The ancestral shape of the access proton path of mitochondrial ATP synthases revealed by a split subunit-a

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Abstract

The passage of protons across membranes through F_1F_o-ATP synthases spins their rotors and drives synthesis of ATP. While the principle of torque generation by proton transfer is known, the mechanisms and routes of proton access and release and their evolution are not fully understood. Here, we show that the entry site and path of protons in the lumenal half-channel of mitochondrial ATP synthases are largely defined by a short N-terminal α-helix of subunit-a. In *Trypanosoma brucei* and other Euglenozoa, the α-helix is part of another polypeptide chain that is a product of subunit-a gene fragmentation. This α-helix and other elements forming the proton pathway are widely conserved across eukaryotes and in Alphaproteobacteria, the closest extant relatives of mitochondria, but not in other bacteria. Thus, the proton entry site, the shape of the access half-channel, and the proton transfer mechanism involving a chain of ordered water molecules, predate eukaryotes and possibly originated in the lineage from which mitochondria evolved by endosymbiosis.

Introduction

F_1F_o-ATP synthases are rotary molecular machines that convert the proton-motive force across energy-transducing membranes of bacteria, chloroplasts and mitochondria to the chemical energy of ATP. The flow of protons down their electrochemical gradient through the membrane-intrinsic F_o subcomplex spins the enzyme's asymmetric rotor, whose rotation changes the conformation of three catalytic sites on the membrane-extrinsic F_1-ATPase subcomplex, resulting in synthesis of ATP. The transmembrane part of the rotor, the c-ring, consists of several copies of subunit-c. Proton translocation is based on sequential protonation of a glutamate residue of each protomer of the c-ring, rotation of the c-ring with the neutralized glutamates exposed to the hydrophobic environment of the phospholipid bilayer, and release of protons on the other side of the membrane. The second element crucial for proton passage is the stator subunit-a (also referred to as ATP6 or subunit-6). Canonical subunits-a contain six α-helices (h_1-h_6), five of which are largely embedded in the membrane. While h_1 is a vertical transmembrane helix, h_3-h_6 are horizontal, positioned at an angle ~20° relative to the plane of the membrane. Helices h_5-h_6 form a hairpin bending around the c-ring. The sites of proton binding and release from the c-ring glutamates are separated by a universally conserved arginine of h_5. The positively charged arginine prevents protons from leaking without c-ring rotation and induces their dissociation at the release site (reviewed in Walker 2017; Kuhlbrandt 2019; Guo and Rubinstein 2022). The proton-binding glutamates on the c-ring are accessible from the surface of the complex by two offset solvent-filled half-channels. In mitochondrial ATP synthases, the access half-channel opens into the lumen of cristae, hence it is called the lumenal half-channel. It is shaped by subunit-a and adjacent membrane elements, including lipids (Klusch, et al. 2017). Recently, it was proposed that delivery of protons to the c-ring is facilitated by a Grotthuss mechanism through a chain of coordinated water molecules and

adjacent amino acid residues in the inner part of the channel in mammalian (Spikes, et al. 2020) and trypanosomal (Gahura, et al. 2022) mitochondrial ATP synthases.

Here, we explore the lumenal half-channel forming elements across species and show that the N-terminal region of subunits-a form a short α-helix common to alphaproteobacterial and vast majority of mitochondrial ATP synthases. In Euglenozoa, this helix is a part of a different polypeptide chain encoded by a nuclear gene that is presumably the product of fragmentation of the mitochondrial gene for subunit-a. We document that the helix defines the proton entry site and contributes to the shaping of the inner part of the half-channel in modern eukaryotes and their common ancestors.

