A cation–π interaction in a transmembrane helix of vacuolar ATPase retains the proton-transporting arginine in a hydrophobic environment

Walter Hohlweg1‡, Gabriel E. Wagner1††, Harald F. Hofbauer1‡, Florian Sarklet1, Martina Setz1, Nina Gubensák1, Sabine Lichtenegger1, Salvatore Fabio Falsone*‡‡, Heimo Wolinski1, Simone Kosol‡‡‡, Chris Oostenbrink1, Sepp D. Kohlwein1 and Klaus Zangger2‡‡

From the Institutes of 1Chemistry and 2‡‡Pharmaceutical Sciences, University of Graz, 8010 Graz, Austria, the 3§Institute of Hygiene, Microbiology, and Environmental Medicine, Medical University of Graz, 8010 Graz, Austria, the 4¶1Institute of Molecular Biosciences, BioTechMed-Graz, University of Graz, 8010 Graz, Austria, the 5‡‡ Institute of Molecular Modeling and Simulation, University of Natural Resources and Life Sciences, 1190 Vienna, Austria, the 6¶¶1Department of Chemistry, University of Warwick, Gibbet Hill, Coventry CV4 7AL, United Kingdom

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Vacuolar ATPases are multisubunit protein complexes that are indispensable for acidification and pH homeostasis in a variety of physiological processes in all eukaryotic cells. An arginine residue (Arg735) in transmembrane helix 7 (TM7) of subunit a of the yeast ATPase is known to be essential for proton translocation. However, the specific mechanism of its involvement in proton transport remains to be determined. Arginine residues are usually assumed to “snorkel” toward the protein surface when exposed to a hydrophobic environment. Here, using solution NMR spectroscopy, molecular dynamics simulations, and in vivo yeast assays, we obtained evidence for the formation of a transient, membrane-embedded cation–π interaction in TM7 between Arg735 and two highly conserved nearby aromatic residues, Tyr273 and Trp373. We propose a mechanism by which the transient, membrane-embedded cation–π complex provides the necessary energy to keep the charged side chain of Arg735 within the hydrophobic membrane. Such cation–π interactions may define a general mechanism to retain charged amino acids in a hydrophobic membrane environment.

Vacuolar ATPases (V-ATPases)3 are proton pumps that utilize energy derived from ATP hydrolysis for proton translocation across membranes. They are involved in a multitude of processes, including membrane trafficking, neurotransmitter release, and tumor metastasis (1–6). V-ATPase malfunction is linked to a wide range of diseases, such as acidosis and osteopetrosis (7–9), which highlights these enzymes as attractive drug targets (10–13).

Eukaryotic V-ATPases are multiprotein complexes (Fig. 1) that consist of a peripheral V1 complex (subunits A–H) and an integral, membrane-embedded V0 complex (subunits a, d, e, c, c’, and c”). (14) ATP hydrolysis by V1 drives the rotation of the central stalk (subunits D and F) and the connected ring of proteolipid subunits (c, c’, and c”). (14, 15) Crystal structures as well as cryo-EM reconstructions show that a glutamic acid residue is crucial for proton transport of the different subunits c (Glu137), c’ (Glu145), and c” (Glu108) (15, 16). These residues might either be directly involved in transport or serve as gatekeepers for the binding pocket. (17–21). However, little structural information is available about the sites of entry and release of the protons into and out of the membrane, respectively.

Subunit a is believed to form two water-filled hemichannels through which the protons are transported. This is supported by a recent cryo-EM structure of an assembled ATPase from Thermus thermophilus (22). Thus, protons may enter the cytoplasmic hemichannel and are transferred to a specific glutamate residue of the proteolipid c subunits. After one ring rotation, the protons are passed on to the second hemichannel and released to the lumen (4, 3, 6). Arg735, located in transmembrane helix 7 (TM7) of subunit a, is essential for proton transport (23). It is thought to interact with the glutamate residues of the proteolipid ring and thereby enables deprotonation and transfer of protons to the luminal hemichannel (20). However, the position of charged amino acids, like arginine and glutamate, in the hydrophobic part of a membrane is energetically unfavorable. Although the negative charge of the glutamate residues in the proteolipid is neutralized by protonation, glyceraldehyde-3-phosphate dehydrogenase; HSCQ, heteronuclear single quantum correlation; TOCSY, total correlation spectroscopy; C-HSCQ, carbon-HSCQ; N-HSCQ, nitrogen-HSCQ; YPD, yeast peptone dextrose; NA, numerical aperture.
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Figure 1. Schematic of the assembly of the V-ATPase complex. ATP hydrolysis in the V₁ subcomplex leads to a rotation of the central stalk (D, F, d) that drives the rotation of the proteolipid c-ring. Protons are believed to be passed and released from the c-ring through two hemichannels in subunit a. Arg⁷³⁵ is essential for proton translocation across the membrane.

the mode of stabilization of Arg⁷³⁵ within subunit a has remained elusive.

