Published in final edited form as:

J Mol Biol. 2020 September 18; 432(20): 5632–5648. doi:10.1016/j.jmb.2020.08.013.

Structure and reconstitution of a MCU-EMRE mitochondrial Ca^{2+} uniporter complex

Chongyuan Wang*, Rozbeh Baradaran*, Stephen Barstow Long

Structural Biology Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA.

Abstract

The proteins MCU and EMRE form the minimal functional unit of the mitochondrial calcium uniporter complex in metazoans, a highly selective and tightly controlled Ca^{2+} channel of the inner mitochondrial membrane that regulates cellular metabolism. Here we present functional reconstitution of an MCU-EMRE complex from the red flour beetle, *Tribolium castaneum*, and a cryo-EM structure of the complex at 3.5 Å resolution. Using a novel assay, we demonstrate robust Ca^{2+} uptake into proteoliposomes containing the purified complex. Uptake is dependent on EMRE and also on the mitochondrial lipid cardiolipin. The structure reveals a tetrameric channel with a single ion pore. EMRE is located at the periphery of the transmembrane domain and associates primarily with the first transmembrane helix of MCU. Coiled coil and juxtamembrane domains within the matrix portion of the complex adopt markedly different conformations than in a structure of a human MCU-EMRE complex, suggesting that the structures represent different conformations of these functionally similar metazoan channels.

Keywords

Ion channel; calcium channel; membrane protein; flux assay; reconstitution

Introduction

The mitochondrial calcium uniporter is a highly selective calcium (Ca^{2+}) channel in the inner mitochondrial membrane that controls the flow of calcium ions into mitochondria and thereby regulates ATP synthesis and allows mitochondrial respiration to respond to cytosolic Ca^{2+} signals [1, 2]. Ca^{2+} uptake by the uniporter is driven by the negative electric potential

Corresponding author: Stephen B. Long, PhD., Longs@mskcc.org, Phone: 347-401-2531.

*equal contribution

Author Contributions C.W. performed cryo-EM and associated studies. R.B. performed functional measurements. S.B.L. assisted and directed the research. All authors contributed to data analysis and the preparation of the manuscript.

Chongyuan Wang: Conceptualization, Investigation, Data Curation, Methodology, Writing – Original Draft. **Rozbeh Baradaran:** Conceptualization, Investigation, Data Curation, Methodology, Writing – Original Draft. **Stephen B Long:** Conceptualization, Investigation, Methodology, Funding Acquisition, Project Administration, Writing – Original Draft.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors declare no competing financial interests.

of the matrix (approximately -160 mV) relative to the cytosol, which is established by the proton gradient across the inner mitochondrial membrane [3]. The transmembrane proteins MCU and EMRE constitute the minimal unit for Ca^{2+} uptake in metazoan organisms [4, 5]. MCU contains two transmembrane helices (TM1 and TM2) and a water-soluble region in the matrix. EMRE is a small (6.5 kDa) protein containing only one transmembrane helix. In metazoans, the MCU-EMRE complex is regulated by an array of factors, which include MICU1-MICU2 or MICU1-MICU3 heterodimers from the intermembrane space [6-8], MCUR1 [9-11], and the MCU-like protein MCUB [12]. Structural and functional studies indicate that a tetrameric assembly of MCU subunits forms the pore through which Ca^{2+} ions flow [13-19]. While EMRE is necessary for Ca^{2+} uptake in metazoans, it is not found in many lower organisms [4, 5]. Studies using mitochondrial Ca^{2+} uptake assays indicate that EMRE associates directly with MCU [4, 20], and a cryo-EM structure of a human MCU-EMRE (hMCU-EMRE) complex shows it can do so with 1:1 stoichiometry [21]. However, a recent study indicates that channels containing as few as one EMRE subunit per MCU tetramer are functional [22]. The requirement for EMRE in metazoan channel function is not fully understood. We sought to obtain additional structural information on the MCU-EMRE complex and to study its function in a reconstituted system.

Understandings of the uniporter and the role of EMRE are advanced by comparative genomics and functional studies of MCU proteins from a variety of species. While homologs of both MCU and EMRE are found in metazoan organisms, certain organisms, including fungi, green plants, and amoeba contain homologous MCU proteins but lack readily recognizable EMRE homologs [4, 23]. MCU homologs from the amoeba *D. discoideum* and the green plant *A. thaliana*, which don't have EMRE subunits, catalyze mitochondrial Ca^{2+} uptake with uptake rates comparable to metazoan MCU-EMRE complexes [5, 20]. In studies of fungal MCU channels a degree of Ca^{2+} flux has been observed when these proteins are exogenously expressed in the cell membranes of bacteria [17, 19]. Using a reconstituted system, Wang et al. have recently observed Ca^{2+} uptake through purified human MCU-EMRE channels into liposomes [21]. In that assay, Ca^{2+} flux was detected using the radioactive isotope $^{45}\text{Ca}^{2+}$ on the time scale of ~ 1 h. A similar amount of Ca^{2+} flux has been observed for purified MCU protein from the fungus *Neosartorya fischeri* using an analogous assay [17]. The ability to reconstitute channel activity using purified protein is a major advance and promises to allow interrogation of properties that are not easily accessible in cell-based systems such as the influence of particular lipids on channel function. However, the radioactivity-based assay is markedly more sensitive than standard fluorescence-based assays used to measure Ca^{2+} uptake by mitochondria and the duration of the assay is dramatically longer than for mitochondrial-based assays, for which Ca^{2+} uptake is complete within a few minutes [4, 5]. It is possible that additional components and/or alternative conditions may be necessary to achieve robust Ca^{2+} uptake for purified MCU/MCU-EMRE complexes in a reconstituted system.

Recent progress has substantially improved our understanding of the structure of MCU channels. Cryo-EM and X-ray structures of four fungal MCU homologs and a low (~ 8 Å) resolution structure of a metazoan MCU channel, from zebrafish, were determined in 2018 [16-19]. In 2019, Wang et al. presented a cryo-EM structure of human MCU in complex with EMRE [21]. An additional structure of a human MCU-EMRE complex is available in

preprint form [24]. The transmembrane regions of the fungal and human MCU structures are similar, which indicates that the architecture of the pore is structurally conserved among different clades of life despite differences in regulation and the metazoan channel's dependence on EMRE. The X-ray and cryo-EM structures show that the channel is formed from a tetrameric assembly of MCU subunits [16-19, 21]. Intriguingly, the cryo-EM analysis of the hMCU-EMRE complexes revealed that a portion of the channels in the purified sample formed dimers comprising a complex of two intact tetrameric channels adjacent to one another [21, 24]. The N-terminal domains (NTDs) of human MCU mediate dimerization [21, 24]. In addition to channel dimers, isolated channels were observed in the recent structure [24], which suggests that channel oligomerization is dynamic. Dimer formation seems to be dependent upon EMRE to some extent because dimerization was not observed in a cryo-EM sample without it [21]. However the effect of dimerization on the function of the channel is not clear because the NTD is not required for Ca^{2+} uptake by the channel or for its dependence on EMRE [16, 21, 25, 26].

In this study, we took an orthogonal approach to obtain structural information for a metazoan MCU-EMRE complex. From fluorescence-detection size exclusion chromatography (FSEC) screening [27] of numerous metazoan MCU homologs, we identified that the MCU homolog from the red flour beetle, *Tribolium castaneum* (*TcMCU*), was well suited for structural studies (Methods). Because it has been shown that the NTD of MCU is not required for Ca^{2+} uptake by metazoan MCU/EMRE channel complexes [16, 21, 25, 26], we removed the NTD for further structural studies. To facilitate structural studies of MCU in complex with EMRE, we capitalized on the observation that fusion proteins comprising MCU and EMRE subunits form functional channels [20] and used an analogous expression construct in which EMRE is connected via a polypeptide linker to the C-terminal end of MCU. This construct is hereafter referred to as *TcMCU-EMRE* (Figure 1A). Using purified *TcMCU-EMRE* protein that had been reconstituted into lipid nanodiscs, we determined a cryo-EM structure at 3.5 Å resolution. For studying the function of the channel *in vitro*, we reconstituted purified *TcMCU-EMRE* channels into liposomes and, using a novel Ca^{2+} uptake assay, observed robust Ca^{2+} uptake that was dependent on both EMRE and the mitochondrial lipid cardiolipin. The cryo-EM structure of *TcMCU-EMRE* has a similar architecture to that observed for hMCU-EMRE [21]. Noteworthy differences in the transmembrane, juxtamembrane, and coiled-coil domains may represent alternative conformations of these functionally similar channels.

Results

***TcMCU-EMRE* catalyzes Ca^{2+} uptake into mitochondria.**

To investigate whether the *TcMCU-EMRE* construct that was used for structural studies (Figure 1A) was capable of catalyzing Ca^{2+} uptake into mitochondria of human cells, it was expressed in the mitochondria of HEK293 MCU/EMRE knockout cells (Methods). Mitochondrial Ca^{2+} uptake measurements were made using a standard assay that measures the depletion of Ca^{2+} from the bath solution using a fluorescent Ca^{2+} indicator [5, 16]. Consistent with previous experiments using human MCU-EMRE fusion proteins [20], uptake of Ca^{2+} into mitochondria was observed when *TcMCU-EMRE* was expressed

(Figure 1B, Supplementary Figure 1B). Uptake was also observed when full length *Tc*MCU or *Tc*MCU without an NTD (*Tc*MCU_{NTD}) was coexpressed with *Tc*EMRE (Figure 1B, Supplementary Figure 1B). These experiments indicate that the NTD is not required for uptake, which has been demonstrated previously for other metazoan channels [16, 21, 25, 26]. Thus, the *Tc*MCU-EMRE construct used for structural studies catalyzes the uptake of Ca^{2+} into human mitochondria.

