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Mitochondrial Ca^{2+} homeostasis in trypanosomes

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Abstract

Mitochondrial calcium ion (Ca^{2+}) uptake is important for buffering cytosolic Ca^{2+} levels, for regulating cell bioenergetics, and for cell death and autophagy. Ca^{2+} uptake is mediated by a mitochondrial Ca^{2+} uniporter (MCU) and the discovery of this channel in trypanosomes has been critical for the identification of the molecular nature of the channel in all eukaryotes. However, the trypanosome uniporter, which has been studied in detail in *Trypanosoma cruzi*, the agent of Chagas disease, and *T. brucei*, the agent of human and animal African trypanosomiasis, has lineage-specific adaptations which include the lack of some homologues to mammalian subunits, the presence of unique subunits, and different functional roles of the common subunits. Here, we review newly emerging insights into the role of mitochondrial Ca^{2+} homeostasis in trypanosomes, the composition of the uniporter, its functional characterization, and its role in general physiology.

Keywords

Acidocalcisome; calcium; cell bioenergetics; inositol phosphate; mitochondria; mitochondrial calcium uniporter; polyphosphate; trypanosomatids

1. INTRODUCTION

Trypanosomatids are the etiologic agents of several neglected tropical diseases that cause significant morbidity and mortality in millions of people and animals in the world. *Trypanosoma cruzi* is the agent of Chagas disease, which is endemic in the Americas. The *Trypanosoma brucei* group causes human African trypanosomiasis or sleeping sickness, and nagana in cattle, and is endemic in Sub-Saharan Africa. *Leishmania* spp. cause cutaneous, mucocutaneous and visceral leishmaniasis in several continents. *T. cruzi* has four main life cycle stages, epimastigotes and metacyclic trypomastigotes in the triatomine vector, and bloodstream trypomastigote and intracellular amastigote in the mammalian host. *T. brucei* has two best studied stages, procyclic trypomastigotes in the *tsetse* fly vector and bloodstream trypomastigotes in the mammalian host. *Leishmania* spp. has two well-studied forms, the promastigote in the sand fly vector and the intracellular amastigote in the mammalian host.

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Trypanosomatids belong to the eukaryotic supergroup Excavata, which is distantly related to the supergroup Opisthokonta, which includes animals and fungi (Adl et al., 2012). However, like animals and fungi, trypanosomatids possess mitochondria, although with lineage-specific adaptations. They have only one mitochondrion per cell that is characterized for the presence of the kinetoplast. The kinetoplast is formed by thousands of concatenated DNA minicircles and a few DNA maxicircles encoding a few mitochondrial proteins and rRNA (Shlomai, 2004). Several mitochondrial mRNA are edited by a complex mechanism, first discovered in these cells (Benne et al., 1986). As the mitochondrial genome does not possess tRNA genes, these molecules have to be imported into the mitochondrion (Seidman et al., 2012). Some respiratory complexes are incomplete (Surve et al., 2012; van Hellemond et al., 2005), or absent in some stages of these parasites, like in the bloodstream form of *T. brucei*, which possesses an alternative oxidase (Clarkson et al., 1989) and also lacks the tricarboxylic acid cycle.

Despite these distinct characteristics, mitochondrial Ca^{2+} homeostasis mechanisms are conserved in trypanosomatids and will be the subject of this review.

2. THE MITOCHONDRIAL Ca^{2+} UNIPORTER: DISCOVERY

The mitochondrial Ca^{2+} uniporter (MCU) was discovered almost 60 years ago in rat kidney mitochondria (Deluca, Engstrom, 1961; Vasington, Murphy, 1962). Ca^{2+} transport was found to be energized by coupled respiration, blocked by respiratory chain inhibitors and oxidative phosphorylation uncouplers, and resulted in large amounts of Ca^{2+} taken up (Vasington, Murphy, 1962). Further work revealed that the process does not require ATP, except when the respiratory chain is inhibited and, in this case, it is sensitive to oligomycin because it is driven by the ATP synthase working in reverse as an ATPase (Lehninger et al., 1963). Phosphate is also needed for Ca^{2+} uptake (Lehninger et al., 1963) and the uniporter is inhibited by ruthenium red (Moore, 1971), or its derivative, Ru360 (Ying et al., 1991). The uniporter is a Ca^{2+}-selective channel (Kirichok et al., 2004), although other cations, such as Mn^{2+} (Bartley, Amoore, 1958) and Sr^{2+} (Greenawalt, Carafoli, 1966) can also be taken up.

The finding that MCU was absent in *Saccharomyces cerevisiae* mitochondria (Carafoli et al., 1970) led to the proposal that this process was absent in non-animal species (Carafoli, Lehninger, 1971; McCormack, 1986). However, functional evidence of a mitochondrial Ca^{2+} uniporter with similar properties to the animal MCU was found in *T. cruzi* (Docampo, Vercesi, 1989a, b). *T. cruzi* mitochondrial Ca^{2+} transport has all the characteristics of the animal MCU: it is electrogenic, has high capacity and low affinity for Ca^{2+}, and is inhibited by ruthenium red (Docampo, Vercesi, 1989a, b).

Other trypanosomatids were later shown to possess an MCU, like several *Leishmania* spp. (Benaim et al., 1990; Vercesi, Docampo, 1992; Vercesi et al., 1990), and *T. brucei* (Moreno et al., 1992; Vercesi et al., 1992; Vercesi et al., 1993; Xiong et al., 1997). Even the mitochondria of the bloodstream form of *T. brucei*, which lacks a respiratory chain and oxidative phosphorylation is able to transport Ca^{2+}, but in this case using the electrochemical gradient generated by the ATP synthase working in reverse, as an ATPase. This Ca^{2+} transport is inhibited by oligomycin (Vercesi et al., 1992).

Interestingly, the lack of MCU in yeast (Carafoli et al., 1970) and its presence in trypanosomes (Docampo, Vercesi, 1989a) together with the availability of several eukaryotic genomes, led to the identification, first of the gene encoding a modulator of MCU in animals, the mitochondrial calcium uptake 1 (MICU1) (Perocchi et al., 2010), and then of the gene encoding the MCU pore subunit (Baughman et al., 2011; De Stefani et al., 2011; Docampo, Lukes, 2012).

3. THE MITOCHONDRIAL Ca^{2+} UNIPORTER OF TRYPANOSOMES: THE PORE SUBUNITS

Following the discovery of the molecular nature of the MCU pore subunit, other components of the MCU complex (known as uniplex or holocomplex) were described in mammals, such as MCU regulator 1 (MCUR1) (Mallilankaraman et al., 2012a), MICU2 and MICU3 (Plovanich et al., 2013), MCUb (Raffaello et al., 2013), and essential MCU regulator or EMRE (Sancak et al., 2013).

The trypanosomatid MCU complex differs from the mammalian one in several aspects. First, some subunits, like MCUR1, MICU3, and EMRE, are absent (Docampo et al., 2014). Second, additional subunits, named MCUc, and MCUd form part of the complex and together with the MCU and MCUb subunits, have Ca^{2+}-transporting roles (Chiurillo et al., 2019; Huang, Docampo, 2018).