There has been great interest concerning the protonation state and localization of charged amino acids, in particular arginine, in a lipid bilayer environment. Molecular dynamics (MD) simulations have revealed that a charged arginine in a transmembrane model would experience a large free energy barrier of ~ 17 kcal/mol to move across a lipid bilayer (24). In contrast to these calculations, a relatively low apparent free energy of ~2.5 kcal/mol was observed in cell biology experiments that studied the membrane partitioning of a hydrophobic protein segment containing an arginine residue (25). The situation is even more complicated by the unknown pKa value of arginine residues in a lipid environment, which usually eludes experimental determination (26). Theoretical as well as experimental work suggests that the guanidinium group of arginine either remains protonated inside the lipid membrane or resides close to the membrane–water interface (26, 27).

To clarify the role of Arg⁷³⁵ in V-ATPase function and to examine the environment and inter- and intramolecular interactions of this residue, we used solution NMR spectroscopy. Combined with MD simulations, this approach allows investigation of peptide structure and dynamics, providing detailed insights into Arg⁷³⁵ function (28, 29). Surprisingly, we found close spatial proximity between Arg⁷³⁵ and the two aromatic residues Tyr⁷³³ and Trp⁷³⁷ with subunit a of the V-ATPase complex. The results suggest a transient “cation–π interaction” (30–33) between Arg⁷³⁵ and these aromatic residues, which keeps the arginine residue in the hydrophobic environment and thus plays a crucial role in the catalytic mechanism. The physiological relevance of Tyr⁷³³ and Trp⁷³⁷ for V-ATPase assembly in the baker’s yeast *Saccharomyces cerevisiae* was indeed shown by growth assays and fluorescence microscopic analyses that demonstrate the essential roles of Tyr⁷³³ and Trp⁷³⁷ for proper V-ATPase function.

Results and discussion

**NMR solution structure and fluorescence spectra of TM7 reveal spatial proximity of the essential Arg⁷³⁵ and the two aromatic residues Tyr⁷³³ and Trp⁷³⁷**

In a previous study of TM7 of V-ATPase in membrane mimetics, we found evidence of Arg⁷³⁵ being located in the hydrophobic environment (34) rather than near the polar surface, as typically found for membrane-bound peptides. To investigate the structural features of TM7 that keep the charged side chain of Arg⁷³⁵ within the nonpolar core, we used a synthetic TM7 peptide with uniformly ¹³C/¹⁵N-labeled Arg⁷³⁵ embedded in a dodecylphosphocholine (DPC) micelle. DPC is a zwitterionic detergent that has been extensively used to mimic eukaryotic membranes (35).

The solution NMR structure (Fig. 2A), NMR statistics summarized in Table 1, PDB code 6HH0, BMRB accession number 34309) shows TM7 to adopt a transmembrane α-helix in the hydrophobic environment. Interestingly, Arg⁷³⁵ is in close contact with two aromatic residues, Tyr⁷³³ and Trp⁷³⁷. We observed NOEs from side chain protons Arg⁷³⁵-He and Hδ to the ring protons Tyr⁷³³-Hδ as well as Trp⁷³⁷-Hζ3 and Hη2. The calculated structure points to a cation–π interaction formed between the cationic side chain of the arginine and the polarizable π electron cloud of the aromatic groups. The close spatial proximity between the arginine side chain and the two aromatic residues at positions i−2 and i+2 would not be found without a specific interaction because the side chains of i±2 are usually on opposite sides of an α-helix. This can be seen in the structure of a double-mutant transmembrane helix peptide, TM7-Y733A-W737L (Fig. S1). Very recently, two cryo-EM structures of the V₀ domain of yeast V-ATPase, reconstituted in amphipole (at 3.9-Å resolution) (15) and nanodiscs (3.5-Å resolution) (16) have been reported. In these structures, Arg⁷³⁵ is embedded in a very long tilted α-helix (a₀CT₇) and extended toward Glu¹₀₈ in the c" subunit, although the resolution of these structures does not allow to unambiguously identify side-chain interactions. The aromatic residues Tyr⁷³³ and Trp⁷³⁷ are not as close to Arg⁷³⁵ as we have found them in the isolated helix (Fig. 3). However, Trp⁷³⁷ is also unusually bent around a₀CT₇ and therefore closer to Arg⁷³⁵ than the typical side-chain orientation in a free helix. Helix a₀CT₇ is kinked in the region around Arg⁷³⁵ and flexible in its N-terminal part, as indicated by the missing density. In both cryo-EM structures, a snapshot of Arg⁷³⁵ being close to Glu¹₀₈ of c" is captured. The increased flexibility of a₀CT₇ could be necessary to accommodate the rotation of the nearby c-ring. As indicated in Ref. 15, as rotation of the ring continues, the glutamate residue encounters Arg⁷³⁵, possibly in a different conformation, causing deprotonation of glutamate via the
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hydrophobic environment. It might just as well alternate with the salt bridge formed between Arg$^{735}$ and Glu$^{198}$ during c-ring rotation.