Development of an assay to measure Ca^{2+} uptake into liposomes

After demonstrating that *Tc*MCU-EMRE was capable of catalyzing Ca^{2+} uptake into mitochondria, we wanted to evaluate whether purified *Tc*MCU-EMRE protein could catalyze Ca^{2+} uptake in a reconstituted system. To do so, we developed a novel Ca^{2+} uptake assay designed to measure Ca^{2+} uptake into liposomes. The mitochondrial lipid cardiolipin was included in the lipid composition (POPE:POPG:cardiolipin, 3:1:0.3) used for the assay because of its prevalence in the mitochondrial inner membrane [28].

Mitochondria are capable of taking up an enormous amount of Ca^{2+} from the cytosol, in part, because high levels of phosphate within the matrix sequester free Ca^{2+} by forming amorphous $\text{Ca}_3(\text{PO}_4)_2$ precipitates [2, 29]. In the assay, phosphate was used as the anionic species within the liposomes in order to mimic the ability that mitochondria have to sequester Ca^{2+} (Figure 2A). The liposomes were formed from a bath solution containing 150 mM potassium phosphate and then they were diluted 100-fold into a solution that contains NMDG-chloride. This dilution established a 100-fold gradient of potassium (K^+) across the proteoliposomal membrane (150 mM K^+ inside the proteoliposomes and 1.5 mM K^+ outside). The NMDG cation was used in the bath solution because it is too large to pass through most ion channels. After dilution, the K^+ ionophore valinomycin, which is highly selective for K^+ , was added. The K^+ flow out of the liposomes due to the 100-fold chemical gradient generated an electric potential of approximately -116 mV, according to the Nernst equation [30], on the inside of the vesicles relative to the bath solution. This voltage could then be used to drive the uptake of Ca^{2+} ions from the bath solution, provided there was an ion channel present in the lipid membrane that permitted their passage. As is typically done in mitochondrial Ca^{2+} uptake assays, the concentration of Ca^{2+} in the bath solution, $[\text{Ca}^{2+}]_{\text{bath}}$, was monitored during the course of the experiment using a fluorescent indicator that is membrane impermeant and remains in the bath (Ca-Green 5N). This indicator was the same one used for mitochondrial uptake assays (Figure 1B) and has a K_d of ~ 14 μM , which enables detection within the ~ 1 – 40 μM range (Invitrogen). At the beginning of the experiment $[\text{Ca}^{2+}]_{\text{bath}}$ was kept low (below 1 μM) by a small concentration of EGTA (1 μM). 30 μM CaCl_2 was then added to raise $[\text{Ca}^{2+}]_{\text{bath}}$ to approximately 29 μM , after which point a time-dependent decrease in fluorescence would indicate Ca^{2+} uptake by the liposomes.

We used the Ca^{2+} ionophore ETH129, a known Ca^{2+} translocator [31], to evaluate the experimental system. Liposomes without protein, which were formed and used in the manner described above, did not take up Ca^{2+} on their own, as was evidenced by a flat fluorescence-vs-time curve following the addition of 30 μM Ca^{2+} to the bath solution (Figure 2B). After the addition of ETH129, however, a time dependent decrease in

fluorescence was observed, which indicated a decreasing concentration of $[Ca^{2+}]_{bath}$ during the course of the experiment (Figure 2B). This result verified that the experimental system could be used to study Ca^{2+} uptake by an entity that was capable of catalyzing Ca^{2+} permeation through membranes.

We found that phosphate was required for substantial Ca^{2+} uptake. When potassium chloride was used in place of potassium phosphate within the liposomes, the $[Ca^{2+}]_{bath}$ concentration hardly changed (Figure 2B). This can be rationalized because calcium chloride is highly soluble in aqueous solutions whereas calcium phosphate is nearly insoluble in water. In both cases, Ca^{2+} influx reaches equilibrium when the concentration of soluble Ca^{2+} ions within the liposomes ($[Ca^{2+}]_{free-inside}$) is approximately 50-fold higher than $[Ca^{2+}]_{bath}$ (corresponding to half of the potassium gradient due to calcium's +2 charge). When chloride is used as the anion within the liposomes, $[Ca^{2+}]_{free-inside}$ is equal to the total concentration of Ca^{2+} ions within the liposomes. Because the internal volume of the liposomes is extremely small in comparison to the volume of the bath solution, the equilibrium concentration gradient of soluble Ca^{2+} ions (50-fold higher on the inside) requires only a relatively small number of ions to enter the liposomes and this results in a only a minute change in $[Ca^{2+}]_{bath}$ when chloride is used. On the other hand, because calcium phosphate is only slightly soluble in water, when phosphate is used as the anion within the liposomes, Ca^{2+} ions that emerge within the liposomes are sequestered by phosphate, presumably as $Ca_3(PO_4)_2$ aggregates. Thus, $[Ca^{2+}]_{free-inside}$ remains low throughout the course of the experiment, as it does in mitochondria because of these aggregates [29]. Therefore, even though the volume of the liposomes is small relative to the volume of the bath solution, the uptake of Ca^{2+} is substantial enough to dramatically lower $[Ca^{2+}]_{bath}$ when phosphate is used. This is analogous to the situation in mitochondrial Ca^{2+} uptake assays. In those assays, the internal volume of mitochondria is very small in comparison to the volume of the bath solution, but the uptake of Ca^{2+} is large enough to substantially reduce the $[Ca^{2+}]_{bath}$.

Purified *TcMCU-EMRE* catalyzes Ca^{2+} uptake into liposomes and its activity depends on cardiolipin

Having established an assay to monitor Ca^{2+} uptake into liposomes, we purified *TcMCU-EMRE* from mammalian cells and reconstituted it into liposomes containing cardiolipin to analyze its ability to catalyze Ca^{2+} uptake. As described for the test case above, a 100-fold gradient of K^+ across the membrane was established by dilution of the proteoliposomes into a bath solution, after which point the potassium ionophore valinomycin was added (Figure 2). Robust Ca^{2+} uptake, observed as a time-dependent decrease in fluorescence, occurred for liposomes containing *TcMCU-EMRE* (Figure 2C). Ca^{2+} uptake was not observed for liposomes devoid of protein. Uptake occurred on a similar time scale as in Ca^{2+} uptake assays using mitochondria, reaching completion within a few minutes. Impressively, proteoliposomes containing *TcMCU-EMRE* deplete the 1 ml bath solution of approximately 30 μM of Ca^{2+} during the assay despite the small internal volume of the vesicles (which we estimate to be < 0.5 μl , assuming a proteoliposomal diameter of 100 nm and ~80,000 lipid molecules per proteoliposome [32, 33]).

To evaluate the dependence of the channel on EMRE in a purified setting, we purified an analogous construct without EMRE (denoted *TcMCU*_{NTD}), reconstituted it into liposomes, and studied its function in the same manner. Ca^{2+} influx was dramatically diminished in the absence of EMRE (Figure 2C). This confirms the requirement for EMRE using a purified system and is a further indication that the function of purified *TcMCU*-EMRE is similar to that observed for the channel in a mitochondrial setting.

We next evaluated the effect of the MCU inhibitor Ruthenium red (Ru-Red), which is known to block the channel from the intermembrane space (IMS) [29, 34–36]. Ru-Red blocked most of the Ca^{2+} influx through *TcMCU*-EMRE when added to the bath solution (Figure 2C). Incomplete block is presumably an indication that channels adopt both orientations relative to the liposomal membrane.

The use of a reconstituted system allowed us to control the lipid composition of the proteoliposomes. We wondered what effect cardiolipin, which comprises approximately 20% of the inner mitochondrial membrane lipid composition [28], might have on the function of the channel. To this end, purified *TcMCU*-EMRE was reconstituted into liposomes with and without cardiolipin. Proteoliposomes without cardiolipin displayed markedly reduced Ca^{2+} uptake in comparison to those containing cardiolipin (Figure 2C). This difference does not seem to stem from different efficiencies for reconstitution, as preparations of proteoliposomes with and without cardiolipin contain approximately the same amount of *TcMCU*-EMRE channel protein (Supplementary Figure 1C). The data suggest that cardiolipin has a direct effect on the function of the channel.

Structure determination

Having determined that *TcMCU*-EMRE protein catalyzes Ca^{2+} uptake into mitochondria and in a reconstituted system, we sought to obtain high-resolution structural information for the channel using cryo-electron microscopy (cryo-EM). For these structural studies, we reconstituted purified *TcMCU*-EMRE into lipid nanodiscs that contained 20% cardiolipin (POPC:POPE:cardiolipin, 2:2:1) in order to mimic the lipid environment of the inner mitochondrial membrane. Initial 3D models of the *TcMCU*-EMRE ion channel complex were deduced *de novo* from the data and a 3D reconstruction was generated without imposing symmetry (Supplementary Figure 3). The assembled channel comprises four MCU subunits and four EMRE subunits (Figure 4). Inspection of the density indicated that the reconstruction had twofold rotational symmetry along the length of the channel (Supplementary Figure 3). Incorporating this C2 symmetry improved the resolution and yielded a final 3D reconstruction at 3.5 Å resolution (Figure 3A). The structure of *TcMCU*-EMRE is fourfold symmetric in the TMD, whereas the matrix regions have C2 symmetry. Similar symmetry transformations are observed in the structures of fungal MCU and hMCU-EMRE channels, in which the TMDs are fourfold symmetric and the matrix regions exhibit lower symmetry [16–19, 21]. Density for *TcMCU*-EMRE is present for the majority of the polypeptide (Supplementary Figure 3 and 4) and for most side chains, which made it possible to build and refine an atomic model that has good stereochemistry and agreement with the density (Table 1, Methods). Local resolution estimates indicate that the core of the

channel has the highest resolution (~ 3.3 Å) while that for EMRE is somewhat lower (~ 3.8 Å) (Supplementary Figure 4).