The MCU subunit was the first MCU complex component described in trypanosomes (Huang et al., 2013b). The gene is single copy in trypanosomes and the encoded protein in *T. brucei* (TbMCU) has only 20% identity and 33% similarity with the human MCU. In contrast, the orthologues in *T. cruzi* (TcMCU) and *Leishmania major* (LmMCU) share 49 and 41% identity, respectively, with the *T. brucei* protein. All these MCU subunits have two transmembrane domains and a mitochondrial targeting signal and localize to the inner mitochondrial membrane. The processed proteins have an apparent molecular weight of ~30 kDa.

Knockdown of *TbMCU* by RNAi significantly affects the growth of procyclic (insect form) and bloodstream (mammalian form) forms, reduces mitochondrial Ca^{2+} uptake, and their ability to accumulate large Ca^{2+} quantities (Huang et al., 2013b). The mitochondrial membrane potential ($\Delta\Psi_m$) is not affected, indicating specific MCU inhibition. Procyclic forms in which *TbMCU* is downregulated by RNAi have a higher AMP/ATP ratio and increased autophagy, as revealed by the increase in the number of autophagosomes per cell and the increase in the autophagy marker Atg8.2-II, orthologue to LC3-II in mammalian cells (Huang et al., 2013b). In contrast, overexpression of *TbMCU* in procyclic forms leads to increased mitochondrial Ca^{2+} uptake and mitochondrial Ca^{2+} overload, changes that make the cells more sensitive to proapoptotic agents like C2-ceramide and H_2O_2, increases production of reactive oxygen species (ROS), and cell death (Huang et al., 2013b).

Conditional knockdown of *TbMCU* in the bloodstream stage greatly affects its growth, mitochondrial Ca^{2+} uptake, and virulence in mice, demonstrating its essentiality (Huang et al., 2013b). The bloodstream form of *T. brucei* uses glucose as the main source of energy, as

it does not have an active tricarboxylic acid cycle or respiratory chain. The end product of glycolysis is pyruvate, that is mostly excreted with protons to maintain their intracellular pH (Vanderheyden et al., 2000). However, some pyruvate is needed in the mitochondria where a pyruvate dehydrogenase (PDH) catalyzes its conversion into acetyl-CoA (Huang et al., 2013b; Zhuo et al., 2017). Acetyl-CoA is used for intramitochondrial fatty acid synthesis (FAS II) to generate lipoic acid and myristic acid (Stephens et al., 2007). Alternatively, acetyl-CoA is used to generate acetate (Van Hellemond et al., 1998) that is transferred to the cytosol where it is converted back to acetyl-CoA by acetyl-CoA synthetase (Millerioux et al., 2012) and can be used for fatty acid synthesis. Pyruvate dehydrogenase is one of the mitochondrial dehydrogenases that has been demonstrated to be regulated by Ca^{2+} (McCormack, 1986). Ca^{2+} stimulates a PDH phosphatase (PDP) that dephosphorylates the E1α subunit of PDH stimulating its activity, which explains the partial rescue of the lethal effect of *TbMCU* downregulation by addition of threonine to the culture medium (Huang et al., 2013b). Bloodstream forms have a threonine dehydrogenase (Linstead et al., 1977) able to bypass the need of a Ca^{2+}-stimulated step for generation of acetyl-CoA. More recent work demonstrated that Ca^{2+} directly stimulate the PDH phosphatase of *T. brucei* (Lander et al., 2018). Fig. 1 shows a scheme of the reactions that occur in these cells.

Screening for genes encoding TbMCU orthologs led to the identification of three putative proteins, each with two transmembrane domains (TMD) that were designated TbMCUb, TbMUCc, and TbMCUd. The open reading frames predict proteins of 254, 249, and 214 amino acids, with apparent molecular weights of 28.4, 27.8, and 24.7 kDa, respectively. TbMCUb, TbMCUc, and TbMCUd have 16 to 19% identity and 28 to 34% similarity with TbMCU and, in addition to two TMD, they have a modified putative Ca^{2+} selectivity filter (WDXXEPXXY) and belong to the MCU family (Pfam: PF04678) (Huang, Docampo, 2018).

The *T. cruzi* ortholog *TcMCUb* was studied first, together with *TcMCU* (Chiurillo et al., 2017). Both genes were knocked out using the CRISPR/Cas9 system (Lander et al., 2015) and the mutants lost their mitochondrial capacity to take up Ca^{2+} without any alteration in their mitochondrial membrane potential. Complementation of *TcMCU*-KO cells with an exogenous *TcMCU* gene, but not with the human orthologue (*HsMCU*) or a *TcMCU* gene mutated in the critical Asp (D) and Glu (E) amino acids of the putative pore region, was able to restore mitochondrial Ca^{2+} uptake.

Overexpression of both genes (*TcMCU*-OE and *TcMUCb*-OE) led to increased mitochondrial Ca^{2+} uptake, also without alterations in $\Delta\Psi_m$. The results suggest that both proteins are Ca^{2+} transporting subunits. This is in contrast with the mammalian ortholog of *TcMCUb*, which encodes a dominant negative subunit that inhibits mitochondrial Ca^{2+} transport when overexpressed (Raffaello et al., 2013). Both proteins, TcMCU and TcMCUb, co-immunoprecipitate indicating that they form oligomers. Growth of both mutants was affected by varying degrees. Growth was slightly slower in *TcMCU*-KO epimastigotes (vector form), especially in a glucose-deficient medium, but recovered during stationary phase, suggesting an alternative source of energy. In agreement with these results, *TcMCU*-KO epimastigotes have more lipid droplets, which suggests that they might use fatty acids as alternative source of energy in the stationary phase. These mutants have higher ability

to differentiate into the infective forms (metacyclic trypomastigotes) and trypomastigotes were able to infect host cells and replicate normally as intracellular amastigotes. In contrast, *TcMCUb*-KO epimastigotes were difficult to differentiate to infective metacyclic trypomastigotes and it was not possible to obtain infected host cells, indicating the relevance of this subunit for infection. Concurrently with the higher mitochondrial Ca^{2+} uptake and overload, *TcMCU*-OE and *TcMCUb*-OE also caused oxidative stress. On the other hand, *TcMCUb*-KO, but not *TcMCU*-KO epimastigotes, had decreased respiratory rate, lower mitochondrial mass, and increased autophagy, suggesting a better adaptation of *TcMCU*-KO parasites to the lower mitochondrial ability to take up Ca^{2+} (Chiurillo et al., 2017).

