (AfCcdA) that mimics the reduced state of the protein\textsuperscript{34}. The two proteins show significant sequence homology (29% identity, 60% similarity) but bear no structural similarity. Second, bioinformatics analysis and membrane-topology assays show a consensus that the two conserved PCxxP motifs are located in TM1 and TM4 (refs. \textsuperscript{10,14,22}), which is in agreement with the structure of TtCcdA. In contrast, the first PCxxP motif of AfCcdA is in the cytoplasmic loop between TM1 and TM2, and the second PCxxP motif is between h and h’. Third,
An inward-facing model of oxidized TtCcdA. Oxidized TtCcdA is in an outward-facing state. In order for the protein to be reduced by cytoplasmic Trx, it needs to switch to an inward-facing state in which the disulfide bond is exposed to the cytoplasm. Imperfect structural symmetry of sequence repeats implies underlying conformational flexibility. An established ‘repeat-swap homology modeling’ approach switches the conformations of the repeats and predicts the inward-facing state from an outward-facing structure or vice versa. Using this approach, we swapped the conformations of TM1–3 and TM4–6 of TtCcdA, which effectively switched the orientations of TM1 and TM4 relative to the O scaffold (Fig. 3a and Supplementary Fig. 4a,b). In the repeat-swap model, the disulfide bond is located at the bottom of a shallow cytoplasmic pocket composed of the N terminus of TM6, TM1b, the C terminus of TM3, and the flexible loop L3. TM1a and TM4b seal the disulfide bond off from the periplasm. This model represents a putative inward-facing state of oxidized TtCcdA.

We validated the model using a cysteine cross-linking experiment. A scaffold residue (F45) and a transport helix residue (A113) are distant (13 Å Cα–Cα) in the outward-facing structure but close (6 Å Cα–Cα) in the inward-facing model. We constructed a F45C A113C mutant. The purified mutant has its native disulfide bond formed, as its 15N–1H HSQC NMR spectrum resembles that of the oxidized wild-type protein (Supplementary Fig. 6a). The two introduced cysteines are free to be alkylated (Fig. 3b, lane 3). Preincubation of the mutant with equimolar HgCl₂ prevents the two free cysteines from alkylation, indicating that a ’S–Hg–S’ bridge is formed between F45C and A113C (Fig. 3b, lane 5). This bridge is intramolecular, because otherwise, the mutant would migrate as a dimer or oligomers on SDS–PAGE. To confirm this, we recorded the 15N–1H HSQC NMR spectrum of the mutant with near equimolar HgCl₂. This spectrum is considerably different from the spectrum of the HgCl₂-free sample, with many peak shifts and a large number of peaks either disappearing or diminishing in intensity (Supplementary Fig. 6a–c). This finding suggests that the HgCl₂ cross-linking traps oxidized TtCcdA in a metastable state, which shows a global difference from the ground outward-facing state. The cross-linking experiment demonstrates that the inward-facing state is accessible to oxidized TtCcdA.

Next, we asked whether the outward- and inward-facing conformational switch happens in vivo. Data for this are available in the form of previous studies of the solvent accessibility of EcDsbD. The residues of TM1 and TM4 of EcDsbD were systematically
mutated to cysteine, and their reactivity with a membrane-impermeable alkylation reagent was measured in spheroplasts. The C-terminal halves of the two transport helices (TM1b and TM4b) are exposed to the aqueous environment, while the N-terminal halves (TM1a and TM4a) are not (Fig. 3c). TtCcdA and EcDsbD have significant sequence homology (28% identity and 57% similarity). We sought to understand the EcDsbD solvent accessibility using the structural models of TtCcdA. For TtCcdA, TM1b is only solvent exposed in the inward-facing state, whereas TM4b is only solvent exposed in the outward-facing state (Fig. 3a). Neither state matches the observed solvent accessibility of EcDsbD; however, if the two states are accessed alternately, the solvent-accessibility data can be explained satisfactorily, suggesting that oxidized CcdA indeed exchanges between the two states in vivo.