The polypeptide linker used to connect MCU and EMRE is not visible in the density and we detected a degree of proteolysis in this linker by SDS-PAGE analysis (Supplementary Figure 1F). We wondered if the structure obtained for *Tc*MCU-EMRE might arise from the portion of channels in which this linker had been cleaved. To investigate this possibility, we reengineered the linker to prevent proteolysis (this construct is denoted *Tc*MCU-EMRE_{EM2}, Supplementary Figure 1A, G). A relatively small cryo-EM dataset of *Tc*MCU-EMRE_{EM2} in lipid nanodiscs yielded a 3D reconstruction at ~ 5.0 Å resolution. The cryo-EM map of *Tc*MCU-EMRE_{EM2}, including the portions for EMRE, is indistinguishable from the structure of the original construct (Supplementary Figure 5). Thus the small amount of proteolysis within the linker did not affect the structure of the complex. We also evaluated the function of *Tc*MCU-EMRE_{EM2} and found that it catalyzed Ca^{2+} uptake by mitochondria and in the reconstitution-based assay (Supplementary Figure 1B, D). The Ca^{2+} uptake rate observed for purified *Tc*MCU-EMRE_{EM2} was slightly more rapid than for *Tc*MCU-EMRE, which could potentially be attributed to the partial proteolysis of *Tc*MCU-EMRE. Like *Tc*MCU-EMRE, *Tc*MCU-EMRE_{EM2} was inhibited by Ru-Red and cardiolipin dramatically increased its Ca^{2+} uptake rate (Supplementary Figure 1D).

The structure of *Tc*MCU-EMRE has three prominent sections: the transmembrane domain (TMD), a coiled-coil domain (CCD), and a linker helix domain (LHD) (Figure 3). *Tc*MCU and *Tc*EMRE share 58 % and 56 % amino acid sequence identity with their human counterparts (not including their mitochondrial targeting sequences), and there are no gaps in their aligned amino acid sequences (Supplementary Figure 2). Amino acid numbering referred to herein is according to the human proteins.

The selectivity filter and pore

The TMD comprises a fourfold symmetric assembly of the TM1 and TM2 helices of MCU and the transmembrane helix of EMRE (Figure 3). A single ion pore, perpendicular to the membrane, is surrounded by four MCU subunits. The walls of the pore are primarily formed by the TM2 helices (Figure 4). As in other structures of MCU channels [16-19, 21], each TM1 helix interacts extensively with an adjacent TM2 helix from another subunit. TM1 makes fewer contacts with TM2 of the same subunit and these two helices are slightly separated within the leaflet of the membrane closest to the matrix (Figure 5A). This separation gives rise to fenestrations (openings) in the molecular surface of the TMD that would be filled by lipids. The fenestrations are markedly smaller in *Tc*MCU-EMRE than they are in the structure of hMCU-EMRE, and this indicates that the pore is less exposed to the lipid membrane in the conformation observed for *Tc*MCU-EMRE (Figure 5).

The region of the pore closest to the IMS contains the selectivity filter (Figure 4B-C). Its conformation is indistinguishable from the selectivity filters in other cryo-EM and X-ray structures of MCU channels [16-19, 21]. As in those structures, an 'E ring' of four glutamate residues, Glu 264 from each subunit, forms a high-affinity Ca^{2+} binding site that directly coordinates Ca^{2+} . Strong density for Ca^{2+} is observed there (Supplementary Figure 4E-F). The conformation of the E-ring is stabilized by tryptophan (W) and proline (P) residues of

the conserved WDXEP signature motif of MCU channels that surround the glutamate residues, but they do not contribute to the pore directly (Figure 4C). A second ring of acidic amino acids (the ‘D ring’), comprising Asp 261 from each subunit, is located above the E-ring at the IMS end of TM2. The D-ring could coordinate Ca^{2+} through water-mediated interactions as observed in certain other structures [16-19]; density for Ca^{2+} is weak in this location in the *Tc*MCU-EMRE.

EMRE

The transmembrane helix of each of the four EMRE subunits is located at the periphery of the TMD and is oriented approximately 45° with respect to the membrane (Figures 4 & 5). The N-terminal end of each EMRE helix is located on the matrix side of the membrane whereas its C-terminal end is on the IMS side. The EMRE helix does not contribute to the walls of the pore and it interacts mostly with the TM1 helix to which it is adjacent, but it also interacts with the TM2 helix of a neighboring subunit. An interaction with TM1 was predicted on the basis of mutagenesis studies [20]. The distribution of amino acids on the EMRE helix corresponds to the transmembrane region of MCU; hydrophobic amino acids are located within the membrane-spanning region and the ends of the helix are flanked by basic and hydrophilic amino acids that could interact with phospholipid head groups (Figure 6A). Density for the N-terminal region of EMRE that precedes its transmembrane helix is not visible in the map, which may be an indication of its flexibility. This region is ordered in the hMCU-EMRE structure [21], but otherwise EMRE adopts a similar conformation as in the human structure. An acidic C-terminal tail of EMRE, which we removed from the expression construct to increase protein yield and because it is predicted to have a low degree of secondary structure, would be located so as to extend into the IMS (Figure 6A). This region has been implicated in binding to the MICU1-MICU2 and/or MICU1-MICU3 regulatory complexes within the IMS [20].

The interactions between EMRE and TM1 are typical of close-packed transmembrane helices that interact, in part, via small amino acids such as glycine or alanine [37]. Amino acids on TM1 that are conserved as glycine or alanine in metazoan channels (Gly 241, Gly 248) and small amino acids on EMRE (Gly 77, Gly 81) participate in the interaction (Figure 6, Supplementary Figure 2). These sites of closest contact are flanked by larger amino acids that form hydrophobic interactions between the helices (e.g. Trp 237, Leu 240, Val 245, and Phe 247 on TM1 and Leu 70, Val 74, Leu 78, and Ile 84 on EMRE) (Figure 6B, C). In addition to contacts with TM1, the N-terminal end of the EMRE helix also makes a smaller contact with a TM2 helix of a neighboring subunit. This contact involves Leu 70 from EMRE and Ala 280 and Leu 284 from MCU (Figure 6B, C). Each EMRE helix bridges two subunits of MCU, which suggests that EMRE may contribute to the overall structural integrity of the channel.

The CCD, JML, and conformational differences with hMCU-EMRE

The CCD, which comprises helices CC1a, CC1b, CC2a and CC2b from each subunit, forms a vestibule at the matrix side of the pore (Figure 7A-B). This matrix vestibule has side openings that would allow Ca^{2+} ions emerging from the transmembrane pore to diffuse into the matrix. The architecture of the CCD appears to be flexible and there are substantial

differences in this region in comparison with the cryo-EM structure of hMCU-EMRE (Figure 7D) [21]. Overall, the CCD in the hMCU-EMRE structure is considerably wider than in the *Tc*MCU-EMRE structure (~ 45 Å versus ~ 65 Å across, measured at MCU residue 219) due to conformational changes in the CC1a, CC1b, CC2a and CC2b helices (Figure 7). Although it is possible that these differences could arise from species-specific distinctions, the metazoan uniporter channels that have been studied to date exhibit conserved functional features. These features include a requirement for EMRE and an ability to catalyze Ca^{2+} uptake into mitochondria that is independent of the NTD of MCU [4, 16, 20, 21, 25, 26]. This functional conservation suggests that the observed structural differences may represent alternative conformations of these similar metazoan channels.

On each of the four subunits of the *Tc*MCU-EMRE complex, TM1 forms a continuous helix with CC1b that extends down into the matrix and contributes to the walls of the matrix vestibule (Figure 7A-B). In the hMCU-EMRE structure, TM1 and CC1b also form a continuous helix, but there is a slight bend between them that places the matrix portions of the CC1b helices further apart and makes the matrix region of the CCD wider than in the *Tc*MCU-EMRE structure (Figure 7D).

Differences between the beetle and human structures are even more dramatic for the portions that extend from TM2 into the matrix. In the hMCU-EMRE structure, the amino acids immediately following TM2 (residues 285-290) form a 'juxtamembrane loop' (JML) at the matrix end of the pore (Figure 7D-E) [21]. The amino acid sequence of this region is highly conserved between the human and beetle channels (Supplementary Figure 2). In the human structure, the C-terminal end of the JML connects to the CC2a and this results in a dramatic bend (~120°) between the TM2 and CC2a helices, such that CC2a occupies the upper part of the matrix vestibule and lies almost horizontally with respect to the membrane (Figure 7D-E). In the structure of hMCU-EMRE, the arrangement of the four JMLs and four CC2a helices (one from each subunit) forms a constriction that is approximately 8 Å in diameter and located at the matrix end of the pore (Figure 7E, F) [21]. As described below, the dramatic differences in the JMLs and CC2a helices make the pore considerably wider in the corresponding region of the *Tc*MCU-EMRE structure (Figures 4A, 1C).

The JML and CC2a helices of *Tc*MCU-EMRE adopt markedly different conformations than in the hMCU-EMRE structure. While the human structure has fourfold symmetry within the JML and CC2a regions [21], the structure of *Tc*MCU-EMRE has twofold symmetry within these regions (Figure 7). On two opposite subunits of *Tc*MCU-EMRE, the TM2, JML, and CC2a regions form a continuous α -helix (Figures 3C, 7A-B; labeled protomer 'A'). Thus, the JML region does not form a constriction at the matrix side of the pore but rather is part of a continuous helix. The region of this helix corresponding to the JML is located at the periphery of the channel, approximately 15 Å from the pore axis (Figure 7A-C). This helix of *Tc*MCU-EMRE is nearly straight and extends approximately 25 Å into the matrix from the membrane, ending at Leu 300. The distal end of this helix, corresponding to the CC2a region, forms coiled coil interactions with CC1b and contributes to the walls of the matrix vestibule (Figure 7A-B).

