Purification and cryoelectron microscopy structure determination of human V-ATPase

Vesicular- or vacuolar-type adenosine triphosphatases (V-ATPases) are multi-component, ATP-driven proton pumps, which play important roles in many physiological processes by acidifying intracellular vesicles, organelles, and the extracellular milieu. Long-standing challenges in purifying mammalian V-ATPases have limited the biochemical and structural study of mammalian V-ATPase. Here, we provide a protocol for purifying milligrams of human V-ATPase and detail procedures for the reconstruction of its structure by cryo-EM. Our method can be applied to any biochemical and biophysical study of human V-ATPase.
Protocol

Purification and cryoelectron microscopy structure determination of human V-ATPase

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SUMMARY

Vesicular- or vacuolar-type adenosine triphosphatases (V-ATPases) are multi-component, ATP-driven proton pumps, which play important roles in many physiological processes by acidifying intracellular vesicles, organelles, and the extracellular milieu. Long-standing challenges in purifying mammalian V-ATPases have limited the biochemical and structural study of mammalian V-ATPase. Here, we provide a protocol for purifying milligrams of human V-ATPase and detail procedures for the reconstruction of its structure by cryo-EM. Our method can be applied to any biochemical and biophysical study of human V-ATPase.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2020).

BEFORE YOU BEGIN

This protocol was used in a recent publication to purify a human V-ATPase and determine its structure by cryo-EM. Prior to the experiment, prepare all the buffers to be used one day in advance and make a recombinant plasmid containing SidK, which encodes a bacteria effector protein that can bind and inhibit the activity of human V-ATPase (Xu et al., 2010).

Buffer preparation

© Timing: 1 day

1. Buffer A
   Prepare 1 L Buffer A containing 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM Imidazole.
2. Buffer B
   Prepare 500 mL Buffer B containing 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 300 mM Imidazole.
3. Buffer C
   Prepare 1 L Buffer C containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM tris(2-carboxyethyl) phosphine (TCEP).
4. Buffer D
   Prepare 1 L Buffer D containing 50 mM HEPES, pH 7.5, 50 mM NaCl.
5. Buffer E
   150 mM NaCl, 1 mM TCEP, 1% lauryl maltose neopentyl glycol (LMNG), 0.1% cholesteryl hemi-succinate (CHS), protease inhibitor cocktail (Sigma-Aldrich), and 50 mM HEPES at pH 7.4.
6. Buffer F
150 mM NaCl, 1 mM TCEP, 0.1% lauryl maltose neopentyl glycol (LMNG), 0.01% cholesteryl hemisuccinate (CHS), and 50 mM HEPES at pH 7.4.

7. Buffer G
150 mM NaCl, 1 mM TCEP, and 20 mM HEPES at pH 7.4.

8. Kanamycin stock
Prepare 10 mL Kanamycin stock with a concentration of 50 mg/mL.

Plasmid encoding the SidK gene

© Timing: 3 days

9. SidK was cloned into pET28a with an N-terminal His<sub>6</sub> tag and a Flag tag (Figure 1). The plasmid can be obtained from Dr. Fu through material transfer assignment.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Freestyle 293 medium | GIBCO | Cat# 12338-018 |
| Fetal bovine serum (FBS) | GIBCO | Cat# 16000-044 |
| Lauryl maltose neopentyl glycol (LMNG) | Anatrace | Cat# NG310 |
| Cholesteryl hemisuccinate (CHS) | Anatrace | Cat# CH210 |
| Kanamycin | Goldbio | Cat# K-120 |
| IPTG | Goldbio | Cat# 12481 |
| Glycerol | Fisher Scientific | Cat# G33500 |
| Protease inhibitor cocktail tablets | Millipore-Sigma | Cat# 58830 |
| Antibiotic-antimycotic (anti-anti) | Thermo Fisher | Cat # 15240-062 |
| 3X FLAG peptide | Millipore-Sigma | Cat # F4799 |
| Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) | Goldbio | Cat # 51805-45-9 |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Superdex 200, 10/300 GL | GE Healthcare | Cat# 17-5175-01 |
| Ni-NTA agarose | QIAGEN | Cat# 30230 |

(Continued on next page)
Expression and purification of flag-tagged Sidk protein

@ Timing: 4–5 days

Purify the flag-tagged SidK protein, which will be used as an affinity bait for the purification of human V-ATPase (Figure 3A).