The outward-facing structure and inward-facing model suggest that TtCcdA uses an elevator-type transport mechanism. The O scaffold is immobile in the membrane, while the A-helices use a rotation movement to transfer the disulfide bond ∼12 Å along the direction of the membrane norm (Supplementary Video 1). This rotation movement minimizes the hydrophobic mismatch as the result of TM1 and TM4 moving in and out of the membrane and maximizes the vertical translation of the disulfide bond. The disulfide bond runs against the inside surface of TM3 and TM6, which is tightly packed and hydrophobic in the middle to prevent solvent leakage while loosely packed and hydrophilic at both ends to encourage water penetration, which is important for the thiol-disulfide-exchange reactions between CcdA and its substrates (Fig. 4a).

The transport helices are loosely packed inside the O-scaffold domain, and the two parts are connected by flexible loops. An interesting question is how the up and down positions of the transport helices are determined. Two conserved glycines, one eight residues preceding the first reactive cysteine (G12) and the other ten residues preceding the second reactive cysteine (G117), were found to be important to CcdA activity (Supplementary Fig. 4d). In TtCcdA, G12 belongs to a conserved Ax3G motif on TM1a, and G117 belongs to a conserved Ax3Gx3A motif on TM4a. In the outward-facing structure, the Ax3G motif on TM1a packs against another conserved Ax3G motif on TM5 (Fig. 4b). A pair of Ax3G motifs and its variations are known to facilitate TM-helix packing. We mutated G154 in the Ax3G motif of TM5 to alanine. Such a small change in this tightly packed area resulted in severe destabilization of the outward-facing state, evidenced by the deteriorated NMR spectrum of this mutant (Supplementary Fig. 6d). In the inward-facing state, the Ax3Gx3A motif on TM4a packs against a conserved glycine, G49, on TM2. The alternate association and dissociation of these glycine-containing motifs may play a major role in the alternating access of TtCcdA. The strengths of these interactions may be one of the major factors in determining the relative population of the inward and outward-facing states of TtCcdA.

**Discussion**

We propose a multiple-step mechanism for the transport of reducing equivalents across the membrane by TtCcdA (Fig. 5). The outward-facing NMR structure probably represents the ground state of oxidized TtCcdA. It is in a state in which TtCcdA has reduced the periplasmic substrates and forms an internal disulfide bond that remains at the periplasmic side of the protein. In this state, C127 is more exposed to the periplasm than C20, suggesting that the reactive cysteine on TM1 directly interacts with Trx. This finding is consistent with previous experimental observations.

Immediately after being reduced by the cytoplasmic Trx, TtCcdA would be in an inward-facing state in which both reactive cysteines remain at the cytoplasmic side. To reduce the periplasmic substrate, it is necessary for reduced TtCcdA to convert to an outward-facing state in which both reactive cysteines move to the periplasmic side. Without the internal disulfide bond, TM1 and TM4 could move independently, resulting in two potential intermediate states with one reactive cysteine at each side of the protein. The NMR spectrum of reduced TtCcdA suggests the reduced protein might exchange between multiple conformations (Fig. 1c). The trypsin domain chain NMR signals of the reduced-state mimic of AfCcdA also suggest that the reduced protein has multiple conformational states. Further studies are needed to delineate the population and conformational exchange of the reduced states of CcdA.

As known from bioinformatics analysis (for example Pfam, http://pfam.xfam.org/clan/LysE/), CcdA belongs to the LysE superfamily of membrane transporters. According to the Transport Classification Database (TCDB, http://www.tcdb.org/superfamily.php?id=9/), the LysE superfamily contains six families of divalent ion trans-
porters, three families of amino acid transporters, one family of peptidoglycolipid transporter, and the CcdA family. Aside from sequence homology, the internal 3-TM repeats are also detected in several families of the LysE superfamily. Bioinformatics analysis and mutagenesis studies have found conserved and functionally important motifs located on TM1 and TM4. The sequence homology, the inverted 3-TM repeats, and the conserved motifs suggest that the members of the LysE superfamily may share a common fold. TtCcdA could serve as a template to model other LysE transporters and to better understand their mechanistic and functional attributes.