On the other two subunits of the *Tc*MCU-EMRE structure (Figures 3C, 7A-B; labeled protomer 'B'), the matrix portion of the TM2 helix extends to residue Arg 286, which is within the JML. The JML regions of these subunits are partially disordered (amino acids 285-286 of the JML comprise part of the helix, whereas amino acids 287-293 are disordered). Density for two CC2a helices, one from each of these two subunits, is observed at the bottom of the matrix vestibule (Figure 7A-B and Supplementary Figure 4C,D). The density for these CC2a helices is relatively weak and there is some ambiguity as to which CC2a helix is associated with which subunit due to the disordered connection between the TM2/JML helix and CC2a. The two CC2a helices lie perpendicular to the axis of the pore and interact with one another in an antiparallel manner (Figure 7A-B). The interaction of these helices appears to be responsible for the twofold symmetry of this region of the channel. Their positioning at the bottom of the matrix vestibule would provide ample space for Ca^{2+} ions emerging from the pore to diffuse into the matrix. Even though the JML regions of these subunits are partially disordered, they could not adopt the conformation observed in the hMCU-EMRE structure in which they form a constriction at the mouth of the pore. In summary, the JML and CC2a portions of *Tc*MCU-EMRE structure are markedly different than in the hMCU-EMRE structure. These differences make the matrix end of the pore dramatically wider in the *Tc*MCU-EMRE structure (Figure 7F).

Helical bundle and LHD

A fourfold symmetric assembly of CC1a and CC2b helices creates a bundle of eight helices that forms the bottom of the matrix vestibule (Figure 7A-B). This helix bundle has substantially larger diameter in the hMCU-EMRE structure, which indicates additional conformational plasticity in this region (Figure 7D). Below the eight-helix bundle resides the LHD, comprising an antiparallel assembly of four linker helices that would connect to the NTD of each subunit. The LHD lies sideways, with one pair of helices closer to the TMD and the other pair further away, which gives the LHD its twofold symmetry. An analogous LHD is observed in the hMCU-EMRE structure [21], and this indicates that the assembly of the LHD is independent of the presence or absence of the NTD (Figure 7D). Thus, the distal ends of the *Tc*MCU-EMRE structure, the selectivity filter and the LHD, adopt analogous conformations as in the hMCU-EMRE structure, but the intervening regions have marked differences.

Discussion

In this study we have determined a 3D structure of a metazoan MCU-EMRE complex and studied the function of this Ca^{2+} channel in a reconstituted system using purified components. Following observations of others that the NTD of MCU is dispensable for Ca^{2+} uptake and that functional channels can be formed by expressing MCU and EMRE as a single polypeptide, we used a construct comprising these features (*Tc*MCU-EMRE) for the functional and structural studies. Using a reconstituted system that was designed to replicate key aspects of mitochondrial Ca^{2+} uptake, we observed robust Ca^{2+} uptake into liposomes containing purified *Tc*MCU-EMRE. Ca^{2+} uptake was dependent on a negative electrostatic potential across the liposomal membrane, dependent on EMRE, and blocked by Ru-red. Additionally, Ca^{2+} uptake was observed on a time scale analogous to that observed using

mitochondria. Thus, purified *Tc*MCU-EMRE protein recapitulated fundamental properties of the uniporter that have been established from studies using mitochondria. Our results confirmed that the NTD is not required for these properties.

A reconstituted system afforded the opportunity to control the lipid composition of the membrane and we found that the Ca^{2+} uptake rate was markedly increased when cardiolipin, which constitutes ~20% of the lipid composition of the inner mitochondrial membrane [28], was included in the proteoliposomes. This demonstration of the channel's dependence on cardiolipin may explain the relatively slow rate of Ca^{2+} uptake observed for purified hMCU-EMRE that had been reconstituted without it [21]. Corroborating our results using purified protein, a recent study indicates that cardiolipin is important for the function of the channel in mitochondria [38]. While the molecular basis of the channel's dependence on cardiolipin is not known, we speculate that it may be due to direct interactions with the channel. It is possible that the fenestrations within the TMD of the channel are involved. In support of this hypothesis, it is thought that fenestrations in TRAAK potassium channels are responsible for the effects of particular lipids on the function of those channels and that the presence or absence of lipids within the fenestrations regulates ion flow [39].

Our proteoliposome-based assay represents a new method to study the mitochondrial Ca^{2+} uniporter, and it could be used to investigate other Ca^{2+} channels. It provides certain advantages over other flux assays. In particular, it allowed us to measure the uptake of Ca^{2+} from a bath solution containing ~30 μM Ca^{2+} . Highly selective Ca^{2+} channels often require at least micromolar levels of Ca^{2+} for robust ion flow [40], which makes the use of more sensitive indicators such as Fura-2 ($K_d \sim 145$ nM) less well-suited for studying those channels in a purified context. A key component of the assay was the inclusion of phosphate within the proteoliposomes. Using a model system we showed that liposomes filled with Cl^- took up only a small amount of Ca^{2+} in comparison to those containing phosphate. We hypothesize that phosphate sequesters free Ca^{2+} ions within the proteoliposomes, as it does within mitochondria [29], and allowed the liposomes to deplete the bath solution of Ca^{2+} despite their small internal volume in comparison to the bath.

The cryo-EM structure of *Tc*MCU-EMRE has similarities and notable differences with the cryo-EM structure of hMCU-EMRE [21]. The selectivity filters within these structures adopt indistinguishable conformations, which suggests that the conformation of the selectivity filter is relatively rigid from a structural perspective. This structural similarity despite the conformational differences observed in the other regions of the channel suggests that Ca^{2+} -selectivity is unlikely to be coupled to conformational changes in other regions of the channel. This would distinguish the Ca^{2+} selectivity mechanism from that of the Ca^{2+} channel Orai, which is also one of the most selective Ca^{2+} channels known [41]. In Orai, as in MCU, a ring of glutamate residues forms a binding site for Ca^{2+} in the selectivity filter, but this ring comprises six glutamates from six subunits in contrast to MCU's four glutamates from four subunits [42]. Unlike what appears to be a relatively rigid selectivity filter in MCU, the selectivity filter of Orai is thought to undergo a conformational change that is associated with the opening (gating) of the channel [43-45].

Differences between the structures of *Tc*MCU-EMRE and hMCU-EMRE are found within the portion of their TMDs that span the inner leaflet of the membrane (the leaflet closest to the matrix) and within their matrix regions. Fenestrations in the molecular surface between TM1 and TM2 are larger in the hMCU-EMRE structure than in *Tc*MCU-EMRE. Because these fenestrations expose the ion pore to the membrane, these differences could influence ion conduction through the channel. For example, smaller fenestrations that seal off lipids from the pore may allow ion conduction more readily due to reduced interactions between permeating ions and hydrophobic lipids.

Other notable differences between the structures of *Tc*MCU-EMRE and hMCU-EMRE are present within the JML and CCD regions. Data indicate that the JML region is particularly important from a functional perspective [21, 46]. Replacing the JML of human MCU with the corresponding region of *D. discoideum* MCU allows the modified channel to conduct Ca^{2+} without EMRE [21]. A preprint from MacEwen and colleagues describes similar observations [46]. In the hMCU-EMRE structure, the four JMLs create a constriction at the matrix end of the pore, approximately 8 Å in diameter, which is large enough for hydrated Ca^{2+} ions to pass through [21]. From comparison of the structure of hMCU-EMRE with a low-resolution structure of hMCU alone, it was hypothesized that the EMRE subunits alter the conformation of the JML such that a constriction that it forms is wider when EMRE is bound [21]. From this hypothesis, one might expect that four EMRE subunits would need to bind to MCU to stabilize the widened JML, but recent functional data suggest that only one EMRE subunit is needed for Ca^{2+} uptake by channel [22]. These new functional results suggest that the mechanism for why MCU channels require EMRE for Ca^{2+} uptake is not yet fully understood. The JML regions do not form a constriction in the *Tc*MCU-EMRE structure. Two JMLs are part of a continuous helix between TM2 and CC2a and the other two, although partially disordered, connect to CC2a helices that are located at the bottom of the matrix vestibule. The implications of the different conformations of the JMLs and CC2a helices will require additional study, but they point to the intricate conformational changes that can occur in MCU-EMRE channels.

It is possible that the conformational differences between the structures of *Tc*MCU-EMRE and hMCU-EMRE result from the absence of the NTD in the *Tc*MCU-EMRE construct and/or differences associated with these species. However, lines of evidence suggest that this may not be the case: First, the NTD is not required for Ca^{2+} -uptake by the channel or for the channel's dependence on EMRE. Second, previous studies indicate that species that contain an EMRE subunit require it for Ca^{2+} uptake, which suggests that there is a conserved mechanism for its effect on the channel [4, 20]. Finally, we have shown that the purified *Tc*MCU-EMRE protein that was used for structural studies recapitulates fundamental functional properties of the channel (Ca^{2+} uptake, Ru-red block, and a requirement for EMRE).

The ion conduction pathway in the structure of *Tc*MCU-EMRE is wide enough to accommodate Ca^{2+} permeation and we have demonstrated that the purified channel catalyzes Ca^{2+} flux. These observations suggest that the structure represents a conductive (open) conformation of the channel. However, we wish to be cautious in assigning a

particular gating state to the observed conformation as history has shown that it can be difficult to do so for other ion channels (e.g. [47, 48]).

What could be the molecular basis for the requirement for EMRE? EMRE interacts primarily with one subunit (on TM1) but it also makes contacts with another subunit through interactions with its TM2 helix. We hypothesize that EMRE provides a degree of structural stabilization to the channel. Structures of metazoan MCU channels without EMRE have only been determined to ~ 8 Å resolution [16, 21], and one should be cautious about drawing conclusions regarding channel gating mechanisms from them. Future high-resolution structural studies of MCU channels without EMRE could be informative. However, it is possible that EMRE acts more like a constitutive component of the channel and does not play a significant role in the opening or closing of a “gate” within the transmembrane region of the pore in a dynamic way. One role of EMRE is to enhance the association of the regulatory MICU1-MICU2 complex (or the MICU1-MICU3 complex in neurons) with the channel and thereby influence Ca^{2+} -dependent control of the channel through the action of those regulatory complexes [6-8, 20, 22, 49-53]. Recent data indicate that the number of EMRE subunits associated with each channel tunes the regulation by these complexes [22]. Ca^{2+} uptake and MICU1-MICU2 dependent gating can be conferred by as few as one EMRE subunit per channel [22]. Functional studies to date suggest that metazoan uniporter channels operate through conserved mechanisms; they all require EMRE for Ca^{2+} uptake and they are regulated by MICU1-3 proteins. These conserved mechanisms suggest that the cryo-EM structures of hMCU-EMRE and TcMCU-EMRE may represent alternative conformations of these functionally similar metazoan channels. The structures and the reconstitution of channel function using purified components lay a foundation for further mechanistic studies.