Results and discussion

The short N-terminal α-helix of subunit-a is a widely conserved structural element of mitochondrial ATP synthases

When examining the proton translocation elements of mitochondrial ATP synthases of two divergent eukaryotes, *Saccharomyces cerevisiae* (Guo, et al. 2017) and *Trypanosoma brucei* (Gahura, et al. 2022), we noticed a remarkable similarity between the N-terminal regions of subunit-a in the yeast and subunit ATPEG3 in the parasite. The N-termini of both proteins form a short single-turn α-helix, hereafter referred to as h_N, which is located at the layer corresponding to the lumenal surface of the membrane and interacts with the horizontal helix h_5 of subunit-a in the same manner in both structures. Downstream of h_N, yeast subunit-a and trypanosomal ATPEG3 follow a similar path; however, after ten residues the yeast chain turns back towards the first transmembrane helix h_1, thereby diverging from ATPEG3, which continues to the periphery of the complex. The canonical trypanosomal subunit-a, which contains h_1 and four characteristic horizontal membrane-intrinsic helices h_3-h_6, begins nine amino acid residues upstream of h_1 in close proximity to the site of divergence between yeast

subunit-a and ATPEG3 (Fig. 1A). Based on the striking structural and sequence (see below) similarity, we conclude that subunit-a in *T. brucei* has been split and ATPEG3 represents its N-terminal fragment. To acknowledge the origin of ATPEG3, we introduce an alternative name subunit-a_N.

The split of subunit-a in *T. brucei* occurred in the region corresponding to the loop involved in ATP synthase dimerization in *S. cerevisiae* (Guo, et al. 2017). In *T. brucei*, the loop is structurally replaced by a two-stranded β-sheet, formed by the N-terminus of subunit-a and a β-strand of subunit-a_N (Fig. 1A). Unlike in yeast, this structure does not contribute to the ATP synthase dimerization interface, which is provided by subunits g and e in this organism (Gahura, et al. 2022). After splitting, subunit-a_N was extended at its C-terminus. The extension interacts exclusively with lineage-specific subunits (ATPTB1, ATPTB6, ATPTB11, ATPTB12) or with lineage-specific extensions of subunits shared with other ATP synthases (subunit-i/j and k; Gahura, et al. 2022). It extends to the periphery of the membrane part of the F_o-moiety, where it co-constitutes a euglenozoan-specific subcomplex, separated from the conserved part by a lipid-filled cavity (Muhleip, et al. 2019; Gahura, et al. 2022). Consistently, fragmented subunit-a is also discernible in the structure of ATP synthase from *Euglena gracilis* (Muhleip, et al. 2019; Gahura, et al. 2021).

The sequence of h_N and flanking residues in yeast is highly similar to the N-terminal sequence of subunit-a in Rickettsiales and other Alphaproteobacteria (Fig. 2, Supplementary table S1 and Supplementary data 1), the closest extant relatives to mitochondria (Roger, et al. 2017). We predicted the structure of subunit-a from *Rickettsia rickettsii* using AlphaFold (Jumper, et al. 2021). The model resembles structures of bacterial and mitochondrial homologs resolved experimentally and includes h_N located at exactly the same position as the h_N in *T. brucei* and *S. cerevisiae* (Fig. 1C). Similarity of this region in ATP synthases from mitochondria of two deeply branching eukaryotic lineages and the group of bacteria from which mitochondria

evolved strongly indicates that the observed architecture of the N-terminal segment of subunit-a is ancestral.

We compared subunit-a sequences from 88 species covering all major eukaryotic lineages, and structures from selected species determined by cryoEM or predicted by AlphaFold. The sequence comparison documented that the split of subunit-a observed in *T. brucei* and *E. gracilis* is restricted to Euglenozoa, because subunits-a from all representatives of the sister lineage Heterolobosea contain a conserved sequence of h_N. Further, the analysis revealed high sequence similarity in the N-terminal region, with most sequences showing a degenerate sequence motif SPLEQF (Fig. 2, Supplementary table S1), supporting the notion that presence of h_N represents an ancestral state. The only organisms without the SPLEQF motif were most alveolates, chlorophycean algae *Polytomella* and *Chlamydomonas*, and all metazoans (Fig. 2, Supplementary table S1). In alveolates and chlorophycean algae, the N-terminal region including h_1 is divergent or absent, as documeted by cryoEM structures of ATP synthases from *Tetrahymena* (Flygaard, et al. 2020), *Polytomella* (Murphy, et al. 2019) and *Toxoplasma* (Muhleip, et al. 2021). Subunit-a in *Tetrahymena* features a highly extended and structurally divergent N-terminus, which interacts predominantly with ciliate-specific ATP synthase components. On the contrary, subunit-a in *Polytomella* lacks the entire N-terminal region including the transmembrane helix h_1, while subunit-a in *Toxoplasma* is even more reduced and contains horizontal helices h_5 and h_6 only (Fig. 1C). The only alveolate with a canonical N-terminus of subunit-a is *Colponema* (Fig. 2), which supports the view that it represents a lineage sister to all other Alveolata (Tikhonenkov, et al. 2020).