CcdA, semiSWEET, Pnu, and MFS transporters share the use of 3-TM repeats as their basic building blocks. semiSWEET sugar transporter is a parallel dimer of 3-TM bundles (Fig. 6a). The three helices of each bundle are connected in a right-handed order (Fig. 6e). Pnu vitamin transporters contain two parallel 3-TM repeats and a linker TM helix in between (3 + 1 + 3 TM, Fig. 6b). Each 3-TM bundle displays a linear arrangement of the three helices (Fig. 6f). A potential domain-swapping evolutionary mechanism connecting the semiSWEET and Pnu transporters has been proposed. Pnu and semiSWEET form an interesting contrast to CcdA that consists of two antiparallel left-handed 3-TM bundles (Fig. 6c,g). So far, Pnu and semiSWEET represent the minimal model of rocker-switch-type transporters, and CcdA is the smallest model of elevator transporters. It will be interesting to see whether the 2 × 3-TM bundle represents the minimal arrangement of natural membrane transporters. A non-natural designed zinc transporter with a 4 × 1-TM arrangement has been developed.

Fig. 5 | Proposed reducing-equivalent transport mechanism of CcdA. CcdA exchanges between four major conformational states during the transport cycle: (I) outward-facing reduced state, (II) outward-facing oxidized state, (III) inward-facing oxidized state, and (IV) inward-facing reduced state. The dark gray and the white boxes represent the O frame and the transport helices of CcdA, respectively. The disulfide bonds are shown as a thick yellow line.

Fig. 6 | Structural comparison of CcdA, semiSWEET, Pnu, and MFS. a–d, Cartoon representations of the structures of semiSWEET (PDB 4QNC), Pnu (PDB 4QTN), TtCcdA, and MFS (PDB 1PV7). Neighboring 3-TM bundles are colored red and blue. e–h, Ribbon representations of the 3-TM bundles of semiSWEET, TtCcdA, Pnu, and MFS. The first, second, and third TM helices of each 3-TM bundle are colored blue, yellow, and red, respectively.
MFS is a large and diverse group of secondary transporters and facilitators that share the common architecture known as the MFS fold[6]. MFS transporters consist of four left-handed 3-TM bundles[7,8] (Fig. 6dh). The N- and C-terminal domains of MFS are pseudo-symmetrical and parallel. Within each domain, the two pseudo-symmetrical and antiparallel 3-TM repeats show a similar arrangement as that in TcCda. Using the N domain as an example, TM1 and TM4 are in the center of the domain and are inserted into the opposite 3-TM bundles. TM3 and TM6 pack against each other. A difference is that TM2 and TM5 are separated from each other and pack against TM11 and TM8, respectively, which are the C-domain counterparts of TM5 and TM2. To our knowledge, the 6-TM folds of Cda and the MFS domains are not observed in other membrane proteins.

Alternating access is a general framework to understand the mechanisms of membrane transporters. It posits that the substrate is alternately exposed to either side of the membrane through conformational changes of the membrane transporter[9]. Structural studies have revealed a spectrum of examples within this framework[10]. At one end, such as in the rocker-switch mechanism, the substrate-binding site is relatively immobile inside the membrane, and the membrane transporter rearranges its conformation around this site. At the other end, such as in the elevator mechanism, the substrate-binding site moves across the membrane via protein conformational change. semiSWEET uses an inter-3-TM bundle rocker-switch movement to transport substrates[11]. MFS transporters use an inter-6-TM-domain rocker-switch movement to alternatively expose the substrates to both sides of the membrane[12,13]. Cda relies on intra-6-TM-domain helix movement to shuttle the substrate to either side of the membrane. MFS and semiSWEET achieve a similar transport mechanism using different combinations of different 3-TM bundles. MFS and Cda provide an intriguing example that different transport mechanisms can be realized using a single or a combination of the same 6-TM fold.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41594-018-0022-z.

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Author contributions
Y.Z. and J.H.B. designed the research; Y.Z. performed the experiments and analyzed the data; Y.Z. and J.H.B. wrote the manuscript.

Competing interests
The authors declare no competing financial interests.