The two other subunits identified in the trypanosome proteome, MCUc and MCUd, have only orthologues in trypanosomatids (Chiurillo et al., 2019; Huang, Docampo, 2018) (Fig. 2). Knockdown of all *T. brucei* proteins (TbMCU, TbMCUb, TbMCUc, and TbMCUd) by RNAi decreased mitochondrial Ca^{2+} uptake without affecting $\Delta\Psi_m$ (Huang, Docampo, 2018) and their overexpression enhanced Ca^{2+} uptake. Therefore, TbMCUb is not a dominant negative subunit as it occurs with the animal MCUb (Raffaello et al., 2013). In addition, knockout of each of the *T. cruzi* subunits by CRISPR/Cas9 suppressed, while their overexpression increased, mitochondrial Ca^{2+} uptake (Chiurillo et al., 2019). Taken together the results indicate that MCU, MCUb, MCUc, and MCUd are all Ca^{2+}-transporting subunits. All the *T. brucei* subunits co-immunoprecipitate and exist in a large protein complex with a net molecular weight of ~380 kDa, suggesting that the complex is a hetero-oligomer (Huang, Docampo, 2018). Further evidence of its hetero-oligomeric structure was provided using the split-ubiquitin membrane-based yeast two hybrid (MYTH) and by co-immunoprecipitation assays. Combining mutagenesis analysis with MYTH assays determined that the transmembrane helices (TMH) of the subunits are involved in these interactions and that the subunits form a hetero-hexamer (Fig. 3A). Mutagenesis of TM helix 1 (TMH1) and especially 2 (TMH2) of each of the four subunits of the complex showed that they are required for their interactions (Huang, Docampo, 2018). These results are apparently different from those reported in animals (Baradaran et al., 2018) and fungi (Baradaran et al., 2018; Fan et al., 2018; Nguyen et al., 2018; Yoo et al., 2018) in which structural studies proposed that the MCU subunit forms homo-tetramers. However, these studies were done using recombinant MCU and how MCU interacts with its membrane partners, like MCUb, and which is their oligomeric structure in vivo remains to be investigated.

Knockdown of *TbMCUc* and *TbMCUd* reduced procyclic trypomastigotes growth in glucose-deficient media (Huang, Docampo, 2018), and increased the AMP/ATP ratio (Huang, Docampo, 2020), in agreement with the importance of mitochondrial metabolism for these stages (Lamour et al., 2005). Similar results were observed after ablation of *TcMCUc* or *TcMCUd* by CRISPR/Cas9 in epimastigotes (Chiurillo et al., 2019). *TcMCUc*-KO and *TcMCUd*-KO epimastigotes have also alterations in their respiratory rate, citrate synthase activity, and AMP/ATP ratio, but they normally differentiate into metacyclic trypomastigotes, while trypomastigotes are less infective and amastigotes have a reduced replication rate. All these results are in agreement with the relevance of mitochondrial metabolism for *T. cruzi* invasion of host cells (Schenkman et al., 1991) and for replication of intracellular amastigotes (Dumoulin, Burleigh, 2018).

Complementation of the knockout of each MCU subunit of *T. cruzi* with mutant genes in which the Glu (E) and Asp (D) of the pore motif (WDXXEPXXY) were mutated revealed that the Glu is essential and the Asp is important for Ca^{2+} uptake (Chiurillo et al., 2019) (Fig. 3B). The results are in agreement with structural studies that have shown that the selectivity filter is formed by symmetrical arrangement of WDXXEPXXY sequences in TMH2 from each monomer around the pore. The Glu residues form an acid mouth (Site 1), and the Asp residues form a second acidic ring (Site 2) (Baradaran et al., 2018). Site 2 is a high-affinity binding site for Ca^{2+} in MCU. Mutations of the Asp residues located in the filter region of TcMCU (D^{223}) or TcMCUb (D^{161}), or the Asp residues located N-terminal to the selectivity filter of TcMCU (D^{221}), TcMCUc (D^{157}) or TcMCUd (D^{141}), reduced but did not completely abolished mitochondrial Ca^{2+} transport (Chiurillo et al., 2019). In contrast, mutations of the Glu residues of each monomer suppressed mitochondrial Ca^{2+} transport. Since mitochondrial Ca^{2+} uptake in *TcMCU*-KO cells can be restored with $TcMCU^{R214W/D219V}$ mutant, these other residues are not important for Ca^{2+} transport in *T. cruzi* (Chiurillo et al., 2017) (Fig. 3B). These residues are located in TMH1 and loop region near the WDXXEPXXY motif but outside the channel entrance (Baradaran et al., 2018; Fan et al., 2018; Nguyen NX, 2018; Oxenoid et al., 2016; Yoo et al., 2018).

Complementation of *TcMCU*-KO epimastigotes by co-expression of *HsMCU* and *HsEMRE* were unsuccessful, possibly because the proteins did not insert properly in the inner membrane or with the right topology, failed to interact, or did not form part of the MCU complex (Chiurillo et al., 2019).

All *T. cruzi* MCU complex monomers lack the serine amino acid (Ser^{259} in the human MCU) (Fig. 3B) that has been proposed to be responsible for the sensitivity of the uniporter to the ruthenium red derivative Ru360 (Baughman et al., 2011). As mitochondrial Ca^{2+} transport in *T. cruzi* is sensitive to Ru360, this suggest that there is another target for Ru360 besides Ser^{259} (Chiurillo et al., 2019).

In conclusion, four pore subunits MCU, MCUb, MCUc, and MCUd form a hetero-oligomer, probably a hetero-hexamer, required for mitochondrial Ca^{2+} uptake in trypanosomes (Chiurillo et al., 2019; Huang, Docampo, 2018). The selectivity filter is formed by symmetrical arrangement of WDXXEPXXY sequences in TMH2 from each subunit around the pore. The lack of any of the subunits abolishes Ca^{2+} uptake. MCUb is not a dominant negative subunit of the uniporter as in animal cells (Chiurillo et al., 2017; Huang, Docampo, 2018). MCUc and MCUd are subunits present only in trypanosomatids (Chiurillo et al., 2019; Huang, Docampo, 2018). All subunits are required for growth under glucose-limited conditions, revealing the importance of the uniporter in mitochondrial metabolism (Chiurillo et al., 2019; Huang, Docampo, 2018). Complementation studies of all the pore subunits with mutant subunits found that the Glu and Asp of the selectivity filter are essential, and important, respectively, for Ca^{2+} uptake (Chiurillo et al., 2019). Complementation of *MCU*-KO cells with human *MCU* (Chiurillo et al., 2017) or human *MCU* and *EMRE* (Chiurillo et al., 2019) or attempts to reconstitute the uniporter in yeast transforming them with *TcMCU* (Chiurillo et al., 2017) failed, suggesting that exogenous subunits could not form part of the trypanosome uniporter and that probably all the endogenous subunits of the pore would be required for reconstitution of its function in yeast. The different function of the MCUb

subunit and the presence of two additional Ca^{2+}-transporting subunits (MCUc and MCUs) in trypanosomes reflect the parallel evolution of the uniporter in different supergroups of eukaryotes (Pittis et al., 2020).

4. THE MITOCHONDRIAL Ca^{2+} UNIPORTER OF TRYPANOSOMES: THE GATEKEEPER SUBUNITS

MICU1 was reported to act as a gatekeeper of the uniporter by inhibiting Ca^{2+} uptake at low cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), thus preventing mitochondrial Ca^{2+} overload under physiological ($[Ca^{2+}]_{cyt}$ (Csordas et al., 2013; Mallilankaraman et al., 2012b). Later work suggested that MICU2, which binds covalently to MICU1,was the most important inhibitor (Patron et al., 2014). Subsequently either MICU1 or MICU2 or both were considered more relevant gatekeepers in a variety of cells (Kamer et al., 2017; Liu et al., 2016; Matesanz-Isabel et al., 2016; Paillard et al., 2017; Payne et al., 2017).