Cation–π interactions were first described about 30 years ago, but their importance was firmly established only recently (30, 36, 37), although these noncovalent interactions can be stronger than hydrogen bonds. The formation of a cation–π interaction involving a tryptophan residue in a protein can be monitored by fluorescence spectroscopy. Thereby, a cation–π formation in solution is characterized by a small red shift of a few nanometers, accompanied by an increase in fluorescence intensity of about 30–40% (38, 39). However, in contrast to spectra of proteins in solution, Peter et al. (40) reported even small blue shifts for a peptide embedded in different membrane mimetics. Comparing the fluorescence spectra of the WT and the R735L mutant of yeast V-ATPase, we observed the expected intensity increase of ~30% (Amax of 339.81 ± 1.40 nm for the WT and 341.66 ± 2.03 nm for R735L (Fig. 2B), which corroborates the spatial proximity of the essential Arg$^{735}$ and the two aromatic residues. The lack of the red shift might be explained by the use of membrane mimetics, as mentioned above.

The Tyr$^{733}$ and Trp$^{737}$ residues influence the immersion depth of the Arg$^{735}$ side chain

We hypothesized that the loss of the cation–π interaction and its charge stabilization of the arginine would directly influence the immersion depth of the Arg$^{735}$ side chain. To investigate the influence of Tyr$^{733}$ and Trp$^{737}$ on membrane immersion, we performed solvent paramagnetic relaxation enhancement (sPRE) measurements. (41–43) Gadolinium-diethylentriamine pentaacetic acid-bismethylamide (Gd(DTPA-BMA)) is an inert, water-soluble, paramagnetic agent (44). Addition to a solution renders the solvent around the micelle paramagnetic because Gd(DTPA-BMA) is unable to penetrate the micelle membrane (34). The interaction of the unpaired electron with a nuclear spin leads to enhanced relaxation (45). This sPRE is proportional to the inverse cube of the distance to the closest paramagnetic center (46, 41) and is, therefore, an immersion depth indicator for nuclei in the micelle. In other words, the closer an atom is to the surface of the micelle, the stronger the decrease in its peak intensity (hence, a high sPRE) after addition of the paramagnetic molecule (47). As we could not use the guanidinium protons for sPRE measurements because of fast chemical exchange, the H6 provides information closest to it and, hence, about its localization within the membrane. sPREs were measured by saturation–recovery $^1$H–$^{13}$C HSQC experiments of the WT peptide and a double mutant lacking the aromats (Y733A/W737L). Both contained uniformly $^{13}$C-labeled arginine for high accuracy and were embedded in DPC micelles. In the WT peptide, H6 of Arg$^{735}$ has a rather low sPRE of 0.32 ± 0.02 s$^{-1}$ mm$^{-1}$. A higher sPRE value, associated with less immersion, was found for the TM7 double mutant (H6 sPRE, 0.42 ± 0.02 s$^{-1}$ mm$^{-1}$). The effect can be explained by a shallower immersion and the absence of additional shielding by the aromatic groups in the double mutant. sPREs below ~0.3 s$^{-1}$ mm$^{-1}$ are typically found for nuclei close to the core of DPC micelles, whereas values above ~0.5 places

Figure 2. A, NMR structure of TM7 embedded in a DPC micelle (PDB code 6HH0). The essential Arg$^{735}$ (green) and the two aromatic residues (Tyr$^{733}$ in yellow and Trp$^{737}$ in orange) show the typical geometric arrangement of a cation–π complex. B, fluorescence emission spectra of TM7 versus a TM7 R735L mutant show the expected intensity decrease, indicative of the loss of a cation–π interaction. C, representative snapshot from MD simulations of NOE-restrained TM7 in a bilayer using the same coloring scheme for Tyr$^{733}$, Arg$^{735}$, and Trp$^{737}$. The MD simulation shows the possibility that water (red/white sticks) penetrates the bilayer, coming close to W737 (orange spheres), which might affect tryptophan fluorescence spectra.

luminal half-channel. We believe that the cation–π interaction observed in our structure constitutes this event, which is the preferred conformation in the absence of the nearby glutamate residue. An isolated transmembrane helix reconstituted in micelles is of course a very simple model of the corresponding helix in the whole-membrane protein. However, it shows that this interaction is strong enough to provide the energy needed to keep an arginine residue in a
them close to the surface. A value of 0.32 s$^{-1}$ mm$^{-1}$ as found for H6 of Arg$^{735}$ corresponds to an immersion depth of $\sim$ 12.1 Å from the surface (34), whereas 0.42 s$^{-1}$ mm$^{-1}$ for the mutant without aromatic groups corresponds to a localization 10.4 Å from the surface, which is just below the zwitterionic headgroup. For comparison, the sPREs of the chain CH$_2$ groups of DPC are 0.12 $\pm$ 0.01 and 0.10 $\pm$ 0.01 s$^{-1}$ mm$^{-1}$ for the solution containing the WT and the aromat-free TM7 peptide, respectively. This indicates that the increase in sPRE of the mutant without aromatic residues is not the result of a rearrangement in the DPC micelle.