Methods

Protein expression and purification

Tribolium castaneum MCU (TcMCU; NCBI: XP_008192975.1) and *Tribolium castaneum* EMRE (TcEMRE; UniProt accession: TcasGA2_TC012057) were selected as candidates for structural and functional studies from among ~30 metazoan MCU orthologs that were evaluated using the fluorescence-detection size-exclusion chromatography (FSEC) screening technique using HEK-293 cells [27]. The TcMCU-EMRE construct comprises amino acids 174-355 of TcMCU (168-349 in hMCU numbering) followed by residues 35-74 of TcEMRE (53-92 in hEMRE numbering) with one of two intervening linkers (Figure 1A, Supplementary Figure 1A). The TcMCU_{NTD} construct comprises amino acids 174-367 (168-361 in hMCU numbering). cDNAs encoding these constructs were synthesized (IDT Inc.) and ligated into a mammalian cell expression vector [27] to encode proteins containing N-terminal Venus tags, which could be removed using PreScission protease. The expression plasmids were transfected into Expi293 cells (Invitrogen) for transient expression. Briefly, 1 mg plasmid and 3 mg PEI25k (Polysciences, Inc.) was mixed in 100 ml OptiMEM media (Invitrogen), incubated at room temperature for 20 min, and the mixture was added into 1 L of Expi293 cells ($3.0\text{-}3.5 \times 10^6$ cells/mL) in Expi293 expression media (Invitrogen). After

incubation at 37° C for 16 hours, 10 mM sodium butyrate (Sigma-Aldrich) was added, and the cells were cultured at 30° C for another 48-72 hours before harvesting.

For purification of *Tc*MCU_{NTD} (for the Ca²⁺ uptake assay), the cell pellet from 1 L of cell culture was resuspended in 100 mL lysis buffer [40 mM HEPES pH7.5, 200 mM NaCl, 0.15 mg/mL DNase I (Sigma-Aldrich), 1.5µg/mL Leupeptin (Sigma-Aldrich), 1.5µg/mL Pepstatin A (Sigma-Aldrich), 1 mM AEBSF (Gold Biotechnology), 1 mM Benzamidine (Sigma-Aldrich), 1 mM PMSF (Acros Organics) and 1:500 dilution of Aprotinin (Sigma-Aldrich)], solubilized by adding lauryl maltose neopentyl glycol (LMNG, Anatrace) to a final concentration of 1%, and stirred at 4 °C for 1 hr. Solubilized proteins were separated from the insoluble fraction by centrifugation at 60,000 g for 1 hr at 4° C and the supernatant was filtered through a 0.22-µm polystyrene membrane (Millipore). GFP nanobody was coupled to CNBr-activated Sepharose Fast Flow beads (GE healthcare) according to manufacturer's protocol. 2 mL GFP nanobody resin was added to the sample and rotated at 4 °C for 1 hr. The beads were washed with 100 mL buffer A (20 mM HEPES pH7.5, 150 mM NaCl, 0.5 mM digitonin (Cayman Chemical Company) and 0.01 mg/mL 18:1 cardiolipin (Avanti Polar Lipids)). MCU proteins were eluted by adding PreScission protease (0.1mg) and incubating for 3 h. at 4 °C. The sample was further purified by size-exclusion chromatography (SEC) using a Superose 6 increase 10/300 GL column (GE Healthcare) in 20 mM HEPES pH 7.5, 150 mM NaCl and 3 mM n-Decyl-β-D-Maltopyranoside and used for reconstitution into liposomes.

For the *Tc*MCU-EMRE fusion constructs, the cell pellet from 1 L of culture was resuspended in 100 mL lysis buffer, solubilized by adding n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) to a final concentration of 1%, and stirred at 4 °C for 1 hr. Solubilized proteins were separated from the insoluble fraction by centrifugation at 60,000 x g at 4° C for 1 hr and the supernatant was filtered through a 0.22-µm polystyrene membrane (Millipore). 2 mL GFP nanobody resin was incubated with the sample with agitation at 4 °C for 1 hr. The beads were washed with 100 mL buffer C (20 mM HEPES pH7.5, 150 mM NaCl, 1 mM DDM and 0.01 mg/mL cardiolipin (1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol)), and then the protein was reconstituted into lipid nanodiscs as follows.

2 ml GFP nanobody resin containing bound MCU-EMRE was mixed with 200 µl lipid/DDM mixture (17 mM DDM, 10 mM lipids: POPC : POPE : cardiolipin with a 2:2:1 weight ratio) and 160 µL MSP1D1 (Sigma, 5 mg/ml, in buffer 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA and 5 mM sodium cholate). After 1hr incubation at 4 °C, with agitation, ~1 g of wet Bio-Beads SM2 (Bio-Rad) were added to the resin, and the sample was rotated at 4 °C for ~ 16 h to remove detergent. The resin/Bio-Bead mixture was transferred into a column and washed with 100mL buffer D (20 mM HEPES pH 7.5, 300 mM NaCl) to remove empty nanodiscs. Channel–nanodisc complexes were then eluted by adding PreScission protease (~0.1 mg) and incubating for 3 h at 4 °C. The sample was further purified by size-exclusion chromatography (SEC) using a Superose 6 increase, 10/300 GL column (GE Healthcare), which was run in buffer E (20 mM HEPES pH7.5, 150 mM NaCl). The peak fractions were pooled, and 1 mM CaCl₂ was added. The sample was then concentrated to 1 mg/ml using a 100 kDa concentrator (Vivaspin-2) and immediately used to prepare cryo-EM grids.

EM sample preparation and data acquisition

5 μ L of purified sample was applied to glow-discharged (10s) Quantifoil R 1.2/1.3 grids (Au 400; Electron Microscopy Sciences) and plunge-frozen in liquid nitrogen-cooled liquid ethane, using a Vitrobot Mark IV (FEI), operated at 4 °C, with a blotting time of 2-3 s, using a blot force of '0', and 100% humidity. Micrographs were collected using a Titan Krios microscope (Thermo) operated at 300 kV using a K2 Summit detector (Gatan) in super-resolution mode (pixel size of 0.544 Å) and a defocus range of -1.0 to -3.0 μ m. The dose rate was 9 electrons/pixel/second and images were recorded for 10 s with 0.25 s subframes (40 total frames) corresponding to a total dose of 76 electrons per Å².

Image processing

Supplementary Figure 3 shows the cryo-EM workflow. For *Tc*MCU-EMRE_{EM1}, 8143 movie stacks were gain-corrected, twofold binned (calibrated pixel size of 1.088 Å), motion corrected, and dose weighted using MotionCor2 [54]. Contrast transfer function (CTF) estimates were performed in CTFFIND4 using non-dose weighted micrographs [55]. 7957 micrographs that had CtfMaxResolution values better than 7 Å were selected. 4,460,801 particles were auto-picked in RELION 3.0 [56] and imported into cryoSPARC v.2 [57] for further processing. 3,021,807 particles were selected from 2D classification, and subjected to Ab initio reconstruction (2 classes requested). One of the Ab initio models (generated from 62% of the particles) generally resembled an MCU-EMRE channel, and particles from it were selected for four sequential rounds of heterogeneous refinement (3D classification) in cryoSPARC in order to remove empty nanodiscs (without channels) and particles that did not yield high resolution reconstructions. This procedure yielded a set of 102,250 particles that was used to obtain high-resolution. While this is a fraction of the initial particle set, we note that a reconstruction generated from the first round of heterogeneous refinement (using 318,497 particles) has an indistinguishable overall structure as the final map (Supplementary Figure 3D). We did not observe evidence of alternative conformations of the channel in the dataset (e.g. from heterogeneous refinement). After the heterogeneous refinement procedure, the selected particles were subjected to non-uniform refinement in cryoSPARC v.2 with C1 or C2 symmetry and yielded reconstructions at 4.7 Å and 4.3 Å overall resolution, respectively. After two rounds of Bayesian polishing in RELION 3.0, the particles were further classified into two classes by heterogeneous refinement in cryoSPARC. 52,593 particles from the class with a more well-defined CC2a region were selected for refinement in RELION 3.0, with C2 symmetry, and yielded the final reconstruction at 3.5 Å overall resolution.

The cryo-EM workflow For *Tc*MCU-EMRE_{EM2} followed a similar procedure. 1323 movie stacks were gain-corrected, twofold binned (calibrated pixel size of 1.088 Å), motion corrected, and dose weighted in MotionCor2. CTF estimates were obtained using CTFFIND4 using non-dose weighted micrographs. 1291 micrographs with CtfMaxResolution values better than 5 Å were selected for further processing. 353,139 particles were auto-picked in RELION 3.0 and imported into cryoSPARC v.2. The particles were cleaned-up by one round of Ab initio reconstruction and two rounds of heterogeneous refinement in cryoSPARC v.2. 34,326 selected particles were subjected to non-uniform refinement in cryoSPARC v.2 and yielded a reconstruction at 6.3 Å overall resolution. The

resolution was improved to 5.0 Å following one round of Bayesian polishing in RELION 3.0.

Model building and refinement

The atomic model was built de novo and refined in real space using the COOT software [58]. Further real-space refinement was carried out in PHENIX [59]. Well-defined density for the amino acid side chains of MCU facilitated model building. Because the side chains of EMRE are less well-defined in the density, we evaluated different possible amino acid registers. Only the final model of the EMRE helix, in which two glycine residues (Gly 77 and Gly 81) interact with the closely-packed TM1 helix, yielded a model without steric clashes. This model agrees with the register of EMRE in the human MCU-EMRE structure (PDB: 6O58). The final *Tc*MCU-EMRE model has good stereochemistry and good Fourier shell correlation with the cryo-EM map (Supplementary Figure 4 and Table 1). Structural Figures were prepared with Pymol (pymol.org) [60], Chimera [61], ChimeraX [62], and HOLE [63].