T. cruzi and *T. brucei* possess orthologs to MICU1 and MICU2, but orthologs to MICU2 are absent in *Leishmania* spp. (Docampo et al., 2014). The *T. cruzi* proteins have been studied in more detail (Bertolini et al., 2019). TcMICU1 and TcMICU2 have estimated molecular masses of 46.7 and 53.2 kDa, respectively, have 20% identity and 38% similarity between them, possess mitochondrial targeting signals, and two canonical and two noncanonical EF-hand domains. TcMICU1 and TcMICU2 have only 22% and 23.9% overall sequence identity (44.4% and 40% of similarity), respectively, to their human orthologs (Bertolini et al., 2019).

Ablation of *TcMICU1* or *TcMICU2* reduces mitochondrial Ca^{2+} uptake and increases the Ca^{2+} concentration needed for opening the uniporter without affecting the $\Delta\Psi_m$. Interestingly, these mitochondria are less efficient in taking up Ca^{2+} across a wide range of Ca^{2+} concentrations and the threshold for Ca^{2+} uptake is elevated (Bertolini et al., 2019). These results indicate that although these proteins have a role in Ca^{2+} sensing in the intermembrane space, they have no role as gatekeepers at low Ca^{2+} concentrations, as it occurs with the mammalian proteins. Ca^{2+} transport at low Ca^{2+} concentrations is already inhibited in the absence of either protein. Both proteins are required for normal growth and respiration of epimastigotes, for normal metacyclogenesis, for trypomastigote invasion of host cells, and for intracellular replication of amastigotes, indicating their relevance for parasite survival (Bertolini et al., 2019). However, these cells do not show changes in mitochondrial mass, AMP/ATP ratio, or autophagy. The lower Ca^{2+} transport correlates with the increase in phosphorylation of the PDH, reflecting the lower stimulation of the *T. cruzi* PDH phosphatase (TcPDP) by Ca^{2+} (Bertolini et al., 2019).

Overexpression of MICU1 or MICU2 in HeLa cells increases or decreases, respectively, mitochondrial Ca^{2+} accumulation (Patron et al., 2014). However, this does not occur in *T. cruzi* where no changes are observed when TcMICU1 or TcMICU2 are overexpressed. Overexpression does not affect epimastigote growth, and these overexpressed proteins do not form covalently bound oligomeric complexes (Bertolini et al., 2019).

In conclusion, trypanosomes appear to also differ from mammalian cells in the ability of either MICU1 or MICU2 to act as gatekeepers of the uniporter at low Ca^{2+} concentrations, in their formation of covalently-bound dimers, and in changes in mitochondrial Ca^{2+} accumulation by their overexpression, although they are still able to sense changes in Ca^{2+} concentration in the mitochondrial intermembrane space and are important for parasite survival and infectivity.

5. THE MITOCHONDRIAL Ca^{2+} UNIPORTER OF TRYPANOSOMES: INTERACTION WITH THE ATP SYNTHASE

Recent work has indicated that the MCU complex of trypanosomes physically interacts with the subunit c of the ATP synthase (Huang, Docampo, 2020). Tandem affinity purification using overexpressed TbMCU, combined with mass spectrometry (MS), resulted in the identification of 19 subunits of the ATP synthase together with the voltage dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), the phosphate carrier (PiC), and the MCU complex components MCU, MCUb and MCUc. The ATP synthase, the ANT, and the PiC constitute what is known as ATP synthasome in mammals (Ko et al., 2003). Similar results were obtained by immunoprecipitation of tagged TbMCU combined with MS. When two subunits of the ATP synthase (TbATPβ, TbATPp18) were in situ tagged they were also able to immunoprecipitate TbMCU. In situ tagged ANT and PiC were not able to do the same, suggesting that TbMCU is closely associated with the ATP synthase but only loosely associated with ANT and PiC.

The split-ubiquitin membrane-based yeast two-hybrid (MYTH) assay was used to validate the interaction of TbMCU with ten of the ATP synthase subunits but only subunit c (TbATPc) was shown to specifically physically interact with TbMCU. Each TbMCU subunit, except TbMCUb, (TbMCU, TbMCUc, and TbMCUd), *T. cruzi* MCU, as well as human MCU, were also able to physically interact with the subunit c of the corresponding species. Expression of truncated or substituted forms of the different TbMCU subunits (TbMCU, TbMCUc, and TbMCUd) or of the TbATPc subunit (without the C- or N-terminal regions, with mutations in conserved residues of the TMHs or with substitutions of TMHs with artificial helices) indicated that TMH1s of each TbMCU complex subunit (except for TbMCUb) specifically interacts with TMH1 of TbATPc via the conserved motifs of the TMH1s. TbMCU subunits (TbMCU, TbMCUc, and TbMCUd) also co-immunoprecipitated with TbATPc. Blue native PAGE and immunodetection analyses showed that the TbMCU complex physically interacts with TbATPc in a large protein complex of ~900 kDa. Further evidence of this interaction was the co-immunoprecipitation of TbMCU and TbMCUc with TbATPb and TbATPp18.

Interestingly, MYTH assays using the human MCU (HsMCU) and ATPc (HsATPc) validated that their specific physical interaction is also through their respective TMH1s. Reciprocal co-immunoprecipitations of HsMCU and HsATPc using HEK-293T and HeLa cells confirmed this interaction in vivo, which was not detected when HsMCU-KO HEK-293T cells were used as control.

In conclusion, three of the *T. brucei* MCU subunits (TbMCU, TbMCUc, and TbMCUd) physically interact with mitochondrial ATP synthase subunit c (TbATPc) when expressed in yeast membranes. These interactions are also observed with the *T. cruzi* (TcMCU) or human MCU (HsMCU) and the corresponding ATP synthase subunit c (TcATPc, HsATPc), and in all cases these results were confirmed by co-immunoprecipitations, and by their co-localizations, as studied by immunofluorescence microscopy. These interactions were also confirmed in trypanosomes or human cells in vivo by their co-immunoprecipitations from cell lysates and in the case of *T. brucei* by blue native PAGE and immunodetection analysis. As a result of these interactions it was possible to pull down the ATP synthase complex together with the ANT and PiC by TbMCU, suggesting the presence of a ATP synthasome megacomplex that includes the TbMCU complex (Huang, Docampo, 2020).