**Table 1**

| NMR restraints | TM7 | TM7-Y733A/W737L |
|----------------|-----|-----------------|
| Distance restraints | 461 | 254 |
| Total NOEs | 254 | 161 |
| Intra-residues NOEs | 93 | 70 |
| Sequential NOEs ($|j| \leq 5$) | 114 | 23 |
| Medium-range NOEs ($|j| < 5$) | 20 | 10 |
| Hydrogen bonds$^a$ | 18 | 13 |

| Average CNS total energy (kcal/mol) | 275.8 $\pm$ 0.9 | 193.5 $\pm$ 1.2 |
| Average bond length energy (kcal/mol) | 11.5 $\pm$ 0.3 | 24.4 $\pm$ 0.2 |
| Average bond angle energy (kcal/mol) | 68.3 $\pm$ 0.6 | 69.0 $\pm$ 0.7 |
| Average van der Waals energy (kcal/mol) | 97.5 $\pm$ 1.1 | 38.9 $\pm$ 0.6 |
| Average NOE energy (kcal/mol) | 67.8 $\pm$ 0.9 | 52.9 $\pm$ 0.5 |
| Average RMSD from idealized bond length geometry (Å) | 0.0052 $\pm$ 0.0002 | 0.0078 $\pm$ 0.0003 |
| Average RMSD from idealized bond angle geometry (°) | 0.768 $\pm$ 0.003 | 0.785 $\pm$ 0.004 |
| Average number of NOE violations ($>0.5$ Å) | 0 | 0 |
| Average number of NOE violations ($>0.2$ Å) | 8 | 2 |
| Average number of major clashes ($>1.0$ Å) | 0 | 0 |
| Average number of minor clashes ($<1.0$ Å)$^b$ | 28 | 18 |

| RMSD from average (Å) | | |
|-----------------------|-------|
| Residues $5\cdots20$ | 0.54 |
| Backbone N,Ca,C' | 0.48 |
| Heavy atoms | 0.86 |
| Overall | 0.88 |

| Ramachandran plot | | |
|-------------------|-------|
| Residues in favored regions | 80.0% |
| Residues in allowed regions | 19.5% |
| Residues in outlier regions$^c$ | 0% |
| Completeness of chemical shift assignment | 96% |
| Overall 1$H$ | 92% |

$^a$ Defined as distance restraints with an upper distance of 2.5 Å.

$^b$ Some of those are close atom encounters within the standard bond geometry of CNS.

$^c$ All residues in outlier regions are near the less well-defined termini.

**Figure 3.** An enlargement of the region containing Tyr$^{733}$ (yellow), Arg$^{735}$ (green), and Trp$^{737}$ (orange) on a$_{CT}$ and Glu$^{108}$ (blue) in the $c'$ subunit of yeast V-ATPase from the cryo-EM structure (PDB code 6C6L) (16) is shown.
A pronounced change in the orientation of Arg\(^{735}\) relative to the aromatic groups was observed, which occurred already after a few hundred picoseconds in the simulations (Fig. 2C). More specifically, the side chains of Arg\(^{735}\) and Leu\(^{736}\) partly exchange positions. Upon this rearrangement, the arginine is positioned favorably to form cation–π interactions with the aromatic groups. The interaction with the tryptophan occurs for 96.2% of the time and is mainly in the stacked conformation, whereas the one with tyrosine in a T-shaped conformation is observed for 95.7% of the time. The tight packing of the bilayer probably enhances the formation of cation–π interactions in the MD simulation. More interestingly, a small number of water molecules were observed to penetrate the bilayer, which further stabilizes Arg\(^{735}\) by microsolvation (Fig. 1B). Taken together, the NMR data and the molecular dynamics simulations show favorable interactions of the guanidium group of Arg\(^{735}\) and the aromatic π systems of Tyr\(^{733}\) and Trp\(^{737}\), indicative of a cation–π interaction.

**Yeast in vivo assays confirm the biological significance of the aromatic residues for V-ATPase activity**

The results from in vitro experiments and MD simulations led us to investigate the relevance of the Tyr\(^{733}\)-Arg\(^{735}\)-Trp\(^{737}\) cation–π interaction within the V-ATPase complex in a physiological context. Residues involved in cation–π interactions are usually highly conserved (49). A BLAST search (50) identified a number of subunit a homologs in various organisms. A multiple sequence alignment of the homologs (ClustalX 2.1 (51)) revealed that both Tyr\(^{733}\) and Trp\(^{737}\), are highly conserved in the eukaryotic kingdom, indicating their biological importance (Fig. S2). Interestingly, similar patterns of conservation can be found for the evolutionarily related Na\(^+\)-ATPase and F-ATP synthase, providing further proof for the functional relevance of the aromatic residues. Replacement of the tyrosine by an aromatic phenylalanine, as observed in some prokaryotes, does not affect V-ATPase activity in S. cerevisiae, ruling out a hydrogen bridge formation as a mechanism of interaction (52).

