Cryo-EM structures of intact V-ATPase from bovine brain

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The vacuolar-type H\(^+\)-ATPases (V-ATPase) hydrolyze ATP to pump protons across the plasma or intracellular membrane, secreting acids to the lumen or acidifying intracellular compartments. It has been implicated in tumor metastasis, renal tubular acidosis, and osteoporosis. Here, we report two cryo-EM structures of the intact V-ATPase from bovine brain with all the subunits including the subunit H, which is essential for ATPase activity. Two type-I transmembrane proteins, Ac45 and (pro)renin receptor, along with subunit c\(^\ast\), constitute the core of the c-ring. Three different conformations of A/B heterodimers suggest a mechanism for ATP hydrolysis that triggers a rotation of subunits DF, inducing spinning of subunit d with respect to the entire c-ring. Moreover, many lipid molecules have been observed in the Vo domain to mediate the interactions between subunit c, c\(^\ast\), (pro)renin receptor, and Ac45. These two structures reveal unique features of mammalian V-ATPase and suggest a mechanism of V1-Vo torque transmission.
The pH homeostasis is essential for the physiological function of cells and cellular organelles. An acidic pH is important for many physiological processes, such as renal acidification, bone resorption, and sperm maturation. V-ATPases localize to the plasma membrane, endosomes, and membrane vesicles and play an important role in proton homeostasis. V-ATPase is involved in autophagy, metabolism, and neurotransmitter secretion. Recycling of cell receptors is facilitated by V-ATPase through endocytosis. For example, the low pH generated by V-ATPase promotes the dissociation of ligands, such as mannose 6-phosphate receptor, which recognizes lysosomal enzymes in Golgi and releases them in prelysosomes by its multiple repeats, facilitating the receptor recycling. Moreover, V-ATPase activity is involved in Wnt, mTOR, and Notch signaling. The aberrant activity of V-ATPases in humans is associated with many pathological processes and diseases, such as tumor metastasis, cutis laxa, distal renal tubular acidosis, autosomal recessive osteopetrosis, infectious diseases, and neurodegenerative diseases.

Mammalian V-ATPase is a rotary machine made up of two domains: the ATP-hydrolysing V1 domain and the proton-translocation Vo domain. The V1 domain consists of three catalytic AB heterodimers that form a heterotrimer with threefold rotational pseudosymmetry, three peripheral stalks each consisting of the subunits EG, one central rotor including subunits D and F, and the regulatory subunits C and H; the proton-translocation domain Vo consists of the proton transport subunit a, a ring of proteolipid subunits c6c", rotary subunit d, and subunits e and Ac45. The subunits B, C, E, G, H, a, d, and e of mammalian V-ATPase have multiple isoforms in various tissues and cells, causing a technical barrier to study its structural and biochemical features due to heterogeneity.

The V-ATPase is structurally and evolutionarily related to the F1F0-ATP synthases and the archaeabacterial ATP synthases termed V/A-ATPases. The composition of the mammalian V1 domain is similar to that of the yeast domain, which is a well-characterized model for V-ATPase studies. The c-ring of yeast's V0 domain contains eight subunit c, one subunit c', and one subunit c". In the V/A-ATPase, there are two subunit E and two subunit G in the V1 domain, and twelve subunits c that form a homogeneous c-ring in the V0 domain. Structural insights into V-ATPase function has been gleaned from structures of the intact V-ATPase from Saccharomyces cerevisiae (scV-ATPase) at 7Å resolution, structures of the intact V/A-ATPase from Thermus thermophilus (ttV/A-ATPase), cryo-EM structures of the scVo domain at atomic resolution, and most recently published, the structure of rat V-ATPase with its bacterial inhibitor SidK.

However, the intact structure of a mammalian V-ATPase with the ATPase activity is still unknown.

In this paper, we purified the intact V-ATPase from bovine brain with the specific ATP hydrolysis activity at about 1.4 μmols of Pi·mg of protein−1·min−1 and report two cryo-EM structures in the distinct rotational states at overall 3.4Å and 3.8Å resolution, respectively. The structures show two unique mammalian V-ATPase components, Ac45 and (pro)renin receptor (PRR) in the V0 domain. This structural work reveals a high-resolution map of the V-type ATPase of bovine brain showing the interaction details between PRR and Ac45. It will also aid in the design of small molecules for the treatment of related human diseases.