These results suggest several intriguing possibilities: 1) As these interactions involve the TMH1s of both the TbMCU subunits and the TbATPc, the results suggest that, if this interaction occurs in situ, the TbMCU complex would be within the c-ring of the *T. brucei* ATP synthase (Fig. 4) (Huang, Docampo, 2020). In this context, the presence of a protein within the c-ring of the porcine ATP synthase, as studied in situ by cryo-electron microscopy, has been reported (Gu et al., 2019), and further work is needed to investigate whether it corresponds to a MCU component; 2) the MCU complex could have a potential role in the formation of the mitochondrial permeability transition pore (mPTP) (Huang, Docampo, 2020). This is because the c-ring of the ATP synthase (Alavian et al., 2014; Bonora et al., 2013), a channel inside the ATP synthase dimers (Bonora et al., 2013; Bonora et al., 2017), or the purified ATP synthase itself (Urbani et al., 2019), have been proposed to form the pore of this channel. 3) the formation of a megacomplex including the ATP synthase, the MCU complex, the ANT, and the PiC suggest the coupling between ADP and Pi transport with ATP synthesis, which is stimulated by Ca^{2+} (Huang, Docampo, 2020). In this regard several reports indicate that the ATP synthase binds or is stimulated by Ca^{2+} (Giorgio et al., 2017; Hubbard, McHugh, 1996; Territo et al., 2001; Territo et al., 2000; Zakharov et al., 1993).

6. MECHANISM OF MITOCHONDRIAL Ca^{2+} EFFLUX IN TRYPANOSOMES

In mammalian cells mitochondrial Ca^{2+} is extruded by either a H^+- or a Na^+-coupled exchanger. While the activity of the Na^+/H^+ exchanger is found in most cell types and is especially important in excitable cells, a Ca^{2+}/H^+ exchanger is mainly found in non-excitable cells (Palty et al., 2010). Early work described the activity of the Na^+/H^+ exchanger (Carafoli et al., 1974) but the molecular identity of the exchanger was discovered more recently (Palty et al., 2010). Trypanosomes lack orthologues to this gene. In contrast, evidence of the presence of a Ca^{2+}/H^+ exchanger was found as judged by the response of *T. cruzi* mitochondria to the additions of Ca^{2+} and EGTA (Docampo, Vercesi, 1989b). A mitochondrial Ca^{2+}/H^+ exchanger named Letm1 was identified in mammalian cells (Jiang et al., 2009; Tsai et al., 2014), and trypanosomes do have orthologues to this gene. Studies in *T. brucei* concluded that TbLETM1 is involved in maintaining mitochondrial volume via K^+/H^+ exchange across the inner membrane (Hashimi et al., 2013) but its function in Ca^{2+} transport was not investigated. Further work is needed to investigate the role of trypanosome Letm1 in Ca^{2+} influx and/or efflux.

7. ROLE OF MITOCHONDRIAL Ca^{2+} UPTAKE

Mitochondrial Ca^{2+} uptake in mammalian cells is important as cytosolic Ca^{2+}-buffering system for regulation of spatially confined cytosolic Ca^{2+} rises, for regulation of mitochondrial metabolism, and for cell survival (Rizzuto et al., 2012). Some of these functions are conserved in trypanosomes.

Cytosolic Ca^{2+} buffering system.

Experiments with *T. brucei* expressing the genetically-encoded Ca^{2+} indicator aequorin targeted to the mitochondria found that intramitochondrial Ca^{2+} concentrations can reach values much higher than cytosolic Ca^{2+} levels when Ca^{2+} influx through the plasma membrane or Ca^{2+} release from acidocalcisomes are stimulated (Xiong et al., 1997). Mitochondrial Ca^{2+} uptake can be induced at both nano- and micromolar Ca^{2+} concentrations (Xiong, Ruben, 1998), suggesting a very close proximity of these organelles and the presence of microdomains of high Ca^{2+} concentration in the vicinity of the plasma membrane or acidocalcisomes (Xiong et al., 1997). Such membrane contact sites between acidocalcisomes and mitochondria were reported in both *T. brucei* (Ramakrishnan et al., 2018) and *T. cruzi* (Miranda et al., 2000). The inositol 1,4,5-trisphosphate receptor is located in acidocalcisomes of both trypanosome species (Huang et al., 2013a; Lander et al., 2016) and close contacts between these organelles facilitate the mitochondrial Ca^{2+} transfer to the mitochondria when the IP_3 receptor is activated (Chiurillo et al., 2020).

Regulation of mitochondrial metabolism.

Mitochondrial Ca^{2+} in vertebrate cells is important for regulation of the activity of several mitochondrial dehydrogenases (McCormack, 1986) and the ATP synthase (Territo et al., 2001). Only one of the dehydrogenases stimulated by Ca^{2+} in vertebrates has been studied in detail in trypanosomatids (Lander et al., 2018). The pyruvate dehydrogenase is activated by dephosphorylation of the E1α subunit catalyzed by a PDH phosphatase (PDP), which is stimulated by Ca^{2+}. Both *T. brucei* and *T. cruzi* recombinant PDPs catalyze the dephosphorylation of a synthetic phosphopeptide from either species containing the phosphorylated sites that regulate PDH activity (Lander et al., 2018). TcPDP and TbPDP exhibit maximal activity at 100 nM and 1 μM Ca^{2+}, respectively, suggesting a physiological response. Interestingly, although the binding site for mammalian PDP is formed in the presence of PDH E2 subunit (Turkan et al., 2004), the parasite enzymes are able to directly dephosphorylate E1α phosphopeptides (Lander et al., 2018), in agreement with earlier studies with the mammalian enzyme that identified an E1α binding site (Teague et al., 1982). Knockout of TcPDP results in reduced growth of epimastigotes, defective metacyclogenesis, and reduced host cell invasion by trypomastigotes (Lander et al., 2018). The epimastigotes have a respiratory deficiency, lower citrate synthase activity, higher AMP/ATP ratio and increased autophagy. These cells have a compensatory increase in amino acid metabolism, as revealed by the increased ammonia production (Lander et al., 2018).

The stimulation by Ca^{2+} of two other dehydrogenases that are stimulated by Ca^{2+} in animals, isocitrate dehydrogenase and the α-ketoglutarate dehydrogenase, has not been

studied in trypanosomes. However, in contrast to the Ca^{2+}-regulated mammalian NAD-dependent enzyme, the mitochondrial isocitrate dehydrogenase present in trypanosomatids is NADP-dependent (Leroux et al., 2011). Another dehydrogenase regulated by Ca^{2+}, the glycerol phosphate dehydrogenase (Denton, 2009) is devoid of the Ca^{2+}-binding EF-hands domains in trypanosomes and presumably insensitive to Ca^{2+}. The aspartate-glutamate carrier (AGC) and the ATP-Mg-Pi carriers (SCaMCs) are known to be regulated by Ca^{2+} in mammalian cells (Satrustegui et al., 2007). However, the trypanosomatid homologues lack EF-hand domains and are therefore potentially Ca^{2+} insensitive. Finally, regulation of the ATP synthase by Ca^{2+} has not been studied in trypanosomes.

Regulation of cell survival.