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**Methods**

TtCcdA expression and purification. The expression vector of TtCcdA was constructed by inserting an *E. coli* codon-optimized sequence (GenScript) encoding TtCcdA (GI 55981378) and a C-terminal ‘PHSHHHHHHHHHH’ tag between the Ndel and XhoI sites of the pET22b vector. An E35A mutation was introduced by site-directed mutagenesis to facilitate the expression of the full-length protein in *E. coli*. All other mutations were introduced on this background. BL21(DE3)pLYS competent cells were transformed, and colonies from freshly transformed plates were used to inoculate two liters of LB medium. Cells were grown at 37 °C with vigorous shaking. When the optical density at 600 nm (OD600) reached 0.7–0.8, cells were harvested and centrifuged, washed 3 times with 0.2 M NaCl, and resuspended in 1.0 L M9 minimal medium supplemented with 10% (v/v) Bioexpress-1000 (Cambridge Isotope Laboratories). Cells were induced by adding IPTG to a final concentration of 1 mM (w/v). The membrane extraction proceeded at 4 °C overnight, during which time TtCcdA became fully oxidized. The DDM-insoluble fraction was removed by a second ultracentrifugation. The DDM-solubilized protein was loaded onto a TALON column (Clontech Laboratory, Inc.). The column was washed with 20 column volumes of the above Tris buffer containing DM (4.2 mM), DPC (1.5 mM), and 0.5 mM EDTA and was degassed before use. Purified oxidized TtCcdA was eluted from the column and concentrated to 0.2 mL by centrifugation using an Amicon Ultra-15 (MWCO 10 kDa, Merck Millipore Inc.) centrifugal filter unit. Subsequently, TtCcdA was exchanged into NMR buffer (25 mM sodium acetate, 25 mM KPO4, pH 5.4, 30 mM NaCl, 0.5 mM DPC, 10% D2O) by four cycles of five-fold dilution and concentration. A TtCcdA NMR sample for saturation transfer experiments was composed of 2.0% (w/v) TtCcdA and [U-15N]-TtCcdA and [U-2H, 13C, 15N]-TtCcdA were expressed using 15N and 13C minimal medium, resuspended in 1.0 L M9 minimal medium supplemented with 1% (w/v) Bioexpress-1000 (Cambridge Isotope Laboratories). Cells were induced by adding Rosetta (DE3) cells grown on M9 minimal medium supplemented with 1% (v/v) Bioexpress-1000 (Cambridge Isotope Laboratories). Cells were induced by adding IPTG to a final concentration of 1 mM (w/v), and cells were harvested and centrifuged. TtCcdA was purified using Ni-NTA chromatography, TEV protease cleavage, and a second Ni-NTA chromatography step to remove the affinity tag. Activity assay. The reaction buffer contains 50 mM Tris, pH 8.0, 100 mM NaCl, and 0.5 mM EDTA and was degassed before use. Purified oxidized TtCcdA was exchanged into the reaction buffer with 4.2 mM DM using a PD-10 column. Purified TtCcdA was reduced with 10 mM DTN on ice for 1 h and then exchanged into the reaction buffer using a PD-10 column. For the activity assay, 10 μL oxidized TtCcdA and 1.5% (w/v) DDM were mixed first, and then 50 μM freshly reduced TtCcdA was added to trigger the reaction at 25 °C. At 20 min, 2, 4, 8, 16, 32, and 64 min time points, 10 μL of the reaction mixture was removed and mixed with 10 μL nonreducing SDS–PAGE loading buffer (contains 2% SDS). SDS quenched the reaction effectively. Samples were then subjected to SDS–PAGE and Coomassie Brilliant Blue staining.

Resonance assignments of TtCcdA. All NMR experiments were conducted at 70 °C on a Bruker Avance III 800 MHz spectrometer equipped with a cryogen-free probe.

NMR chemical-shift assignments of backbone 1H, 15N, 13Cα, 13Cβ and side chain 15N were achieved using TROSY versions of HNCA, HN(CO)CA, and HNCACB experiments. The assignments in the α-helical regions were confirmed by a 3D TROSY-based 13C-edited NOESY-HSQC experiment. These backbone resonances were used to perform a Cα φ/ω map. In addition, seven selectively labeled samples ([U-15N], [U-13C], [U-15N, 13C]-TtCcdA) were used to facilitate the assignment (see “TtCcdA expression and purification” for the list of the seven samples).