In Metazoa, the N-terminal sequence of subunit-a differs from h_N in other groups, but invariably contains the motif FxxF (Fig. 2 and Supplementary table S1 and Supplementary Data 1). In experimentally determined and predicted structures of mammalian (Pinke, et al. 2020; Spikes, et al. 2020) and *Drosophila* subunit-a, respectively, this motif adopts the canonical fold

of h_N (Fig. 1C), indicating that the ancestral architecture is retained despite sequence divergence. The only exception is the group Nematoda, represented by *Caenorhabditis elegans*, whose subunit-a begins with helix h_1, like subunit-a in trypanosomes. This notable exception is in line with a recent cryoelectron tomography study that showed that the nematode ATP synthase diverged architecturally from canonical ATP synthases from yeasts and mammals and might lack subunits 8, k and i/j (Buzzard, et al. 2023), which are otherwise widely conserved in eukaryotes (Sinha and Wideman 2022). Due to high conservancy of h_N across eukaryotes, we wondered whether an equivalent of h_N could be retained in nematodes, possibly as a product of subunit-a gene split analogous to that observed in *T. brucei*. Such evolutionary independent event of fission of a mitochondrial gene would not be unprecedented. The gene encoding the mitoribosomal subunit uS3m has been fragmented in Ciliata (Swart, et al. 2012; Tobiasson and Amunts 2020) and some Heterolobosea (Fu, et al. 2014), and the gene encoding Cox2, a subunit of cytochrome oxidase, has been independently split at least three times in evolution, specifically in alveolates, chlorophycean algae (Perez-Martinez, et al. 2001; Waller and Keeling 2006; Rodriguez-Salinas, et al. 2012), and the hymenopteran insect *Campsomeris* (Szafranski 2017a). Therefore, we searched the proteomes of Nematoda using several approaches (blast, motif search, structure similarity search in AlphaFold database) to identify the protein constituting the missing region including h_N, but we found no candidate, presumably due to the short length of the region and limited sequence conservancy.

In summary, the vast majority of eukaryotes with the exception of most Alveolata and a group of chlorophycean algae have retained the ancestral architecture of the region corresponding to the N-terminus of subunit-a.

Loss of transmembrane helix h_1 predetermines the transfer of subunit-a from the mitochondrial to the nuclear genome

While the gene encoding subunit-a_N in Euglenozoa was transferred to the nuclear genome, the subunit-a gene has been retained in the mitochondrial genome. Likewise, subunit-a in nematodes, also starting with the first transmembrane helix h_1, is encoded mitochondrially. In contrast, the gene of subunit-a in *Polytomella* and *Chlamydomonas*, which lacks h_1, has been transferred to the nuclear genome. This suggests that while loss of h_1 allows the transfer of the gene from the mitochondrial to the nuclear genome, the removal of the N-terminal fragment upstream of h_1 is not sufficient for the relocation. Consistently, the reduced subunit-a in *Toxoplasma* and related species are also nuclear encoded (Huet, et al. 2018; Muhleip, et al. 2021; Fig. 2). Together, these observations indicate that barriers associated with the insertion of h_1-containing subunit-a to the inner mitochondrial membrane from the intermembrane space (or with translocation of the protein into mitochondria prior its insertion from inside) may be a factor limiting the transfer of the subunit-a gene from mitochondrial DNA to the nuclear genome. We expanded a previous analysis of hydropathy of subunit-a (Muhleip, et al. 2021) to all species included in our dataset. It revealed that some subunits-a (e.g., from *Nicotiana* or *Tetrahymena*) retained in mitochondrial genomes show relatively low hydrophobicity similar to those transferred to the nuclear genome (Fig. 2, Supplementary table S1), suggesting that the reduced hydrophobicity is not the only prerequisite for the intracellular subunit-a gene transfer. Regardless of the factor that allowed the subunit-a gene to be transferred, this is very rare event that has most likely occurred only twice in the evolution of eukaryotes described to date, namely in the ancestors of the present-day myzozoans and Chlamydomonadales, an order of chlorophycean algae including *Polytomella* and *Chlamydomonas* (Fig. 2).