Mitochondrial Ca^{2+} is important for programmed cell death (PCD), or apoptosis, in trypanosomatids. Early work on the effects of naphthoquinones on *T. cruzi* demonstrated changes in their morphology that can be attributed to PCD, such as shrinking, membrane blebbing, mitochondrial alterations and chromatin condensation (Docampo et al., 1977). However, trypanosomes lack some key regulatory or effector molecules involved in apoptosis in mammalian cells, such as the tumor necrosis factor (TNF)-related family of receptors, Bcl-2 family members, and caspases (Kaczanowski et al., 2011; Smirlis et al., 2010). Mitochondrial Ca^{2+} overload affects the mitochondrial membrane potential, induces the generation of reactive oxygen species (ROS) generation and releases cytochrome *c* in trypanosomatids (Smirlis, Soteriadou, 2011). The production of ROS in *T. brucei* impairs mitochondrial Ca^{2+} uptake, and leads to its accumulation in the nucleus, resulting in cell death (Ridgley et al., 1999). A mitochondrial endonuclease G is released and translocated to the nucleus in *Leishmania* spp. (Gannavaram et al., 2008) and this change stimulates a caspase-independent, apoptosis-like cell death (reviewed in (Smirlis, Soteriadou, 2011)). *T. cruzi* is highly resistant to mitochondrial permeability transition (Docampo, Vercesi, 1989b), and apoptosis-like death upon mitochondrial Ca^{2+} overload is dependent on superoxide anion generation (Irigoin et al., 2009).

In conclusion, mitochondrial Ca^{2+} uptake in trypanosomatids has a role in shaping the amplitude of cytosolic Ca^{2+} increases after influx through the plasma membrane or release from acidocalcisomes, in the regulation of ATP production, and in apoptosis-like death.

8. CONCLUSIONS AND OPEN QUESTIONS

The mitochondria of trypanosomes possess a Ca^{2+} uniporter for Ca^{2+} uptake and a putative Ca^{2+}/H^{+} exchanger for Ca^{2+} release. The finding of a Ca^{2+} transporting mechanism in trypanosomes with similar characteristics to those of the mammalian uniporter and its absence in yeast, together with the elucidation of the genomes of these and mammalian organisms, led to the discovery of the molecular nature of MICU1 and MCU subunits. However, the MCU complex of trypanosomes has lineage-specific adaptations not seen in the vertebrate uniporter complex. For example, some subunits present in vertebrate cells, such and MCUR1, MICU3, and EMRE, are absent in trypanosomatids. The homologous to vertebrate subunit MCUb does not have a dominant negative effect but has Ca^{2+}-transporting activity. Trypanosomes possess four Ca^{2+}-transporting subunits (MCU, MCUb, MCUc,

and MCUd) that form hetero-oligomers where each subunit contributes to the formation of the pore of the channel, interacting through their TMHs. Interestingly, the MCUc and MCUd subunits are exclusive components of the MCU complex of trypanosomatids. In addition, MICU1 and MICU2 do not form covalently-bound dimers and do not act as gatekeepers of the channel at low Ca^{2+} concentrations. Concerning trypanosome biology, the MCU complex is essential for normal growth in vitro and in vivo. Downregulation of the uniporter expression leads to increase in the AMP/ATP ratio and autophagy while overexpression leads to Ca^{2+} overload, reactive oxygen species (ROS) generation, and cell death. The MCU complex interacts with the subunit c of the ATP synthase and contributes to the formation of a megacomplex including the phosphate carrier (PiC) and the adenine nucleotide translocator (ANT), which couples ADP and Pi uptake with ATP synthesis stimulated by Ca^{2+}. Finally, Ca^{2+} has a direct role in the stimulation of the mitochondrial PDH activity by dephosphorylation of the E1α subunit catalyzed by a Ca^{2+}-sensitive PDH phosphatase (PDP).

It has not been possible to reconstitute the trypanosome uniporter in yeast, or to complement the lack of the MCU subunit in trypanosomes by either *HsMCU* alone or together with *HsEMRE*, suggesting that the four Ca^{2+}-transporting subunits would be needed for reconstitution and that the human MCU subunit is not compatible with the trypanosome-specific subunits. Further work is needed to identify whether Letm1 is the Ca^{2+}/H^{+} exchanger in trypanosomes and to determine how MICU1/MICU2 interact with the pore of the channel, given the apparent absence of an EMRE ortholog. It will also be important to confirm, by structural studies, whether the Ca^{2+}-transporting subunits form hetero-hexamers in situ, and the nature of the megacomplex involving the ATP synthase in situ. The regulation of the activity of the MCU complex in vivo also needs to be investigated.

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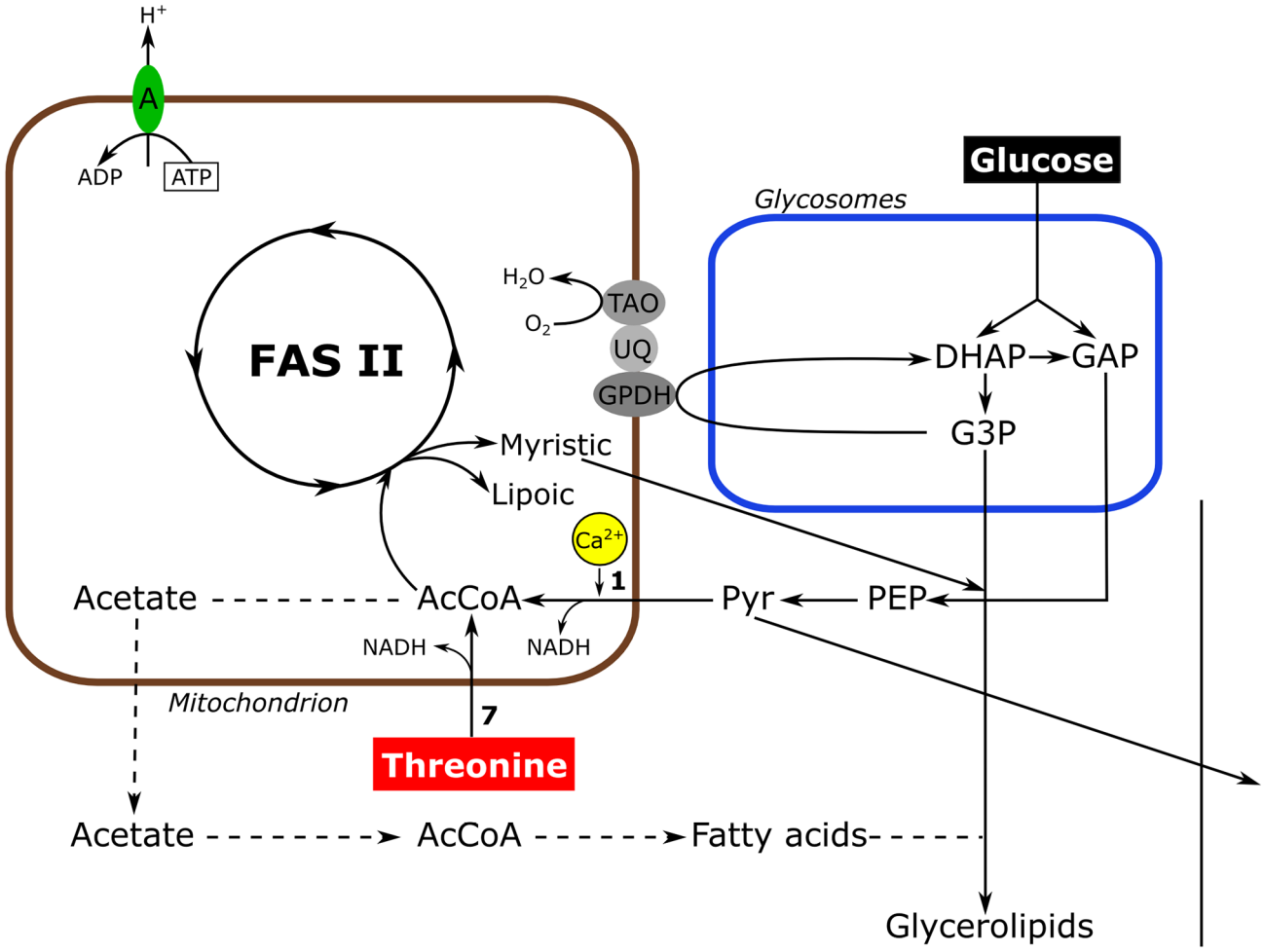

Figure 1.