Mitochondrial Ca^{2+} uptake measurements

The cDNA encoding for the constructs used for mitochondrial Ca^{2+} uptake measurements were cloned into the pCGFP-EU mammalian expression vector with a C-terminal 1D4 tag (and without GFP) [64]. These constructs are as follows: full-length *Hs*MCU-EMRE fusion (*Dd*MTS₁₋₃₀-*Hs*MCU₅₇₋₃₅₁-*Hs*EMRE₃₁₋₁₀₇ fusion), full-length *Tc*MCU (*Dd*MTS₁₋₉₀-*Tc*MCU₇₄₋₃₆₇), full-length *Tc*MCU-EMRE fusion (*Dd*MTS₁₋₉₀-*Tc*MCU₇₄₋₃₆₇-*Tc*EMRE₃₅₋₉₀), *Tc*MCU_{NTD} (*Dd*MTS₁₋₉₀-*Tc*MCU₁₇₄₋₃₆₇), *Tc*MCU-EMRE (the construct used for structural studies preceded by *Dd*MTS₁₋₉₀), *Tc*MCU-EMRE_{EM2} (*Dd*MTS₁₋₉₀-*Tc*MCU-EMRE_{EM2}), and *Tc*EMRE (*Tc*EMRE₁₋₉₀), where *Tc*, *Hs*, *Dr*, and *Dd* indicate *Tribolium castaneum*, *Homo sapiens*, *Danio rerio*, and *Dictyostelium discoideum* respectively, and subscripts denote amino acid numbers. The mitochondrial targeting sequences from *Danio rerio* MCU (*Dd*MTS₁₋₉₀) and *Dictyostelium discoideum* MCU (*Dd*MTS₁₋₃₀) were used to target proteins to mitochondria, as indicated. For MCU-EMRE fusion constructs, 3.0 µg plasmid was transfected into $\sim 2 \times 10^6$ MCU/EMRE knockout cells [16] using Lipofectamine 3000 (Invitrogen). For MCU/EMRE co-expression, 1.5 µg MCU plasmid and 1.5 µg EMRE plasmid were co-transfected into $\sim 2 \times 10^6$ MCU/EMRE knockout cells. After transfection, the cells were cultured at 37 °C for ~48 hours and then harvested for mitochondrial Ca^{2+} uptake assays.

Mitochondrial Ca^{2+} uptake assays were performed according to published procedures [5, 16]. In brief, cells were resuspended in 1 mL of assay buffer: 125 mM KCl, 2 mM K_2HPO_4 , 1 mM MgCl_2 , 10 µM EGTA, 5 mM malate, 5 mM glutamate, 5 mM succinate, and 20 mM HEPES, pH 7.4, then loaded into a stirred quartz cuvette in a Hitachi F-2500 spectrophotometer (excitation: 506 nm, slit length: 3 nm, emission: 531 nm, slit length: 3 nm, sampling frequency: 2 Hz), with the temperature maintained at 22 °C. Reagents were added into the cuvette in the following order: 1 µM cell-impermeable Calcium Green-5N (Life Technologies, from a 1 mM stock solution in water), 0.005% (w/v) digitonin (Sigma-Aldrich, from a 0.5% stock solution in water), 5 µM CaCl_2 , and either 1 µM Ru-red (Sigma-

Aldrich, from a 0.2 mM stock solution in water), or 10 μ M ETH129 (Calcium ionophore II, Sigma-Aldrich, from a 2 mM stock solution in DMSO).

Ca²⁺ uptake assay using purified components.

Purified *Tc*MCU_{NTD} and *Tc*MCU-EMRE proteins were incorporated into lipid vesicles in the following manner. All lipids were obtained from Avanti Lipids. A lipid mixture containing 15 mg/ml POPE, 5 mg/ml POPG, with or without 1.5 mg/ml cardiolipin was prepared in water and solubilized using 8% (w/vol) n-octyl- β -D-maltopyranoside (Anatrace). Protein that had been purified by SEC in 20 mM HEPES pH 7.5, 150 mM NaCl and 3 mM n-Decyl- β -D-Maltopyranoside was mixed, in equal volume, with the solubilized lipids to obtain a final protein concentration of 0.1 mg/ml and a lipid concentration of 10 mg/ml. Detergent was removed by dialysis (8 kDa molecular weight cutoff) at 4 °C for 4 days against a reconstitution buffer containing 10 mM HEPES pH 7.5, 150 mM potassium phosphate (buffered at pH 7.5 by mixing mono and dibasic potassium phosphate) and 20 μ M ethylene glycol tetraacetic acid (EGTA), with daily buffer exchanges, using a total volume of 16 L of reconstitution buffer. The reconstituted sample was sonicated (~30 s), aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C until use. Liposomes without protein ("empty vesicles") were prepared in the same manner but without protein. For control experiments in Figure 2B using chloride, the 150 mM KCl was used in place of potassium phosphate. For SEC analysis of proteoliposomes (Supplementary Figure 1C), 50 μ l of proteoliposomes were thawed, combined with 200 μ l of solubilization buffer (20 mM HEPES pH 7.5, 150 mM NaCl and 20 mM DDM), agitated for 30 min at 4 °C, and run on SEC (in buffer 20 mM HEPES pH 7.5, 150 mM NaCl and 1 mM DDM).

For flux assay measurements, vesicles were thawed rapidly (using a water bath at 37 °C), sonicated for 5 s, and incubated at room temperature for 2-4 hours before use. 10 μ l of vesicles were added into 1 ml of flux assay buffer: 200 mM n-methyl-d-glucamine (NMDG), 10 mM HEPES, 1 μ M EGTA, pH 7.5 (pH adjusted with NaOH), 0.5 mg/mL bovine serum albumin (Sigma) and 1 μ M Calcium Green-5N hexapotassium salt (Life Technologies; from a 500 μ M stock solution in water). For experiments with Ru-red, 2 μ M Ru-red (Sigma; from a 200 μ M stock in water) was included in the flux assay buffer. Fluorescence data were collected every 20 sec over the span of the experiment using a SpectraMax M5 fluorometer (Molecular Devices; excitation and emission set to 506 nm and 531 nm, respectively) and Softmax Pro 5 software. The potassium ionophore valinomycin (Sigma; 20 nM final concentration from a 20 μ M stock in DMSO) was added at the indicated time and the sample was mixed briefly by pipette. CaCl₂ (30 μ M final concentration from 10 mM stock solution in water) was added at the indicated time sec and the sample was mixed briefly by pipette. For experiments using empty vesicles (Figure 2B), 10 μ M ETH129 (Calcium ionophore II, Sigma-Aldrich, from a 2 mM stock solution in DMSO) was added at the indicated time.

The atomic coordinates and cryo-EM map have been deposited in the Protein Data Bank and the EMDB with accession codes 6X4S and EMD-22042, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank R.K. Hite, C.D. Lima, members of the Long laboratory (B.D. Delgado, T. Benz, and A. Jacewicz), and N. Paknejad for discussions. We thank the staff of the New York Structural Biology Center Simons Electron Microscopy Center, M. Ebrahim, and M.J. de la Cruz of the Memorial Sloan Kettering Cancer Center Cryo-EM facility for help with data collection. We thank J. Goldberg for spectrophotometer use. This work was supported, in part, by an NIH core facilities grant to MSKCC (P30CA008748) and by NIH grant R35GM131921 to S.B.L.

References

- [1]. Kamer KJ, Mootha VK. The molecular era of the mitochondrial calcium uniporter. *Nature reviews Molecular cell biology*. 2015;16:545–53. [PubMed: 26285678]
- [2]. Pathak T, Trebak M. Mitochondrial Ca²⁺ signaling. *Pharmacology & Therapeutics*. 2018;192:112–23. [PubMed: 30036491]
- [3]. Gunter KK, Gunter TE. Transport of calcium by mitochondria. *J Bioenerg Biomembr*. 1994;26:471–85. [PubMed: 7896763]
- [4]. Sancak Y, Markhard AL, Kitami T, Kovács-Bogdan E, Kamer KJ, Udeshi ND, et al. EMRE is an essential component of the mitochondrial calcium uniporter complex. *Science (New York, NY)*. 2013;342:1379–82.
- [5]. Kovács-Bogdan E, Sancak Y, Kamer KJ, Plovanich M, Jambhekar A, Huber RJ, et al. Reconstitution of the mitochondrial calcium uniporter in yeast. *Proceedings of the National Academy of Sciences*. 2014;111:8985–90.
- [6]. Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, et al. MICU1 encodes a mitochondrial EF hand protein required for Ca(2+) uptake. *Nature*. 2010;467:291–6. [PubMed: 20693986]
- [7]. Plovanich M, Bogorad RL, Sancak Y, Kamer KJ, Strittmatter L, Li AA, et al. MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling. *PloS one*. 2013;8:e55785. [PubMed: 23409044]
- [8]. Patron M, Granatiero V, Espino J, Rizzuto R, De Stefani D. MICU3 is a tissue-specific enhancer of mitochondrial calcium uptake. *Cell Death Differ*. 2019;26:179–95. [PubMed: 29725115]
- [9]. Mallilankaraman K, Cardenas C, Doonan PJ, Chandramoorthy HC, Irrinki KM, Golenar T, et al. MCUR1 is an essential component of mitochondrial Ca²⁺ uptake that regulates cellular metabolism. *Nat Cell Biol*. 2012;14:1336–43. [PubMed: 23178883]
- [10]. Vais H, Tanis JE, Muller M, Payne R, Mallilankaraman K, Foskett JK. MCUR1, CCDC90A, Is a Regulator of the Mitochondrial Calcium Uniporter. *Cell Metab*. 2015;22:533–5. [PubMed: 26445506]
- [11]. Chaudhuri D, Artiga DJ, Abiria SA, Clapham DE. Mitochondrial calcium uniporter regulator 1 (MCUR1) regulates the calcium threshold for the mitochondrial permeability transition. *Proceedings of the National Academy of Sciences*. 2016;113:E1872–80.
- [12]. Raffaello A, De Stefani D, Sabbadin D, Teardo E, Merli G, Picard A, et al. The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. *The EMBO Journal*. 2013;32:2362–76. [PubMed: 23900286]
- [13]. Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, et al. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature*. 2011;476:341–5. [PubMed: 21685886]
- [14]. De Stefani D, Raffaello A, Teardo E, Szabò I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature*. 2011;476:336–40. [PubMed: 21685888]
- [15]. Chaudhuri D, Sancak Y, Mootha VK, Clapham DE. MCU encodes the pore conducting mitochondrial calcium currents. *eLife*. 2013;2:e00704. [PubMed: 23755363]