The entry point of the proton access half-channel in mitochondrial and alphaproteobacterial ATP synthases is defined by the N-terminal helix h_N of subunit-a

The wide conservancy of h_N and its flanking residues (Fig. 2), whether within subunit-a or as part of a fission product, implies the functional importance of this region. Indeed, h_N contributes to the lining of the inner constricted portion of the lumenal proton half-channel in structurally characterized ATP synthases from *Trypanosoma* (Gahura, et al. 2022), *S. cerevisiae* (Guo, et al. 2017) and mammals (Pinke, et al. 2020; Spikes, et al. 2020; Fig. 1B) and, as can be inferred from its conserved nature, presumably also in most other eukaryotic lineages and Alphaproteobacteria. Thus, the segment of the half-channel proximal to the c-ring most likely does not differ between these organisms. Indeed, several residues proposed to be involved directly in the proton transfer or to coordinate ordered proton-transporting water molecules (Spikes, et al. 2020; Gahura, et al. 2022) are largely conserved in the h_N-containing subunits including those from Alphaproteobacteria. These include a protonatable glutamate or aspartate residue in helix h_6 (Glu203, Glu233 and Asp202 in bovine, *S. cerevisiae* and *T. brucei* numbering, respectively) and a histidine in h_5 (His169/195/155 in bovine/*S. cerevisiae*/*T. brucei*; Fig. 2, Supplementary table S1). In addition, the position of histidine 19 in h_N in *T. brucei* subunit-a_N, which coordinates one of the molecules in the Grotthuss water chain, is occupied by another hydrogen bonding residue, glutamine, in subunits-a of vast majority of mitochondrial ATP synthases (Gln15 in *S. cerevisiae*; Fig. 1B&2 and Supplementary table S1). Therefore, we reason that the Grotthuss mechanism represents an ancestral mode of proton transfer in mitochondrial ATP synthases that has been conserved in most, if not all, eukaryotic lineages.

Notably, ATP synthase in bacteria from various groups outside Alphaproteobacteria (*Escherichia coli;* Sobti, et al. 2019, *Mycobacterium*; Guo, et al. 2021, Montgomery, et al. 2021, and *Acinetobacter*; Demmer, et al. 2022) contain an α-helix at the N-terminus of the subunit-a, which is longer and sequentially dissimilar to h_N in mitochondrial ATP synthases and positionally shifted further from the loop connecting helices h_3 and h_4 (Fig. 1C). As proposed

based on the structure of the *Acinetobacter* ATP synthase, the position of the N-terminal helix determines whether protons enter to the half-channel using the "front" or "rear" entry site (Demmer, et al. 2022). In *Acinetobacter* and some other bacteria, the protons enter from the side facing the c-ring through the "front" entry located between the N-terminal helix and h_5 of subunit-a. In yeast and bovine mitochondrial ATP synthases protons use the "rear" opening, a cleft between h_N and the C-terminal tail of subunit-f (Spikes, et al. 2020; Demmer, et al. 2022; Fig. 3A,B). In the latter two structures, as well as in the structure from *T. brucei* and the predicted structure from *Rickettsia*, the position of the front entry is blocked by h_N (Fig 3B,C), specifically by the phenylalanine residue of the SPLEQF motif that is nearly invariably conserved in all h_N sequences included in our analysis (the only exception is *Fonticula*, where it is substituted by other bulky and aromatic residue, tyrosine; Fig 2 and Supplementary table S1). Therefore, we reason that the rear entry is common to all h_N-containing ATP synthases. Taken together, h_N contributes to shaping of both the surface-exposed and the inner portions of the access proton half-channel, underlying its almost universal occurrence in mitochondrial ATP synthases.