Complete loss of V-ATPase activity is lethal in all eukaryotes except in fungi, which show distinct phenotypes under defined experimental conditions (4). Thus, we chose S. cerevisiae to study the role of Tyr\(^{733}\) and Trp\(^{737}\) for proper V-ATPase functionality. Malfunction disables acidification of vacuoles, leading to a Vma\(^-\) growth phenotype, which is characterized by sensitivity to elevated pH or calcium levels. (4, 53).

We hypothesized that the removal of the aromatic residues near Arg\(^{735}\) would prevent the formation of the cation–π interaction and thus impair proton transport in Y733L and W737L mutants. To test this hypothesis, an S. cerevisiae V-ATPase subunit a knockout strain (lacking both subunit a isoforms encoded by VPH1 and STV1) (54) was complemented either with an empty vector (EV) or plasmids carrying genes encoding the WT VPH1 or the mutations Y733L, W737L, and Y733L/W737L, respectively. Furthermore, the vph1 mutant variant R735K and R735L were used, as they are known to show either a mild or severe Vma\(^-\) phenotype, respectively (23). To monitor the impact of the various mutations on V-ATPase activity, we performed viability tests on rich culture medium at pH 5.5 and pH 7.5, both with varying calcium concentrations (Fig. S3A).

The W737L exchange led to a more severe growth defect than the Y733L mutation, suggesting a more critical role of the tryptophan residue on V-ATPase activity. The more severe effect of the Trp\(^{737}\) over the Tyr\(^{733}\) mutation is consistent with cation–π complex formation; its likelihood of formation and strength decreases from tryptophan to tyrosine and phenylalanine. (31) Importantly, the simultaneous exchange of both aromatic residues, Y733L and W737L, led to the same growth defects as for the EV indicating total loss of V-ATPase activity. As expected, increasing CaCl\(_2\) concentrations, especially in combination with elevated pH levels, resulted in more pronounced growth defects for all tested constructs.

Consistent with published data (23) and the proposed formation of a cation–π interaction, the strain bearing the chargeless R735L mutation led to a Vma\(^-\) phenotype at a level comparable with the vph1 stv1 strain expressing the EV. Notably, replacement of the cationic arginine residue by a cationic lysine (R735K, which forms weaker cation–π interactions) also led to a Vma\(^-\) growth phenotype at elevated pH, especially in the presence of CaCl\(_2\), pointing out the importance of the highly conserved arginine residue within the Vph1 protein in vivo.
To corroborate these results in greater detail, we performed quinacrine staining to monitor V-ATPase activity in living cells (Fig. 4B). Quinacrine accumulates only in acidified vacuoles and thus indicates proper V-ATPase function in vivo (55). The vph1 stv1 strains expressing the native VPH1 and the Y733L mutant were capable to acidify vacuoles at elevated pH levels. The other mutants (W737L, Y733L/W737L, R735K, and R735L) and cells bearing the EV showed no quinacrine staining of the vacuoles, indicative of loss of vacuolar acidification and, thus, V-ATPase function (Fig. 4B).

To rule out that the loss of V-ATPase function caused by distinct mutations in VPH1 was due to disassembly of the V-ATPase complex, we used chromosomally integrated Vma6-mGFP and Vma2-mGFP fusion proteins to monitor their cellular localization in the vph1 stv1 double knockout strain bearing the empty vector or the distinct VPH1 mutant variants (Fig. 5). Vma6 represents subunit d of the V_o integral membrane domain and is required for V_1 domain assembly on the vacuolar membrane, whereas Vma2 represents subunit B of the V_1 peripheral membrane domain of the V-ATPase complex. Colocalization studies of Vma6-mGFP and the vacuolar membrane dye FM4-64 (Fig. 5A) showed a high Pearson’s colocalization coefficient (PCC) for the native Vph1 (0.86 ± 0.01) and almost no colocalization for the empty vector control strain (0.08 ± 0.05). Mutation of either Y733L or W737L did not significantly affect V-ATPase complex assembly, whereas the double mutant Y733L/W737L showed a reduced PCC (0.53 ± 0.02). The R735K mutant was also slightly impaired in V-ATPase...
assembly (PCC = 0.64 ± 0.03), and the published loss-of-function mutant R735L showed the least colocalization (PCC = 0.45 ± 0.02). Immunoblot analyses showed comparable protein levels for native Vph1 and the Y733L, W737L, and R735K mutants in the strain expressing Vma6-GFP, whereas decreased Vph1 protein levels were detected for the Y733L/W737L and R735L mutants, which may be a result of lower expression or decreased protein stability (Fig. 5B). Vma1 levels were not affected by the various Vph1 mutant variants (Fig. 5B). Colocalization studies of Vma2-mGFP and the vacuolar membrane dye FM4-64 (Fig. 5C) showed similar PCC values as for Vma6-mGFP (Fig. 5A), except for the vph1 stv1 mutant strain bearing the EV. Immunoblot analyses of the WT and mutant strains expressing Vma2-mGFP (Fig. 5D) showed almost identical protein levels for the various Vph1 mutants as for the strains expressing Vma6-mGFP (Fig. 5B). It should be noted that the protein levels were analyzed from whole-cell lysates and thus only serve as a control for proper (or improper) expression of the different Vph1 variants and not for analysis of V-ATPase complex assembly. Because Vma2 is a peripheral subunit of V-ATPase, disassembly of the complex leads to cytosolic localization of Vma2 and, thus, to an unspecific overlap with vacuolar membranes. This could explain the higher PCC value for Vma2 in the vph1 stv1 strain bearing the empty vector (PCC = 0.41 ± 0.10) compared with Vma6, which is an integral subunit that remains localized on organelar membranes in the vph1 stv1 strain bearing the empty vector but stays strictly separated from vacuolar membranes (PCC = 0.08 ± 0.05).