- [16]. Baradaran R, Wang C, Siliciano AF, Long SB. Cryo-EM structures of fungal and metazoan mitochondrial calcium uniporters. *Nature*. 2018;559:580–4. [PubMed: 29995857]
- [17]. Nguyen NX, Armache JP, Lee C, Yang Y, Zeng W, Mootha VK, et al. Cryo-EM structure of a fungal mitochondrial calcium uniporter. *Nature*. 2018;559:570–4. [PubMed: 29995855]
- [18]. Yoo J, Wu M, Yin Y, Herzik MA, Jr., Lander GC, Lee SY. Cryo-EM structure of a mitochondrial calcium uniporter. *Science*. 2018;361:506–11. [PubMed: 29954988]
- [19]. Fan C, Fan M, Orlando BJ, Fastman NM, Zhang J, Xu Y, et al. X-ray and cryo-EM structures of the mitochondrial calcium uniporter. *Nature*. 2018;559:575–9. [PubMed: 29995856]
- [20]. Tsai M-F, Phillips CB, Ranaghan M, Tsai C-W, Wu Y, Williams C, et al. Dual functions of a small regulatory subunit in the mitochondrial calcium uniporter complex. *eLife*. 2016;5.
- [21]. Wang Y, Nguyen NX, She J, Zeng W, Yang Y, Bai XC, et al. Structural Mechanism of EMRE-Dependent Gating of the Human Mitochondrial Calcium Uniporter. *Cell*. 2019;177:1252–61 e13. [PubMed: 31080062]
- [22]. Payne R, Li C, Foscett JK. Variable Assembly of EMRE and MCU Creates Functional Channels with Distinct Gatekeeping Profiles. *iScience*. 2020;23:101037. [PubMed: 32315830]
- [23]. Bick AG, Calvo SE, Mootha VK. Evolutionary diversity of the mitochondrial calcium uniporter. *Science (New York, NY)*. 2012;336:886.
- [24]. Zhuo W, Zhou H, Guo R, Yi J, Yu L, Sui Y, et al. Structure of intact human MCU supercomplex with the auxiliary MICU subunits. *bioRxiv*. 2020.
- [25]. Lee Y, Min CK, Kim TG, Song HK, Lim Y, Kim D, et al. Structure and function of the N-terminal domain of the human mitochondrial calcium uniporter. *EMBO reports*. 2015.
- [26]. Oxenoid K, Dong Y, Cao C, Cui T, Sancak Y, Markhard AL, et al. Architecture of the mitochondrial calcium uniporter. *Nature*. 2016;533:269–73. [PubMed: 27135929]
- [27]. Goehring A, Lee C-H, Wang KH, Michel JC, Claxton DP, Bacongus I, et al. Screening and large-scale expression of membrane proteins in mammalian cells for structural studies. *Nature protocols*. 2014;9:2574–85. [PubMed: 25299155]
- [28]. Daum G Lipids of mitochondria. *Biochim Biophys Acta*. 1985;822:1–42. [PubMed: 2408671]
- [29]. Lehninger AL, Carafoli E, Rossi CS. Energy-linked ion movements in mitochondrial systems. *Adv Enzymol Relat Areas Mol Biol*. 1967;29:259–320. [PubMed: 4881885]
- [30]. Hille B Ionic channels of excitable membranes. 2nd ed. Sunderland, Mass: Sinauer Associates; 1992.
- [31]. Prestipino G, Falugi C, Falchetto R, Gazzotti P. The ionophore ETH 129 as Ca²⁺ translocator in artificial and natural membranes. *Anal Biochem*. 1993;210:119–22. [PubMed: 8489006]
- [32]. Miller C Ion channel reconstitution. New York: Plenum Press; 1986.
- [33]. Lee C, Guo J, Zeng W, Kim S, She J, Cang C, et al. The lysosomal potassium channel TMEM175 adopts a novel tetrameric architecture. *Nature*. 2017;547:472–5. [PubMed: 28723891]
- [34]. Deluca HF, Engstrom GW. Calcium uptake by rat kidney mitochondria. *Proc Natl Acad Sci U S A*. 1961;47:1744–50. [PubMed: 13885269]
- [35]. Ying WL, Emerson J, Clarke MJ, Sanadi DR. Inhibition of mitochondrial calcium ion transport by an oxo-bridged dinuclear ruthenium ammine complex. *Biochemistry*. 1991;30:4949–52. [PubMed: 2036363]
- [36]. Kirichok Y, Krapivinsky G, Clapham DE. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature*. 2004;427:360–4. [PubMed: 14737170]
- [37]. Russ WP, Engelman DM. The GxxxG motif: a framework for transmembrane helix-helix association. *Journal of Molecular Biology*. 2000;296:911–9. [PubMed: 10677291]
- [38]. Ghosh S, Basu Ball W, Madaris TR, Srikantan S, Madesh M, Mootha VK, et al. An essential role for cardiolipin in the stability and function of the mitochondrial calcium uniporter. *Proc Natl Acad Sci U S A*. 2020;117:16383–90. [PubMed: 32601238]
- [39]. Brohawn SG, Campbell EB, MacKinnon R. Physical mechanism for gating and mechanosensitivity of the human TRAAK K⁺ channel. *Nature*. 2014;516:126–30. [PubMed: 25471887]
- [40]. Sather WA, McCleskey EW. PERMEATION AND SELECTIVITY IN CALCIUM CHANNELS. *Annual review of physiology*. 2003;65:133–59.

- [41]. Hogan PG, Lewis RS, Rao A. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annual review of immunology*. 2010;28:491–533.
- [42]. Hou X, Pedi L, Diver MM, Long SB. Crystal structure of the calcium release-activated calcium channel Orai. *Science (New York, NY)*. 2012;338:1308–13.
- [43]. McNally BA, Somasundaram A, Yamashita M, Prakriya M. Gated regulation of CRAC channel ion selectivity by STIM1. *Nature*. 2012;482:241–5. [PubMed: 22278058]
- [44]. Gudlur A, Quintana A, Zhou Y, Hirve N, Mahapatra S, Hogan PG. STIM1 triggers a gating rearrangement at the extracellular mouth of the ORAI1 channel. *Nature communications*. 2014;5:5164.
- [45]. Hou X, Burstein SR, Long SB. Structures reveal opening of the store-operated calcium channel Orai. *Elife*. 2018;7.
- [46]. MacEwen MJS, Markhard AL, Bozbeyoglu M, Bradford F, Goldberger O, Mootha VK, et al. Molecular basis of EMRE-dependence of the human mitochondrial calcium uniporter. *bioRxiv*. 2019.
- [47]. Jiang Y, Lee A, Chen J, Cadene M, Chait BT, MacKinnon R. The open pore conformation of potassium channels. *Nature*. 2002;417:523–6. [PubMed: 12037560]
- [48]. Miller AN, Vaisey G, Long SB. Molecular mechanisms of gating in the calcium-activated chloride channel bestrophin. *Elife*. 2019;8.
- [49]. Patron M, Checchetto V, Raffaello A, Teardo E, Vecellio Reane D, Mantoan M, et al. MICU1 and MICU2 finely tune the mitochondrial Ca²⁺ uniporter by exerting opposite effects on MCU activity. *Molecular cell*. 2014;53:726–37. [PubMed: 24560927]
- [50]. Ashrafi G, de Juan-Sanz J, Farrell RJ, Ryan TA. Molecular Tuning of the Axonal Mitochondrial Ca(2+) Uniporter Ensures Metabolic Flexibility of Neurotransmission. *Neuron*. 2019.
- [51]. Payne R, Hoff H, Roskowski A, Foskett JK. MICU2 Restricts Spatial Crosstalk between InsP3R and MCU Channels by Regulating Threshold and Gain of MICU1-Mediated Inhibition and Activation of MCU. *Cell Rep*. 2017;21:3141–54. [PubMed: 29241542]
- [52]. Kamer KJ, Mootha VK. MICU1 and MICU2 play nonredundant roles in the regulation of the mitochondrial calcium uniporter. *EMBO reports*. 2014;15:299–307. [PubMed: 24503055]
- [53]. Kamer KJ, Grabarek Z, Mootha VK. High-affinity cooperative Ca(2+) binding by MICU1-MICU2 serves as an on-off switch for the uniporter. *EMBO Rep*. 2017;18:1397–411. [PubMed: 28615291]
- [54]. Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng Y, Agard DA. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods*. 2017;14:331–2. [PubMed: 28250466]
- [55]. Rohou A, Grigorieff N. CTFFIND4: Fast and accurate defocus estimation from electron micrographs. *J Struct Biol*. 2015;192:216–21. [PubMed: 26278980]
- [56]. Zivanov J, Nakane T, Forsberg BO, Kimanius D, Hagen WJ, Lindahl E, et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife*. 2018;7.
- [57]. Punjani A, Rubinstein JL, Fleet DJ, Brubaker MA. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods*. 2017;14:290–6. [PubMed: 28165473]
- [58]. Coot: Beyond the Basics: Tools for Low Resolution and EM Map Fitting. 2014:1–94.
- [59]. Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Cryst*. 2010;D66:213–21.
- [60]. DeLano W Pymol reference guide. Pymol reference guide. 2009:1–4.
- [61]. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25:1605–12. [PubMed: 15264254]
- [62]. Goddard TD, Huang CC, Meng EC, Pettersen EF, Couch GS, Morris JH, et al. UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci*. 2018;27:14–25. [PubMed: 28710774]

- [63]. Smart OS, Neduvelil JG, Wang X, Wallace BA, Sansom MS. HOLE: a program for the analysis of the pore dimensions of ion channel structural models. *J Mol Graph*. 1996;14:354–60, 76. [PubMed: 9195488]
- [64]. Kawate T, Gouaux E. Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins. *Structure (London, England : 1993)*. 2006;14:673–81.