Concluding remarks

By combination of analyses of available ATP synthase structures with sequence comparison and structure prediction across species, we documented that the proton access path and proton transfer mechanism in the access half-channel is conserved in majority of mitochondrial ATP synthases. The key structural determinant, so far largely overlooked, is h_N of subunit-a, which defines the entry to the access half channel and contributes to hydrogen bonding of water molecules in the Grotthuss chain (Fig. 1C). Although the shape of the inner access half-channel in bacteria resembles that of mitochondrial ATP synthase (Montgomery, et al. 2021), water molecules have not been resolved in cryoEM maps of bacterial ATP synthases. In addition, the

key residues involved in water coordination and proton transfer are not present in bacteria outside Alphaproteobacteria (columns H and D/E, Fig. 2). Therefore, the mechanism of the proton transfer to the c-ring in bacterial complexes has not been unambiguously determined yet. We speculate that the use of Grotthuss mechanism for the proton transfer emerged in Alphaproteobacteria and may represent a preadaptation to eukaryogenesis.

Uniquely, F_1F_o-ATP synthase in *T. brucei* mitochondria contains two functionally central components, the subunits α and a, that are both constituted of two separate polypeptide chains. While the subunit-α is cleaved into two fragments posttranslationally (Gahura, et al. 2018; Montgomery, et al. 2018), the subunit-a is split on the gene level. Our finding of a split subunit-a in *T. brucei* complements recently reported cases of mitochondrial gene fragmentation revealed by structures of mitochondrial ribosomes (uS4m and uS7m in *Polytomella*; Tobiasson, et al. 2022) and respiratory chain supercomplexes (Cox3 and Nad5 in *Tetrahymena*; Mühleip, et al. 2022; Zhou, et al. 2022), and several occurrences reported earlier (reviewed in Szafranski 2017b). The transfer of the same gene from the mitochondrial to the nuclear genome independently in several lineages facilitates the inference of properties of encoded proteins that are incompatible with such events. We inferred that the transmembrane helix h_1 might represent such an obstacle in the case of subunit-a.

Material and Methods

Subunit-a sequences were searched by hmmsearch using the alignment of subunit-a sequences from *Bos taurus*, *Saccharomyces cerevisiae*, *Rickettsia rickettsii* and *Colponema vietnamica* as a query. The sequences from Ciliata and Apicomplexa were searched by phmmer using sequences from *Tetrahymena* and *Toxoplasma*, respectively, as queries. Both searches were performed on the HMMER web server (Potter, et al. 2018) in the Uniprot database. Amino acid sequence alignments were constructed by MUSCLE (Edgar 2004) and corrected manually

using available structures as references. Structures were predicted by AlphaFold (Jumper, et al. 2021), and superposed by the Matchmaker tool and visualized in ChimeraX (Goddard, et al. 2018). Hydrophobicity was calculated as grand average of hydropathy using hydropathy indices from Kyte and Doolittle 1982.

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Author contributions

Conceptualization: O.G. and A.Z.; investigation and data analyses: J.E.W. and O.G.; writing – original draft: O.G.; writing – review and editing: J.E.W, A.Z., O.G.; funding acquisition: O.G. and A.Z.

Declaration of interests

They authors declare they have no competing interests.