Scheme of metabolic pathway in *T. brucei* bloodstream forms. Rectangles indicate steps of glucose and threonine metabolism; dashed arrows indicate steps for which no evidence of flux is available. A, ATPase; AcCoA, acetyl-CoA; FAS II, type II fatty-acid biosynthesis pathway; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; GPDH, glycerol 3-phosphate dehydrogenase; PEP, phosphoenolpyruvate; Pyr, pyruvate; UQ, ubiquinone; TAO, trypanosome alternative oxidase. Enzymes are: 1. Pyruvate dehydrogenase; 7, threonine dehydrogenase. Activity stimulated by Ca^{2+} is in yellow. Figure 2. Phylogenetic tree of trypanosomatid and human MCU complex subunits. Thee scale bar corresponds to a distance of 20 changes per 100 amino acid positions. Reproduced with permission from reference (Huang et al., 2013b).

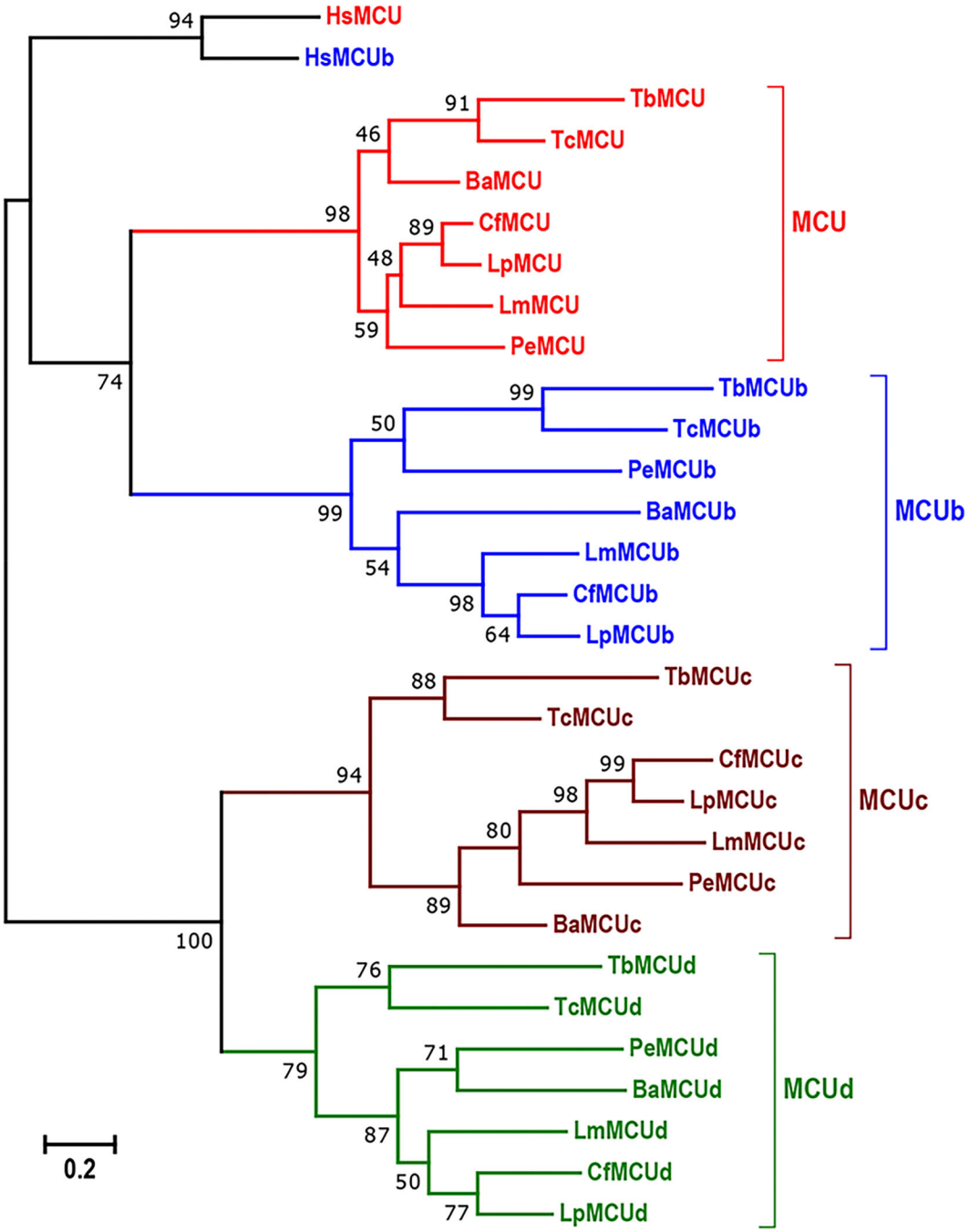

Figure 2.

Phylogenetic tree of trypanosomatid and human MCU complex subunits. The TriTrpDB and GenBank accession numbers for 30 MCUC subunits were described in (Huang, Docampo, 2018). The scale bar corresponds to a distance of 20 changes per 100 amino acid positions. Reproduced with permission from reference (Huang, Docampo, 2018).