PRE restraints. Nitroxide spin labels were introduced into the wild-type TtCcdA through single-cysteine mutagenesis. Wild-type TtCcdA has two native cysteines, which form an internal disulfide bond during expression and purification. Eight mutation sites (L44C, F55C, V78C, L93C, A134C, F147C, F165C, and L186C) were used in the structure determination of oxidized TtCcdA. Purified TtCcdA mutants were labeled with two aliquots of a ten-fold excess of MTSL (1-(1-oxyl-2,2,5,5-tetramethyl-1-d) pyrroline-3-methyl) methanethiosulfonate, Toronto Research Chemicals) at ambient temperature separated by one hour. Unreacted label was removed using a TALON column. Two TROSY-HSQC spectra for each mutant were recorded before and after the nitroxide label was reduced by a five-fold excess of ascorbic acid. The native disulfide bond formation in these samples was checked by (1) the close resemblance of the paramagnetic NMR spectra of the mutants to that of the oxidized wild-type protein and (2) the lack of a reduced protein band on SDS–PAGE. The completeness of the labeling was assessed by the total disappearance of certain peaks in the paramagnetic NMR spectra corresponding to residues in proximity to the site of labeling. PRE distance restraints were derived from the intensity ratios of peaks in the paramagnetic and diamagnetic spectra (Ipure/Imag). The global rotational correlation time of TtCcdA (30 ns) was determined using the [15N,1H] TROSY-HSQC experiment. 

RDCs were composed of 2.0% (w/v, final concentration) acrylamide and 2.0% (w/v, final concentration) (3-acrylamidopropyl)-trimethylammonium chloride (APTMAC) and was vertically compressed from 21 mm to 16 mm in a Shigemi NMR tube, as described previously.24 The 0.15% (w/v) neutral acrylamide gel was stretched from 6.0 mm diameter to 4.24 mm diameter inside an open-end NMR tube. Both gels with high-temperature measurements without significant shrinking. [15N-H RDCs were recorded using the ARTSY experiment on perdeuterated samples.23 The error of the RDC restraints was estimated by the noise of the spectra and a minimal error of ±2–3 Hz was imposed. The initial alignment amplitudes and NOE distances were estimated by powder pattern analysis and were allowed to change during structure calculations.

Structure calculation. The structures were calculated using XPLOR-NIH. A two-step folding and refinement protocol was used. In the folding step, initial structures were folded from an extended conformation using dihedral angle restraints, short-range NOE restraints, hydrogen bond restraints, and PRE restraints. The backbone φ and ω dihedral angle restraints were derived from the chemical shifts using TALOS-N (ref.19). The errors were set using TALOS-N default values: 20° or 2x the TALOS-derived s.d., whichever is larger, for strong predictions; 30° or 3x the TALOS-derived s.d., whichever is larger, for generous predictions. The short-range NOE restraints were derived from a 3D N-(ω to 220°) PREX experiment and were only observed between i and i + 1, i + 2 backbone residues. Residues which hydrogen bond restraints were used were identified using a hydrogen/deuterium exchange (HDX) experiment. For residues that resisted HDX and were in the TALOS-N-predicted α-helical regions, standard XPLOR-NIH α-helical backbone hydrogen bond restraints were imposed. Structures were calculated using 100 ps of torsion angle dynamics at 3.500 K, then ~260 ps of slow cooling in torsion angle space. In total, 200 structures were calculated, and ten structures of lowest energy were used as the refinement stage (Supplementary Fig. 2d). In the refinement step, RDC restraints and an implicit membrane potential were used.7 Structures were calculated using 40 ps of torsion angle dynamics at 3.000 K, then ~160 ps of slow cooling in torsion angle space. In total, 200 structures were calculated and ten structures of lowest energy were chosen to represent the ensemble structure of TtCcdA. From this ensemble, the lowest-energy structure was used as the representative structure. Molprobity reported 95% of all residues were in favored regions, and 99% of all residues were in allowed regions of the Ramachandran plot.