Taken together, the in vivo experiments clearly support the physiological importance of the Tyr733-Arg735-Trp737 cation–π complex in Vph1 TM7; the two single mutant variants Y733L and W737L both showed a Vma− phenotype with a more severe impact of W737L (Fig. 4A) even though the assembly of the V0V1 complex was not impaired, as monitored with live-cell fluorescence microscopy (Fig. 5C). The double mutation of Y733L/W737L in Vph1 results in total loss of V-ATPase activity (Fig. 4), as shown previously for the R735L loss-of-function mutation (23). The mislocalization of Vma6-mGFP and Vma2-mGFP in the vph1 stv1 strain expressing the Y733L/W737L double mutant or the R735L variant indicates disassembly of the V0V1 complex. It is not clear yet whether the functional impairment of the cation–π interaction in Vph1 leads to V-ATPase disassembly or whether these two point mutations in Vph1 impair V-ATPase assembly, and thus its function, per se.

The in vivo experiments confirm the biological significance of the highly conserved aromatic residues for V-ATPase activity and point out the importance of the two aromatic residues for proton transport, complementing the results from MD simulations and NMR spectroscopy. Our results are also corroborated by a study using disulfide-mediated cross-linking that identified a Y733C mutation that compromises growth at pH 7.5 (56), which, according to our model, can be attributed to disruption of the cation–π complex by this mutation. Furthermore, Toei et al. (52) found a Y733F mutation to show WT growth at pH 7.5, confirming the need for an aromatic amino acid at position 733 but not the demand for a hydrogen bond donor.

Despite significant recent progress in understanding V-ATPase function, the role of Arg735 in V-ATPase proton transport is not entirely clear. We propose a novel mechanism (Fig. 6) by which the cation–π interaction helps to keep the positive charge of Arg735 within the membrane increasing its immersion depth, as confirmed by solvent PRE measurements. This, in turn, enables interactions with the glutamic acid residues involved in proton transport. The cation–π interaction might accommodate the arginine residue during the periods when no ionic interactions take place and create a counteracting force weakening the salt bridge between the arginine and the glutamate. The energy involved in the formation of a cation–π interaction (57) is in the same range as the calculated free energy change (~17 kcal/mol) of moving an arginine through a lipid membrane (24). Considering the high degree of conservation of aromatic residues near the arginines in ATPase-related enzymes, cation–π interactions might be a general mechanism to allow the localization of charged arginine residues in membrane environments.

**Conclusion**

We found that the catalytically active arginine residue Arg735 of yeast V-ATPase is positioned in the hydrophobic environment close to two aromatic residues (Tyr733 and Trp737) when embedded in membrane mimetics. These two aromatic residues in the yeast V-ATPase subunit a were identified to play a crucial role in V-ATPase activity in vivo. The findings provide strong support for a cation–π complex formed between Arg735 and its aromatic neighbors, Tyr733 and Trp737. Experimental
evidence indeed suggests that thermodynamic stabilization resulting from cation–π interaction is sufficient to overcome the energetic cost of transferring a positive charge of the guanidinium group of an arginine residue into the hydrophobic membrane environment. Based on the highly conserved nature of the residues identified in this study, similar mechanisms for proton or sodium translocation in evolutionarily related enzymes such as ATP synthases or sodium-transporting ATPases, respectively, appear plausible.

**Experimental procedures**

**Peptides and chemicals**

The peptide TM7 (KKSHTASYRLWALSLAHAQLSSKK) labeled with 13C/15N arginine was purchased from EZBiolab Inc. Mutant peptide TM7 R735L was synthesized by Pepnome Limited Inc. and Arg735 13C/15N-labeled Y733A/W735L TM7 by Chinapeptides. DPC-d38 (98%) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Gd(DTPA-BMA) was purified from the commercially available MRI contrast agent Omniscan as described previously (37). All other chemicals were purchased from Sigma-Aldrich in the highest purity available.