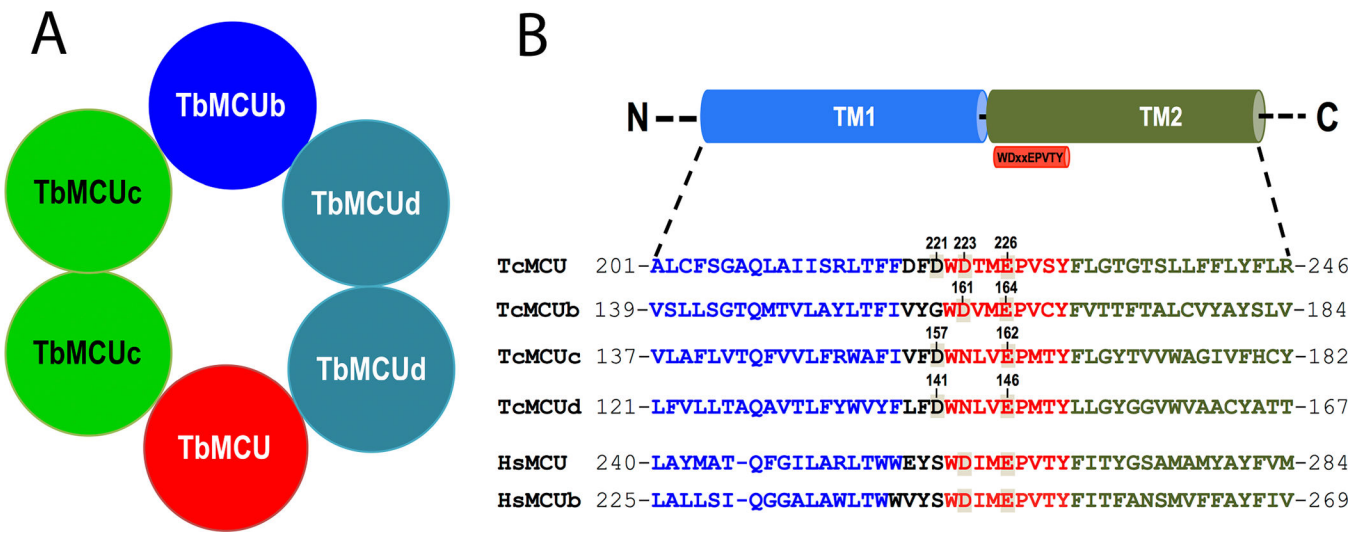

Figure 3.

A. Scheme depicting the putative organization and composition of a hetero-hexameric TbMCU complex. Reproduced with permission from reference (Huang, Docampo, 2018). **B.** conserved WDXXEPXTY motif in TcMCU complex subunits. Alignment of the C-terminal fragment of the first transmembrane domain (TM1, in *blue*) and the N-terminal fragment of the second transmembrane domain (TM2, *in green*), including conserved WDXXEPXTY motif (*in red*), of TcMCU complex subunits with human MCU and MCUb. Conserved putative critical acidic residues in or near the WDXXEPXTY selectivity filter are indicated. TcMCU, TcMCUb, TcMCUc and TcMCUd exhibit a substitution of the Ru360-sensitive residue S to D/G in the pore region. Residues in each TcMCU complex subunit that were subjected to substitutions are indicated with the corresponding number in their sequence. Reproduced with permission from reference (Chiurillo et al., 2019).

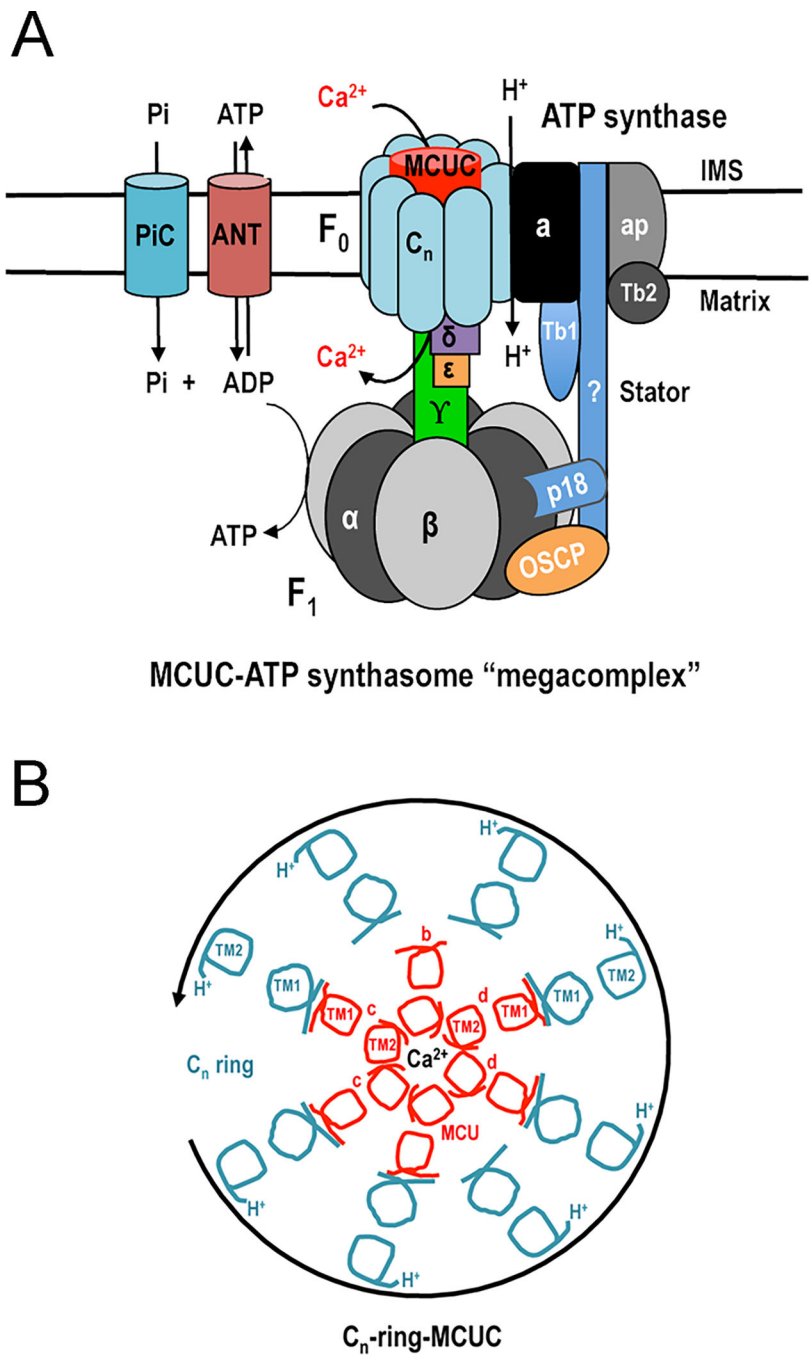

Figure 4.

Models showing organization of putative MCU complex-ATP synthasome megacomplex in trypanosomes. **A**. The MCU complex physically interacts with the ATP synthasome (ATP synthase, ANT, and PiC) via the c-ring of the F_0 ATP synthase. In trypanosomes, ATP synthase consists of F_1 region with the central stalk (α3β3, γ, δ and ε) for ATP synthesis, F_0 region with the putative stator (c_n, a, p18, Tb1, Tb2, and OSCP) for proton (H^+) translocation, and trypanosome-specific associated proteins (ap), while the molecular identity of the peripheral stalk is unknown. OSCP, oligomycin sensitivity-conferring protein;

ANT, adenine nucleotide translocator; PiC, phosphate carrier. **B**. Cross section model to hypothetical *T. brucei* c_n-ring-MCU complex. The *T. brucei* heterohexameric MCU complex consisting of 4 different subunits (MCU, MCUb, MCUc, and MCUd), with a molecular weight of approximately 145 kDa, is within the c-ring of ATP synthase. TMH1 of each MCU complex subunit (excluding MCUb) interacts with TMH1 of ATPc. The c-ring rotates in counterclockwise direction and translocates H^+ from the intermembrane space to matrix during ATP synthesis. Reproduced with permission from reference (Huang, Docampo, 2020).