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Figures and legends

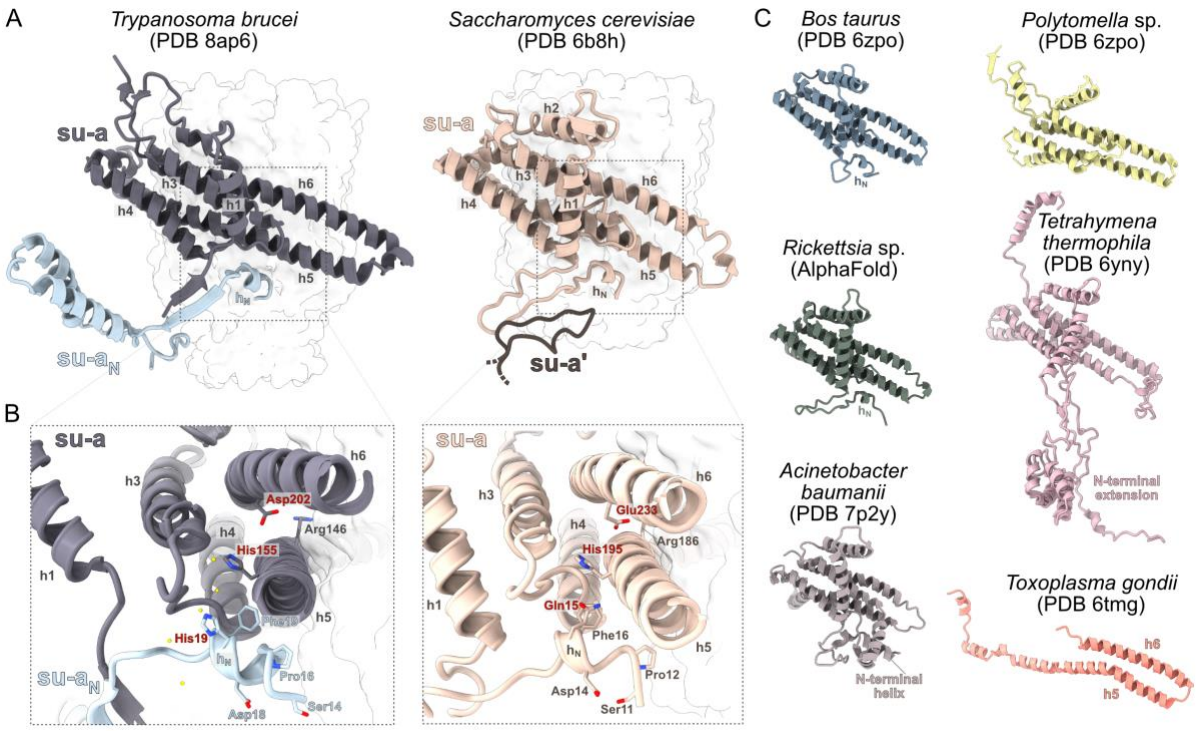

Figure 1. Split subunit-a of mitochondrial ATP synthase in *Trypanosoma brucei* retains ancestral structure and shapes the access proton half-channel (A) Comparison of the structures of subunit-a in *S. cerevisiae* and subunits a and a_N in *T. brucei*. The c-rings are shown as transparent surface. In the structure from *S. cerevisiae*, a fragment of the subunit-a from the other ATP synthase protomer is shown (su-a'). **(B)** The close-up view of the structures in (A). Conserved residues of h_N and other selected conserved residues are shown as sticks. The residues labeled in red are directly involved in proton translocation or coordinate water molecules in the Grotthus chain. Water molecules inferred from cryoEM density are shown as yellow balls in the *T. brucei* structure. **(C)** Structures of subunit-a of mitochondrial and bacterial ATP synthases from selected species. The structure from *Rickettsia* was predicted by AlphaFold