**NMR spectroscopy**

All NMR experiments for the solvent PRE determination, assignment, and solution structure determination were carried out at 25 °C on a Bruker Avance III (Bruker BioSpin, Karlsruhe, Germany) 700-MHz spectrometer equipped with a TCI cryoprobe. Solvent PREs were determined for all peptides dissolved at a concentration of 1 mM in potassium phosphate buffer (50 mM, pH 5.0) and 0.02% sodium azide containing 100 mM perdeuterated dodecylphosphocholine (DPC-d38). Proton T1 relaxation times were determined by titrating the samples with Gd(DTPA-BMA) (60 mM) to final concentrations of 0.5, 1, 2, and 3 mM. Proton T1 relaxation times were obtained from a series of C-HSQC spectra with a saturation recovery sequence as described previously (46). Peak intensities were then fitted to

\[ I = I_0 (1 - e^{-t/T_1}) \]  
(Eq. 1)

Paramagnetic relaxation enhancements were then obtained by fitting longitudinal relaxation rates as a function of gadolinium concentration as described previously (46, 45). For the assignment and solution structure determination, TOCSY, NOESY C-HSQC, N-HSQC, NOESY–N-HSQC, and NOESY–C-HSQC-spectra were acquired. Data were processed with NMR-Pipe (58). Peaks were assigned manually.

**Structure determination**

Peak intensities in NOESY spectra were translated by the program NMRView (59) into distance restraints using the built-in median method. The median intensity was set to a distance of 2.7 Å. Additionally, Φ and ψ dihedral angle restraints were obtained using the program TALOS, (60) based on Hα proton as well as Ca and Cβ carbon chemical shifts. 461 NOEs and 20 dihedral angle restraints were used for the structure determination of WT TM7, whereas 254 NOEs and 10 dihedral angle restraints were used for the Y733A/W737L double mutant (Table S1). NMR solution structure calculations were performed using CNS 1.2 (61). The structure calculation was carried out using the full simulated annealing method. In the final round of structure calculations, hydrogen bonds were imposed as distance restraints exclusively for regions of regular α-helical structure identified in previous structure calculation runs. For each peptide, 30 structures were calculated, and the 10 lowest energy conformations, which did not show any NOE violations larger than 0.5 Å, were selected. The Ramachandran map, hydrogen bonds, secondary structure elements, and root mean square deviations were calculated and analyzed using Molmol (11) and RAMPAGE (62).

**Molecular dynamics simulation**

The MD simulation was performed using the GROMOS11 software package for biomolecular simulations (63) in conjunction with the 54A8 parameter set to describe interactions (64). In this force field, the partial charges for aromatic systems were fitted to reproduce the quadrupole of e.g. benzene. This means that there is a small negative charge on the aromatic carbons and a small positive charge on the aromatic hydrogens. The aromatic carbons then allow interactions with cations to potentially enable the formation of cation–π interactions. The initial structure of TM7 was selected from the NMR structure bundle obtained by NOESY experiments described in this work. To mimic experimental conditions as described by Hesselink et al. (65) as closely as possible, a bilayer consisting of 51 dioleoylphosphatidylcholine and 13 dioleoylphosphatidylglycerol molecules in each leaflet was constructed and equilibrated for 30 ns in simple point charge water, and the peptide was manually inserted after removing two dioleoylphosphatidylcholine molecules in each leaflet. All four lysines and Arg735 were fully protonated to reflect a pH of 7.0, corresponding to experimental conditions. The simulation box was solvated with approximately 5,500 simple point charge water molecules. No counterions were added in the simulation system. Nonbonded interactions were calculated using a cutoff of 14 Å, complemented with a reaction field contribution to account for a homogeneous medium outside the cutoff sphere (66). Atomic motion was integrated with the leapfrog algorithm, a time step of 2 fs, and SHAKE (67) constraints on all bond lengths. Agreement with NOE intensities was enforced through 309 time-averaged distance restraints (68) with an attractive harmonic force constant of 2000 kJ mol⁻¹ nm⁻². Interproton distances were averaged as \( r^{-3} \) with a memory decay time of 30 ps. Because of this averaging, the NOEs do not have to be fulfilled all the time but only a fraction of the time, so the individual snapshots of the simulations can be much more diverse than observed in a single-structure determination. The simulation was performed at a constant temperature of 300 K and a pressure of 1 atmosphere with the weak coupling scheme (69) with relaxation times of 0.1 ps and 0.5 ps, respectively. Semianisotropic pressure scaling was used with a decoupled z axis, and the isothermal compressibility was estimated at 4.575 × 10⁻⁴ (kJ mol⁻¹ nm⁻²)⁻¹. The simulation was performed for 100 ns. Cation–π interactions were defined using the distance between the geometrical centers of the guanidinium, indole, and hydroxyphenyl groups and the angle...
between their respective planes. The angle was defined as the angle between the normal vectors on the planes of the side-chain rings or the guanidinium group. To qualify as a cation–π interaction, the distance had to be below 5.5 Å. If the side chains encompassed an angle between 45° and 135°, the cation–π interaction was defined as “T-shaped” or otherwise as “stacked” (70). The secondary structure of TM7 was classified with DISICL (71).

**Tryptophan fluorescence spectroscopy**

Fluorescence emission spectra of a 1 μM solution of TM7 and the TM7 R735A mutant in potassium phosphate buffer (50 mM pH 5.0) containing 100 mM DPC-d38 were recorded between 300 and 400 nm in a Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, CA) at 25 °C and an excitation wavelength of 295 nm. The slit widths were 5 nm and 10 nm for excitation and emission, respectively. Each spectrum is a background-corrected average of three accumulations.