### Results

**Overall structure of the Bos taurus V-ATPase.** The *Bos taurus* V-ATPase (btV-ATPase) was purified from bovine brain as previously reported. The resulting complex was further purified by gel filtration in the presence of 0.1% CHAPS and 0.004% glyco-diosgenin (GDN) (Supplementary Fig. 1a). The purified protein exhibits high ATPase activity and can be inhibited by baflomycin A1 in vitro (Supplementary Fig. 1b). The identity of each component of the btV-ATPase was confirmed by mass spectrometry following SDS-PAGE (Supplementary Table 1). This complex was prepared on grids and subject to cryo-EM. The btV-ATPase particles are homogenous showing clear features in the cryo-EM images, making them suitable for high-resolution structure determination (Supplementary Fig. 2). The 3D classification enabled us to distinguish two different states. The 3D refinement of individual classes yielded a resolution of 3.4 Å (state 1) and 3.8 Å (state 2) (Fig. 1a–d). The homology models for subunits A-H of V1 and subunits a, c, c", d, e of Vo were generated by MODELLER based on the multiple sequence alignments between the bovine subunits and the corresponding subunits of previously determined structures (Supplementary Fig. 3).

The local resolution of the V1 domain is better than that of the Vo domain in both states 1 and 2 (Supplementary Fig. 4). To improve the quality of the Vo and V1 density, we performed a local refinement by specific Vo and V1 masks, respectively, which resulted in a density that shows clear features of the side chains, helping the unambiguous assignment of most residues (Supplementary Figs. 5 and 6). We built B2, C1, E1, G2, a1, d1, and c2 into the final model based on mass spectrometry results and the expression distribution in brain tissues. The maps after local refinement were merged by “phenix.combine_focused _maps” for model building (Supplementary Fig. 6 and Supplementary Table 2). The overall dimensions of btV-ATPase are 160 Å×100 Å×270 Å. Due to the higher resolution of state 1, we discuss the structure of state 1 in the following section, except for the structural comparison of state 1 and state 2.

The subunit H is crucial for the V-ATPase activity. A structural study showed that without the Vo domain, subunit H can bind to the A3B3 hexamer to prevent the rotation of V1 domain, thereby blocking ATPase activity. In both states of our study, subunit H is well determined in the cryo-EM map (Fig. 1a, d). Structural comparison did not reveal notable conformational changes in subunit H with a root-mean-square deviation (RMSD) of 0.656 Å for 365 Cα atoms. The N-terminal helices of subunit H (HNT) bind to the subunits E1 and G2 (dash box in Fig. 1c) and along with the C-terminal helices of subunit H (HCT)—interact with the cytosolic domain of subunit a1 (Fig. 1c). This contact may keep the subunit a1 in a certain conformation conducive for proton translocation.