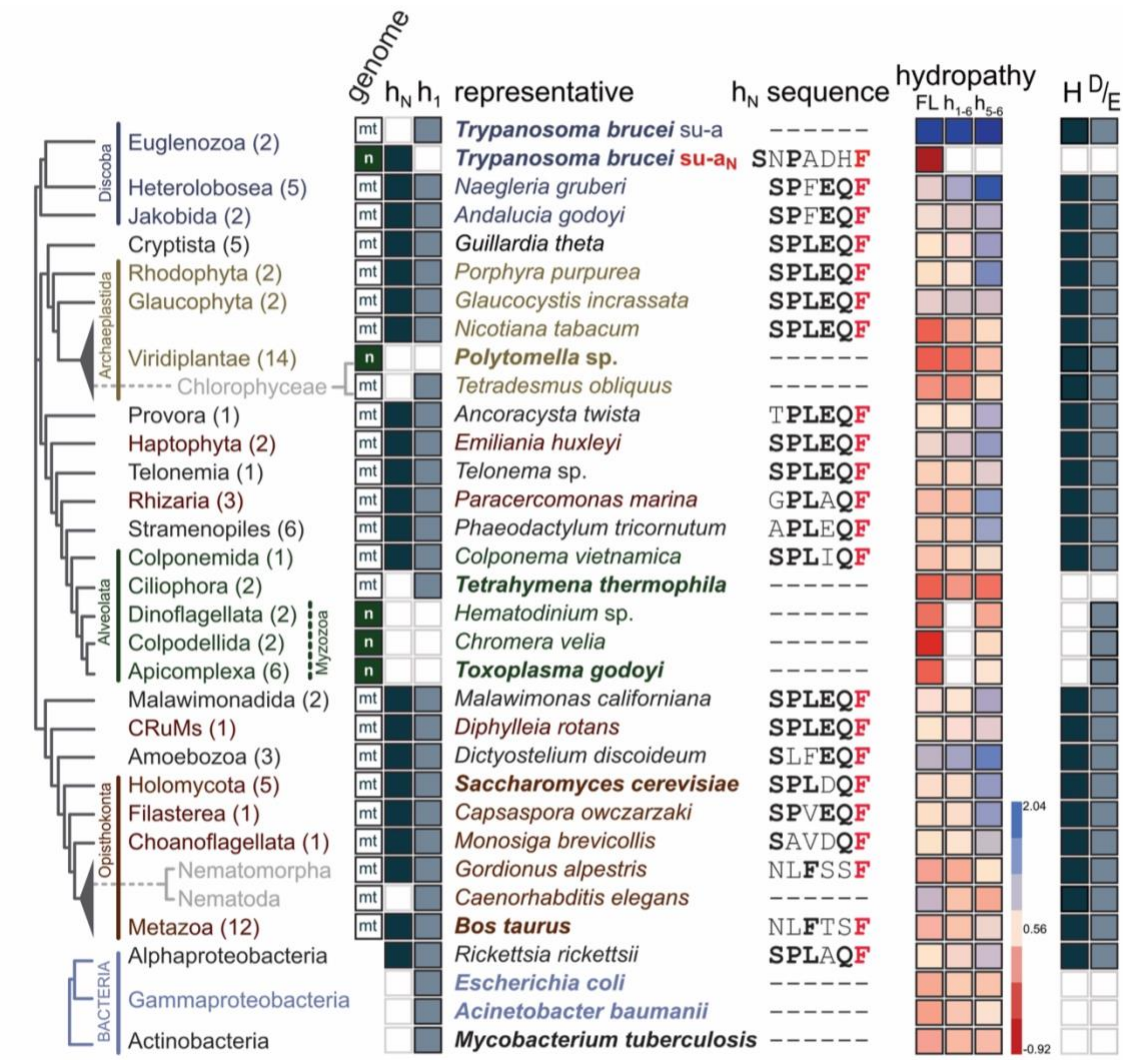

Figure 2. Helix h_N is conserved in most mitochondrial ATP synthases and Alphaproteobacteria. For individual lineages mapped on the phylogenetic tree based on Burki, et al. 2020 and Tikhonenkov, et al. 2022 following features are shown: number of species included in the analyses (in parentheses); genome, in which subunit-a/a_N is encoded ("mt" – mitochondrial, "n" – nuclear); presence or absence of helices h_N and h_1 of subunit-a/a_N; selected representative species used in the analyses presented in the following columns (for species shown in bold a cryoEM structure of ATP synthase has been reported); sequence of h_N and flanking residues (universally conserved phenylalanine is shown in red, other conserved residues of the SPLEQF motif, or the FXXF motif in Metazoa, are shown in bold); average hydrophobicity of the entire subunit-a (FL), region h_1-h_6, and region h_5-h_6 (blue – more

hydrophobic, red – less hydrophobic); presence or absence of the histidine residue shown to coordinate a water molecule in the Grotthuss chain in trypanosomal (His155) and bovine (His168) ATP synthase (column H), and of the acidic residue (aspartate or glutamate) involved in proton translocation (Asp202 in *T. brucei*; column D/E).