**Yeast strains, plasmids, and media**

The WT strain BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was from EUROSCARF (Frankfurt, Germany). The vph1 stv1 double deletion strain MM112 (MATα vph1:LEU2 stv1:LYS2 his3–200 leu2Δ0 lys2Δ0 ura3–52) and the plasmid MM322 (VPH1 in pRS316; containing a CEN sequence and a URA3 marker) (54) were kindly provided by Dr. Michael Forgac.

Rich medium (YPD) contained (per liter) 10 g of yeast extract, 20 g of peptone (both from BD Biosciences) and 20 g of glucose. Synthetic defined selective medium lacking uracil (SD-ura) contained (per liter) 6.7 g of Difco yeast nitrogen base without amino acids (BD Biosciences), 20 g of glucose, 20 mg of adenine, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 300 mg of threonine, and 20 mg of tryptophan. Media for drop tests were buffered with 100 mM MES to pH 5.5 or with 100 mM sodium succinate to pH 7.5 and solidified with 2% agar (BD Biosciences). Quinacrine dihydrochloride was purchased from Sigma and FM 4-64 from Life Technologies.

**Cultivation conditions and viability test**

Cells were grown at 30 °C overnight in SD-ura medium to late logarithmic growth phase and then shifted to 5 ml of YPD medium with a starting optical density at 600 nm (A600) of 1/ml and grown at 30 °C to the logarithmic phase for an additional 3 h. Cells were harvested, washed, and resuspended in sterile water (A600 = 1/ml). 5–μl aliquots of serial 1:10 dilutions starting with A600 = 1/ml were spotted on the indicated plates, and growth phenotypes were analyzed after 2 days of incubation at 30 °C.

**Quinacrine staining**

Cells were grown as described under “Cultivation conditions and viability test.” A cell aliquot at A600 = 0.5 was harvested and incubated for 10 min in 1 ml of YPD medium buffered with 100 mM sodium succinate to pH 7.5 containing 200 μM quinacrine. Cells were washed with 1 ml of ice-cold glucose solution (20 g/liter) buffered with 100 mM sodium succinate to pH 7.5 and mounted on solid agar slides for fluorescence microscopy (73, 74). Quinacrine fluorescence was excited at 488 nm, and fluorescence emission was detected between 500–550 nm using a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) and an HCX PL APO ×63 oil immersion objective (NA = 1.4). Images were exported using Leica LAS Lite software (Leica Microsystems), and brightness and contrast were adjusted for improved representation.

**Microscopic analysis of the V-ATPase complex**

Cells were grown as described under “Cultivation conditions and viability test,” with the exception that the YPD medium contained 1 μg/ml FM 4-64 dye to stain the vacuolar membrane. As this dye is taken up via endocytosis, cells were grown for a total of 6 h to deplete dye from the plasma membrane and endocytic vesicles and to visualize only vacuolar membranes (75). Cells were harvested and mounted on solid agar slides for fluorescence microscopy. Fluorescence of monomeric GFP was excited at 488 nm, and fluorescence emission was detected between 500–550 nm. FM 4-64 dye was excited at 488 nm, and fluorescence emission was detected between 590–700 nm. Experiments were performed using a Leica TCS SP5 confocal microscope and an HCX PL APO ×63 oil immersion objective (NA = 1.4). Images were exported using the Leica LAS Lite software (Leica Microsystems), and brightness and contrast were adjusted for improved representation. The PCC was determined using the open-source Fiji software (76) following the instructions in the manual. PCC values are presented as

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**A cation–π interaction facilitates proton transport**

Fluorescence emission spectra of a 1 μM solution of TM7 and the TM7 vph1 stv1 strains was performed using the lithium acetate method (72). Selection of mutants was performed on YPD plates containing 100 μg/ml nourseothricin (clonNat; Werner BioAgents, Jena, Germany). Transformation of plasmids bearing WT VPH1 or mutated versions of VPH1 into BY4742 and vph1 stv1 strains (with or without chromosomally expressed Vma2-mGFP or Vma6-mGFP fusion protein) was conducted using the lithium acetate method, and cells were selected on SD-ura plates.
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mean ± S.D. of three independent images comprising about 50 cells per image.

**Immunoblot analysis of V-ATPase subunits**

Cells were grown as described under “Cultivation conditions and viability test.” A cell aliquot of A_{600} = 3 was harvested, and proteins were isolated according to Baerends et al. [77], separated on 10% SDS-polyacrylamide gels, and blotted onto nitrocellulose membranes. The protein levels of V-ATPase subunits were monitored using the mouse monoclonal antibodies 8B1-F3 against Vma1 and 10D7 against Vph1 ([22]), followed by a horseradish peroxidase– conjugated secondary anti-mouse antibody from goat (31430, Thermo Scientific, Waltham, MA). Yeast glyceroldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for equal protein loading. Signals were detected by the SuperSignal® WestPico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA).

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