**A3B3 hexamer for ATP hydrolysis.** The A3B3 hexamer hydrolyzes ATP to provide energy for the rotation of the V0 domain (Fig. 2a, b). Both the A and B subunits have three domains: an N-terminal β-barrel domain, a middle nucleotide-binding α/β domain, and a C-terminal α-helical domain (Fig. 2c). ATP hydrolysis occurs at the interface between the nucleotide-binding α/β domains of subunits A and B. In state 1, three AB heterodimers exhibit distinct conformations (ABsemiclosed, ABclosed, and ABopen) (Fig. 2c; Supplementary Fig. 7). The structural comparison of the AB heterodimers with those of ttV/A-ATPase shows that the three AB heterodimers share a similar conformation with ABsemiclosed (ADP-bound) and ABopen of ttV/A-ATPase with RMSD at 2.0 Å, 0.8 Å, and 0.9 Å, respectively (Supplementary Fig. 8). ABopen has a pocket open to the cytosol showing a high affinity to accommodate an ATP molecule. Density representing the di-phosphate of ADP with magnesium was found in ABclosed (Fig. 2d) that binds ADP in ttV/A-ATPase. Therefore, we built an ADP with a magnesium ion into ABclosed (Fig. 2c, d).
Notably, an unknown density was observed in ABsemi (Fig. 2d). This unknown molecule binds to a hydrophobic area of subunit A of ABsemi and the Walker A motif (G250AFGCQ257) of this subunit in the cryo-EM map is not clear (Fig. 2d). The residues 258–264 in the ABsemi form a loop to accommodate this unknown molecule; by contrast, these residues form an α-helix in ABclosed and ABopen (Fig. 2d). It is possible that it represents a nucleotide, since the ABsemi exhibits an intermediate conformation for releasing ADP. This small molecule also may be a CHAPS detergent or another unknown molecule. Further investigation is required for identification of this molecule. The C-terminal α-helical domains of ABsemi and ABclosed are shifted by ~10 Å compared with those in ABopen (Fig. 2c), demonstrating substantial conformational plasticity in the A3B3 hexamer. The structural analysis shows that the ABsemi, ABclosed, and ABopen heterodimers share similar conformations in states 1 and 2 (Supplementary Fig. 7).

Overall structure of Vo domain. The subunit composition of the Vo domain considerably differs among species. The Vo domain of ttV/A-ATPase includes subunits adc12, whereas the yeast V-ATPase contains subunits acecedf. However, the Vo domain of bovine V-ATPase possesses subunits acecedAc45, as previously reported17,27. Three extra TMs (transmembrane helices) in the center of btVo domain have been observed at the edge of the c-ring (Figs. 1c and 3a). Because two analogous α-helices have been identified as subunit f in the scVo structure, we followed this nomenclature and named these helices as subunit f. To date, the function of subunit f is unknown, and knockout of this subunit does not affect the function of V-ATPase in yeast. Mass spectrometry results and our cryo-EM data of the btV-ATPase indicate that subunit f may be the RNaseK that was shown to associate with V-ATPase32. The specific function of RNaseK in V-ATPase still requires further characterization.

Interaction details of Vo domain. The N-terminus of Ac45 contains two luminal domains (Fig. 4b). A previous study showed that furin protease can cleave the linker between the two domains33. Consistent with this observation, only the second luminal domain and TM domain (residues 251–455) are present in our cryo-EM map. Glycosylation sites in this domain facilitated unambiguous residue assignment (Supplementary Fig. 6c). The second luminal domain of Ac45 was also found in a low-resolution cryo-EM map of bovine V-ATPase, supporting our
observation. Similarly, the luminal domain of PRR is not observed in the map, since it has been cleaved by furin protease. The residues 293–313 of renin receptor binds to the luminal domain of Ac45 that contains 14 β-strands and a disulfide bridge between Cys369 and Cys416 (Fig. 4b, c). The structural analysis shows that PRR engages in several hydrophobic interactions with the TM of Ac45 and TM1 of c" (Fig. 4c). Besides the hydrophobic contacts between PRR, Ac45, and subunit c", Lys299 of PRR interacts with Gln270 of Ac45 and Glu43 of subunit c" have hydrophilic interactions with Gln358 of Ac45 to further stabilize the core of the c-ring (Fig. 4c).

Many phospholipid molecules were observed in the Vo domain, particularly in the luminal leaflet, indicating that they may play a role in stabilizing the core of the Vo domain (Fig. 3b; Supplementary Fig. 6c). From the density, we can distinguish that these densities belong to the phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS). The previous studies showed that purified
endogenous btV-ATPase could be associated with a mixture for these lipids. Reconstitution of the V-ATPase complex with proton-pumping activity requires the liposomes containing a mixture of PC, PE, PS, and cholesterol. Since we could not identify these phospholipids in each map, we tentatively docked PC as a lipid representative into our cryo-EM map. These lipids may provide a hydrophobic environment to maintain the conformation of the c-ring core (Fig. 3). Interestingly, a sterol-like density was observed at Trp330 of PRR in the cryo-EM map (Fig. 4d), suggesting that a lipid molecule may stabilize the interaction of PRR with subunit c₂ in Vo.