Highlights

- MCU and EMRE are components of the mitochondrial Ca^{2+} uniporter ion channel.
- Using a novel assay, we interrogate Ca^{2+} uptake by purified MCU-EMRE.
- A cryo-EM structure of the MCU-EMRE complex is determined at 3.5 Å resolution.
- The structure has noteworthy differences from a human MCU-EMRE structure.
- The structures may represent alternate conformations.

Figure 1.

*Tc*MCU-EMRE catalyzes Ca^{2+} uptake into human mitochondria. **(a)** Schematic showing the *Tc*MCU-EMRE construct. Human amino acid numbering is shown, with *Tc* numbering in parentheses. **(b)** Mitochondrial Ca^{2+} uptake experiments for digitonin-permeabilized MCU/EMRE double knockout cells expressing various constructs (as indicated). A decrease in fluorescence is indicative of Ca^{2+} uptake. Fluorescence data are normalized to the value after an addition of Ca^{2+} to the bath solution at time = 0 sec, which corresponds to time = 230 sec in the full trace shown in Supplementary Figure 1B.

Figure 2.

Proteoliposome-based Ca^{2+} uptake assay. **(a)** Assay schematic. Vesicles containing purified protein, or those prepared without protein (empty vesicles) were loaded with 150 mM potassium phosphate and diluted 100-fold into a bath solution containing a fluorescent Ca^{2+} indicator (Calcium Green-5N) and 200 mM n-methyl-d-glucamine (NMDG) to establish a K^{+} gradient. A K^{+} ionophore (valinomycin) was added to allow K^{+} efflux and to generate negative electric potential on the inside of the vesicles. The fluorescence of Calcium Green-5N, which is membrane impermeant, is used to monitor $[\text{Ca}^{2+}]_{\text{bath}}$. The addition of $30 \mu\text{M}$ Ca^{2+} (as CaCl_2) causes a rapid rise in fluorescence. Uptake of Ca^{2+} by the vesicles is detected as a subsequent decrease in fluorescence. **(b)** Internal phosphate is necessary for robust Ca^{2+} uptake in a model system. Here, using vesicles without protein we tested the effects of phosphate and chloride as the anionic species within liposomes. $30 \mu\text{M}$ Ca^{2+} , valinomycin, and the Ca^{2+} ionophore ETH129 were added at the indicated times. Liposomes containing phosphate took up markedly more Ca^{2+} from the bath solution than those containing chloride, as evidenced by the decrease in fluorescence following the addition of ETH129. **(c)** Fluorescence measurements show robust Ca^{2+} uptake by purified $TcMCU$ -EMRE into liposomes. Uptake is inhibited by Ru-Red. Measurements were also made for purified $TcMCU_{\text{NTD}}$ alone (the analogous construct without EMRE) and for $TcMCU$ -EMRE that has been reconstituted into liposomes without cardiolipin. In (b) and (c) fluorescence data were normalized by dividing by the maximum value after the addition of Ca^{2+} , where fluorescence readings are within $\pm 10\%$ for each experiment. Analogous uptake measurements for the $TcMCU$ -EMRE_{EM2} construct are shown in Supplementary Figure 1.

Figure 3.

Overall structure of the *Tc*MCU-EMRE complex. (a) Cryo-EM map of *Tc*MCU-EMRE in nanodiscs. MCU subunits are in colors, EMRE is gray, and nanodisc density is transparent. (b) Cartoon representation of the complex, colored as in (a). Two orthogonal orientations are shown, from the side. Approximate boundaries of the inner mitochondrial membrane are shown as gray bars. (c) Superposition of protomers “A” and “B” (red and blue, respectively).

Figure 4.

The TMD and selectivity filter. **(a)** Ion pore. Within a ribbon representation of two subunits is a representation (gray surface) of the minimal radial distance from the center of the pore to the nearest van der Waals protein contact. EMRE helices are red, TM1 helices are gray, and TM2 helices are yellow. Pore-lining amino acids on TM2 are shown as sticks. Asp 261 and Glu 264 are labeled “D” and “E”, respectively. A calcium ion in the filter is shown as a green sphere. **(b)** Selectivity filter. A side view is shown with two subunits depicted as ribbons and the WDXEP signature sequence as sticks. **(c)** Top view of the filter showing MCU and EMRE subunits. The EMRE subunits contact the TM1 helices almost exclusively and are located at the periphery of the TMD where they do not contribute to the pore.

Figure 5.

Comparison of *Tc*MCU-EMRE and hMCU-EMRE structures within the TMD. **(a-b)** Surface representations of the TMDs from the *Tc*MCU-EMRE and hMCU-EMRE (PDB: 6O58) structures, drawn on the same scale). For each channel, the two subunits nearest the viewer are shown. The molecular surfaces are semi-transparent. EMRE helices are red, TM1 helices are gray, and TM2 helices are yellow. Fenestrations (membrane openings) in the molecular surfaces within the matrix leaflet of the membrane are denoted. **(c)** Superposition of *Tc*MCU-EMRE (in color: EMRE, red; TM1, pale blue; TM2, yellow) and the hMCU-EMRE structure (gray; PDB: 6O58), shown in two orientations. Ca^{2+} is shown as a green sphere. A double-ended arrow highlights the greater separation between TM1 and TM2 in the hMCU-EMRE structure.

Figure 6.

EMRE-MCU interface. **(a)** EMRE residues. The amino acids of EMRE are shown as sticks on a cartoon representation of its transmembrane helix on the subunit closest to the viewer. EMRE is red; MCU is gray (TM1) and yellow (TM2). A hypothetical location of the acidic C-terminal tail of EMRE is sketched as a dashed line. **(b-c)** The EMRE-MCU interface. Residues in contact are depicted as sticks and labeled. Glycine residues are drawn as spheres.

Figure 7. Comparison of *Tc*MCU-EMRE and hMCU-EMRE structures, highlighting differences within the matrix. **(a-b)** Structure of *Tc*MCU-EMRE; shown in ribbon representation from two perspectives and colored as indicated. In two of the subunits, denoted by subscript “(A)”, the JML region (magenta, boxed) forms part of a continuous helix connecting TM2_(A) and CC2a_(A). In the other two subunits, denoted by subscript “(B)”, most of the JML region is disordered and CC2a_(B) lies at the bottom of the matrix vestibule. A dashed line indicates the disordered region between JML_(B) and CC2a_(B). **(c)** Orthogonal view of *Tc*MCU-EMRE showing the matrix end of the pore. The perspective is from within the matrix and depicts a slab of approximately 25 Å. *Tc*MCU-EMRE is colored and depicted as in (a-b). Amino acids on the JML are drawn as sticks (magenta). **(d)** Structure of hMCU-EMRE (PDB: 6O58), shown and colored as for *Tc*MCU-EMRE in (a-b). One channel from the dimeric channel assembly is depicted. The NTD, which is located below the LHD, has been removed for clarity. **(e)** Orthogonal view of hMCU-EMRE showing the matrix end of the pore and the constriction formed by the JMLs (magenta, stick representation). The view is analogous to that for *Tc*MCU-EMRE in (c). **(f)** Dimensions along the pore in the structures of *Tc*MCU-EMRE and hMCU-EMRE, calculated with HOLE [63].

Table 1.

Data collection, refinement, and validation statistics.

	<i>Tc</i> MCU-EMRE	<i>Tc</i> MCU-EMRE _{EM2}
Data collection and processing		
Microscope	FEI Titan Krios (at MSKCC)	FEI Titan Krios (at MSKCC)
Camera	Gatan K2 Summit	Gatan K2 Summit
Magnification	22,500×	22,500×
Voltage (kV)	300	300
Electron exposure (e ⁻ /Å ²)	76	76
Defocus range (μm)	−1.0 ~ −3.0	−1.0 ~ −3.0
Pixel size (Å)	1.088 (0.544) [*]	1.088 (0.544) [*]
Software	RELION 3.0, cryoSPARC v2	RELION 3.0, cryoSPARC v2
Symmetry imposed	C2	C2
Initial particle images (no.)	4,460,801	353,139
Final particle images (no.)	52,493	34,326
Overall map resolution (Å)	3.5	5.0
FSC threshold 0.143		
Local map resolution range (Å)	3.3-5.0	N/A
Refinement		
Software	Phenix 1.13 real-space-refine	
Initial model used (PDB code)	N/A	
Model resolution (Å)	3.9	
FSC threshold 0.5		
Map sharpening <i>B</i> factor (Å ²)	−80	−244
Model composition		
Non-hydrogen atoms	5,373	
Protein residues	706	
Ligands	1 (Calcium)	
Water	0	
<i>B</i> factors (Å²)		
Protein	127.3	
Ligand	88.4	
R.m.s. deviations		
Bond lengths (Å)	0.007	
Bond angles (°)	0.76	
Validation		
MolProbity score	1.6	
Clashscore	10.9	
Poor rotamers (%)	0.97	
Ramachandran plot		
Favored (%)	98.1	
Allowed (%)	1.9	

	<i>Tc</i> MCU-EMRE	<i>Tc</i> MCU-EMRE _{EM2}
Disallowed (%)	0.0	

* Super-resolution pixel size.