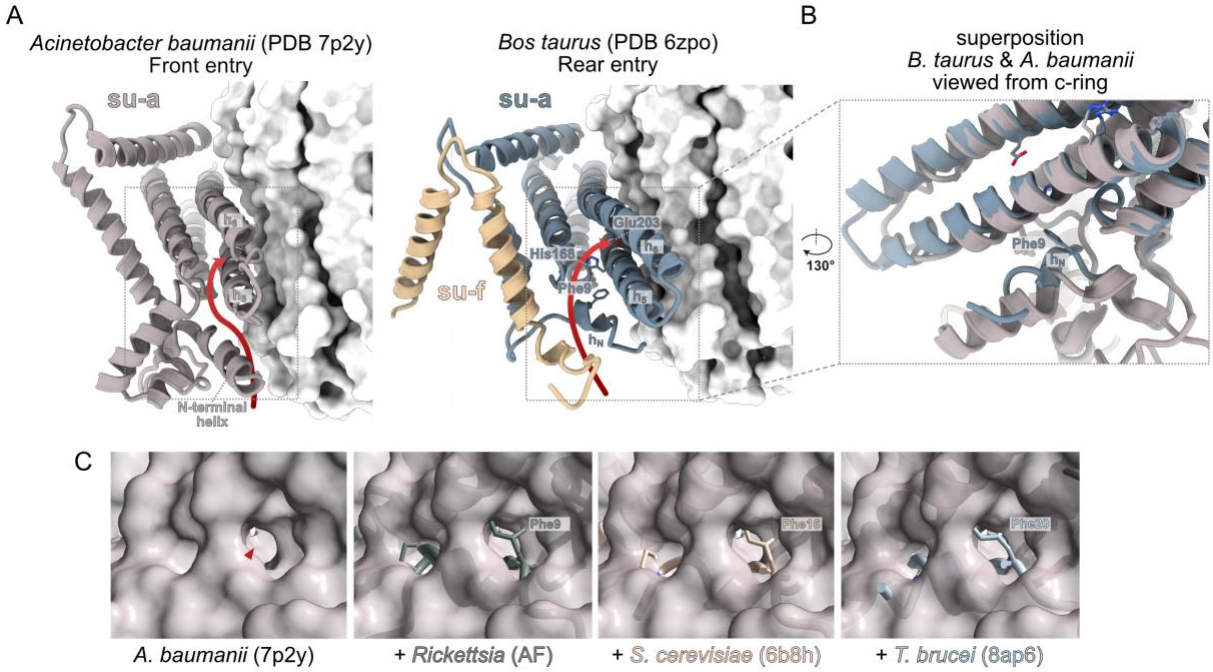

Figure 3. The proton entry point and path in the access half-channel is co-determined by h_N. (A) Comparison of proton paths in the access half-channel of the *Acinetobacter baumanii* and bovine mitochondrial ATP synthases. The c-ring is shown as white surface. **(B)** Superposition of the structures from (A) shows that hN is positionally shifted compared to the N-terminal helix of subunit-a in *A. baumanii*. **(C)** The position of the proposed "front" proton entry to the access half-channel in *A. baumanii* ATP synthase (Demmer, et al. 2022) is blocked by universally conserved phenylalanine in h_N of mitochondrial and alphaproteobacterial ATP synthases. A view of the entry (red arrowhead) to the half channel in the *A. baumanii* subunit-a (surface representation) from the c-ring shown individually and superposed with subunit-a from other ATP synthases (cartoon representation): *S. cerevisiae*, the AlphaFold model of *Rickettsia*, and subunits a and a_N from *T. brucei*. The conserved phenylalanine is shown as sticks.