Proton translocation by Vo. In the state 1, one pair of subunit EG interacts with subunit a, while the other pair engages with subunit C, which binds to subunit d; moreover, subunit d binds to subunits DF to connect the V1 and Vo domains (Fig. 1a). The interaction between subunits d and C will be released during the rotation (Fig. 1e). The C-terminal TM of Ac45 interacts with subunit d and subunit c₁ (Fig. 5a). The subunit d also makes several contacts with c_, c₁, c₂, c₇, and c₈ (Fig. 5a). These interactions are retained in the two states, suggesting that the fixed conformations of the peripheral subunits can facilitate the rotation of subunits DFd and of the c-ring during ATP hydrolysis and proton translocation. These observations imply that the subunit d is the key component to connect the c-ring with subunits DF of the V1 domain.

The subunit a contains two domains: an N-terminal cytoplasmic region (aNT) and eight C-terminal TMs (aCT) (Fig. 5b). Comparing the structure to yeast Vo, the yeast Vo domain is in an autoinhibit state after dissociation with V1 with subunit a N-terminal domain (residues 186–258) bound to subunits c and d, the conformational change on the N-terminus of subunit a in non-inhibitory state induces it interacting with subunits H, C, and two peripheral stalks which facilitate the V1 and Vo assembly and ATP hydrolysis coupled proton translocation. There are two aqueous half-channels in subunit a near the interface to the c-ring providing access for protons from the cytoplasm to lumen or extracellular space. We hypothesize that a proton accesses the lipid bilayer through the cytoplasmic half-channel to neutralize the negative charge of a conserved residue Glu139 of subunit a that would directly transfer the proton to Arg741 of subunit a; then, the protonated Arg741 would deliver the proton into the luminal/extracellular half-channel. Proton release may be facilitated by the residue Glu795 of subunit a, which is conserved in eukaryotic V-ATPases.

The structures of the A₃B₃ hexamer in the two states showed that ATP hydrolysis triggers a conformational change in the subunit DF, which further induces a shift of subunit d. The c-ring is subsequently rotated and results in a continuous proton translocation across the membrane. Notably, Glu98 of the subunit c disrupts the pattern that is created by the key Glu residues of the nine c subunits in the c-ring, causing an asymmetric distribution of these Glu residues. The structural analysis shows that the TM1 of subunit c, Ac45, and PRR do not change their relative positions in these two states. However, they have rotated ~120° with the c-ring, suggesting that the entire c-ring including subunits c, c̃, Ac45, and PRR function as a rigid domain in proton translocation.
**Discussion**

We compared the structures of state 1 and state 2, with the previously reported model of V/A-ATPase to elucidate the rotational mechanism. We found that the switch of AB heterodimers (Fig. 2b) provides the energy necessary to trigger a rotation of subunits DF resulting in the spinning of subunit d coupled with the entire c-ring region, and each state will rotate ~120° (Fig. 5e). Remarkably, the complex interaction network between subunit d, TM1 of subunit c, Ac45, PRR, and lipids in our cryo-EM data, provides multiple fulcrums to facilitate the rotation of the entire c-ring region between different states.

Three different rotational states of yeast V-ATPase and ttV/A-ATPase had been found in the cryo-EM maps; in contrast, our cryo-EM maps reveal only two rotational states of btV-ATPase (Figs. 1a, d and 5e). The previous structural studies revealed that the endogenous ATPase exhibits the unequal population for three different classes, such as the yeast V-ATPase with state 1 (47%, 6.9 Å), state 2 (36%, 7.6 Å), state 3 (17%, 8.3 Å), and the ttV/A-ATPase with state 1 (66%, 5.0 Å), state 2 (16%, 6.7 Å), state 3 (7%, 7.5 Å), and 11% bad class. These findings imply that state 3 shows the least population and the worst resolution after 3D refinement. We speculate that the third state, where the subunit c faces subunit a, may not be stable in our bovine tissue preparation or exists in very few populations that could not be captured, as the similar results observed in some studies.