Structure validation. To assess the robustness of the TtCcdA structure, we evaluated the robustness of the TtCcdA structure using root mean square deviations (RMSDs) for backbone atoms of the ensemble structure of TtCcdA. From this ensemble, the lowest-energy structure was used as the representative structure. Molprobity reported 95% of all residues were in favored regions, and 99% of all residues were in allowed regions of the Ramachandran plot.
were compared with the representative structure, and the average r.m.s.d. was reported (Supplementary Fig. 2e). They were also checked against the withheld data from the neutral gel, and the average $Q_{\text{free}}$ was reported. When all RDC restraints and one set of the PRE restraints were removed (PREx7, ~30% long-range structural restraints removed), the calculated structures still resembled the reported structure.

**Solvent accessibility of TtCcdA.** Solvent accessibility of TtCcdA was measured by a titration experiment with Gd-DTPA (diethylenetriaminepentaacetic acid gadolinium). Gd-DTPA was dissolved at 200 mM in NMR buffer and the pH was adjusted back to pH 5.4 using NaOH. Two TtCcdA samples with and without 20 mM Gd-DTPA were prepared, and TROSY-HSQC spectra were recorded. The PRE effects were defined as the signal-intensity ratio of the paramagnetic spectrum to the reference spectrum.

**Model building of inward-facing TtCcdA.** We followed an established repeat-swap homology modeling protocol to construct the inward-facing model of oxidized TtCcdA. The structural repeating units are TM1–3 (residues ~4–91) and TM4–6 (residues ~109–199). Their sequence alignment was generated using T-coffee. The initial alignment was further adjusted by shifting the TM1a by –1 residue. The full-length alignment of the model and the template was constructed by joining the TM1–3 and TM4–6 fragment alignment (Supplementary Fig. 5a,b). The nonrepeating membrane peripheral elements (residues 92–94, 200–222) were aligned to themselves. The flexible loops (residues 1–3, 32–39, 95–108, 141–145) were not aligned, and their conformations were generated de novo. To conserve the secondary structure of the template, both ends of TM1 and TM4 and the N termini of TM2 and TM5 were constrained to be $\alpha$-helical. To conserve the conformation of the O scaffold and H1 helix, distance restraints between Cz atoms were introduced in these regions (residues 50–91, 155–199, and 207–214). The inward-facing model was built using MODELLER (version 9.17). A total of 200 models were calculated, and a representative model was chosen on the basis of low molPDF and DOPE scores.

**Cysteine cross-linking experiment.** The purified TtCcdA was exchanged into the degassed cross-linking reaction buffer (the activity buffer without EDTA) using a PD-10 column. To induce cross-linking, 20 μM protein was mixed with 22 μM HgCl₂, and the sample was incubated on ice for 15 min. To label the free cysteines, the sample was mixed with 2.0 mM 5 kDa PEG maleimide (malPEG5K), and the reaction was kept at room temperature for 30 min. The reaction products were analyzed using nonreducing SDS–PAGE (4–20% gradient gel).

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** The accession number for the coordinates and structural restraints of TtCcdA is PDB 5VKV and BMRB 30286. The PMDB accession code for the inward-facing model of TtCcdA is PM0081003.

**References**

1. Shen, Y. & Bax, A. Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. *J. Biomol. NMR* 56, 227–241 (2013).
2. Tian, Y., Schwieters, C. D., Opella, S. J. & Marassi, F. M. A practical implicit membrane potential for NMR structure calculations of membrane proteins. *Biophys. J.* 109, 574–585 (2015).
### Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - N/A

2. **Data exclusions**
   - Describe any data exclusions.
   - No structural data excluded

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - The NMR structure of TtCcdA has been validated using (1) partial structural restraints (See Supplementary Figure 3e and Methods for detail) and (2) solvent access experiment (See Supplementary Figure 4a-b).

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - N/A

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - N/A

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
   - n/a Confirmed

- The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. \(P\) values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

- NMR data acquisition: Bruker Topspin
- NMR data process: NMRPipe, CcpNMR analysis
- NMR structure calculation: Xplor-NIH
- Structure modeling: Modeller v9.17
- Sequence alignment: LALIGN

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

- No restriction.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- N/A

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

- N/A

b. Describe the method of cell line authentication used.

- N/A
c. Report whether the cell lines were tested for mycoplasma contamination.

- N/A
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

- N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

- N/A

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

- N/A