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**Fig. 5 Proton translocation and the rotation of Vo domain.**

- **a** Overall structure showing the connection between subunit d and c-ring.
- **b** The structural elements of subunit a1 with subunit H, e2, and f. The transmembrane helices of a1 have been labeled.
- **c** Structural comparison with auto-inhibitory yeast Vo domain (pdb: 6O7T).
- **d** The putative proton-translocation mechanism. The residues for the proton transfer are shown in sticks. The cryo-EM map of subunit a is shown.
- **e** The cytosolic view of Vo showing the rotation of c-ring in two states. The Glu residues for protonation in state 1 are shown in stick.
The structures of rat V-ATPase with SidK, a bacterial inhibitor of V-ATPase, has been reported recently. This remarkable work showed that Ac45, PRR, and RNAseK are essential components of Vo, consistent with our analysis. In the rat V-ATPase structures, three conformationally different AB heterodimers have been reported: one is bound to ADP and the other two represent nucleotide-free states. When comparing our structure with the rat V-ATPase structure, we find that their conformations of AB\text{open} and AB\text{closed} share similar conformations with our AB heterodimers (Supplementary Fig. 1a; b); however, the C-terminal helical domain of subunit A in the AB\text{closed} state of SidK bound V-ATPase has a -3 Å shift to make the AB\text{closed} state more compact than the active form (Supplementary Fig. 1c). The structural comparison reveals that the subunits EG directly (Supplementary Fig. 1d). The V₁ and Vo structures of rat V-ATPase adopt a similar conformation to those in bovine V-ATPase structures state 1 and state 2, respectively. Due to the limitation of the resolution of the rat V-ATPase structure, the luminal domain of Ac45 could not be modeled; in contrast, we built the entire second luminal domain of Ac45 showing the interaction details of the Ac45 and PRR (Fig. 1c; Supplementary Fig. 1e). In summary, we report the mammalian V-ATPase structure including all the essential subunits, revealing a more complete map of this important rotary machine.

**Methods**

**Gaining clathrin-coated vesicles.** About three defatted bovine brains were rinsed and blended with 0.1 M Na-MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, and 3 mM NaN₃ (buffer A). Usually, one kilogram of the cleaned tissue is homogenized in 900 ml of buffer A. The homogenate is centrifuged in a GSA (Sorvall) rotor for 50 min at 20,000 g, and the supernatant is centrifuged at 140,000 g for 1 h to sediment the membrane vesicles. The pellet containing enriched coated vesicles is resuspended in buffer B at a protein concentration of 15 mg/ml, frozen in liquid nitrogen, and stored at -80 °C.

**Purification of V-ATPase.** The V-ATPase was solubilized and purified using an established protocol. Briefly, the membrane vesicles incubated with 0.75 M Tris-HCl pH 8.0 for 1 min to strip clathrin. After centrifugation at 150,000 g for 35 min, the pellet was resuspended in 0.5% Na-cholate and incubated at 0 °C for 30 min. After centrifugation at 150,000 g for 35 min, the pellet was resuspended in 0.75% Na-cholate and 10 mM Tris-MES pH 6.75, incubated on ice for 60 min and centrifuged at 150,000 g for 90 min.

The supernatant was applied to a hydroxylapatite column which had been equilibrated with 0.1% C₆H₁₂O₇, 10% glycerol, 0.5 mM DTT, 10 mM Tris-MES pH 7.0 (buffer B). The column was washed with 2CV of buffer B and eluted with 0–0.3 M Na-phosphate in buffer B. The active hydroxylapatite fractions (selected by either SDS-PAGE or ATPase assay), to a final concentration of 1.65 M. The mixture was centrifuged at 100,000 g for 30 min, the pellet was harvested and dissolved in buffer B without glycerol.

Then the sample was loaded on to a glycerol gradient (12 ml, 10–30% prepared in buffer B). The gradient was centrifuged at 170,000 g for 20 h, and fractions were collected. The fractions containing V-ATPase were concentrated and further purified by gel filtration using a Superose 6 10/300 column (GE Healthcare) pre-equilibrated with buffer C (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% CHAPS, 0.001% sodium deoxycholate (Anatrace)). Mass spectrometry confirmed the identity of the subunits of V-ATPase (Supplementary Table 1). The peak fractions were collected and concentrated to 2.5–3.5 mg/ml for cryo-EM grid preparation.

**V-ATPase activity assay.** ATPase activity was measured as the liberation of 32P from [γ-32P]ATP. The assay was carried out in a total volume of 200 μl under the following conditions: 4 μl phosphatidylserine (26 μM concentration), 5 μl V-ATPase in buffer C (at 10 nM concentration), and 189 μl ATPase assay solution A (30 mM KCl, 50 mM Tris-MES, pH 7.0, 0.3 mM MgCl₂, and 3 mM [γ-32P]ATP (400 pmol/ml)) and 2 μl of ethanol or ethanol dissolved bafilomycin A1 (Sigma-Aldrich, 1 μM concentration), The V-ATPase was first incubated with phosphatidylserine for 2 min, and then the reaction was started by addition ATPase assay solution A and ethanol/bafilomycin A1, and continued for 10 min at 37 °C. The ATP hydrolysis reaction was terminated by adding 1.0 ml of 1.25 N perchloric acid, and the released 32P was extracted and counted in a Beckman scintillation counter. The results were expressed as specific activity (μmol of Pi/min per mg of protein). The experiment has been repeated twice, and the results were performed using GraphPad Prism.

**EM sample preparation and imaging for 200 kV Cryo-TEM.** Freshly purified 2.3–3.5 mg/ml V-ATPase in buffer C was applied to Quantifoil R1.2/1.3 300 or 400 mesh holes carbon grids (Quantifoil). The grids were then blotted and plunged into liquid ethane for flash freezing using a Vitrobot Mark IV (FEI). The grids were imaged in a 200 kV Talos Arctica (FEI) with a Gatan K3 Summit direct electron detector (Gatan) in super-resolution mode using the data-collection software Serial EM. Data were collected at 0.735 Å/pixel with a counted rate of 42 electrons per physical pixel per second. Images were recorded for 1 s in 50 subframes with a total dose of 80 electrons per Å² and a defocus range of -1.0 to -2.0 μm.

**Imaging processing and 3D reconstruction for 300 kV Cryo-TEM.** The images were collected in two sessions (images from 400 mesh, images from 300 mesh Au holey carbon grids). The images used for the second session were selected using the following criteria: Two rotational states were separated by the obvious position of subunits D and F. One class comprised of state 1, including 61,520 particles, provided a 3.79 Å map after 3D refinement with a mask and postprocess in RELION-3, then CTF refinement and Bayesian polishing of particles were performed using RELION-3 for once followed by 3D refinement using a soft mask. The next, the second 3D classification was performed, two rotational states were separated by the obvious position of subunits D and F. One class comprised of state 1, including 61,520 particles, provided a 2.61 Å map after 3D refinement with a mask and postprocess in RELION-3. After the second CTF refinement with beam tilt correction, the resulting particles of state 1 were used for the final 3D refinement with a soft mask and solvent-flattened FSCs yielded a reconstruction at 7.22 Å revealing clear secondary structural elements. The resolution was estimated using “post-processing” with the FSC criteria of 0.143.
For rotational state 1, after 3D refinement, applying a full mask in RELION-3 post processing yielded a 3.37 Å overall resolution based on the Fourier shell correlation (FSC) 0.143 criterion. But due to the flexibility of V1 and Vo, Vo was not well resolved. Several focused refinements with different masks were attempted to get a high-quality local map. These focused refinements included subunits $A_b$, $D_b$, $E_b$, $F_b$, subunits $A_b$, $C_b$, $D_b$, $E_b$, $F_b$, subunits $A$, $B$, $C$, $D$, $E$, $F$, $G$, $H$. The generated by MODELLER22, Ac45 and subunit 8 NATURE COMMUNICATIONS| (2020) 11:3921 | https://doi.org/10.1038/s41467-020-17762-9 | www.nature.com/naturecommunications

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