1. Expression of flag-tagged SidK in E. coli
   a. Transform the recombinant plasmid of pET28a containing flag-tagged SidK into E. coli BL21(DE3) RIPL and grow single colonies on an LB plate with 50 μg/mL Kanamycin.
   b. Inoculate a single colony to 25 mL LB broth with 50 μg/mL Kanamycin for overnight culture at 37°C with shaking at 200 rpm.
   c. The 25 mL culture was diluted into 1 L LB broth with 50 μg/mL Kanamycin for further growing at 37°C with shaking at 200 rpm to a density of OD ~0.6.
   d. Add 0.5 mM IPTG to the culture for the induction of protein expression at 18°C for 16–20 h.
   e. Harvest the cultured cells by centrifugation at 180 rcf for 10 min.
2. Purification of flag-tagged SidK
   a. The harvested cells were resuspended in 30 mL buffer A containing 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM Imidazole that was pre-cold at 4°C.
   b. The cells were disrupted by sonication (50% power, 5 s on, 5 s off) for 4 min on ice.
   c. The cell lysate was centrifuged at 20,000 rcf for 40 min to remove cell debris.
   d. After centrifugation, the supernatant was incubated with 1 mL Ni-NTA resin pre-equilibrated by buffer A at 4°C for 30 min.
   e. The resin was then washed with 10 mL buffer A containing 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM Imidazole.
   f. The protein was eluted using 4 mL buffer B containing 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 300 mM Imidazole.
   g. The eluted flag-tagged SidK was further purified by gel filtration in buffer C containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM TCEP.
   h. Analyze the purity of the protein by SDS-PAGE (Figure 3B).
   i. Pool the protein fractions and concentrate to 2 mg/mL using spin filter concentrator (Company). Protein concentration was quantified by measuring the UV280 absorbance using NanoDrop Spectrophotometer (Thermo Fisher Scientific).
j. Freeze 100 μL aliquots of SidK protein in liquid nitrogen and store the protein in −80°C freezer.

**Note:** During the wash step in step 2e, up to 20 mL buffer A may be used to wash the resin, which will help to maximally eliminate non-specific bound proteins.

**Purification of human V-ATPase**

© Timing: 2–3 weeks

Purify human V-ATPase from HEK293S cells by affinity purification and glycerol gradient ultracentrifugation (Figure 4A).

3. **293S cell culture**
   a. One vial of frozen 293S cells were diluted into 25 mL Freestyle 293 medium for centrifugation at 180 rcf for 5 min.
   b. The cell pellets were resuspended using 30 mL fresh medium of Freestyle 293 medium supplemented with 10% FBS and 1% antibiotics-antimycotic (anti-anti) in a 200 mL Baffled flask and cultured at 37°C with shaking at 100 rpm in a mammalian cell culture shaker.
   c. The cells were diluted into 1.0 million/mL using fresh Freestyle 293 medium with 1% anti-anti when the cell density arrived at 2.0 million/mL to prepare 5 L 293F cell culture.
   d. 293S cells were harvested by centrifugation at 180 rcf for 10 min.

4. **V-ATPase Purification**
   a. **Isolation of Membrane Fraction**
      i. The harvested cells were resuspended in 150 mL buffer D containing 50 mM HEPES, pH 7.5, 50 mM NaCl supplemented with protease inhibitor cocktail and incubated for 2 h on ice.
      ii. The cells were further disrupted by hand-driven Dounce homogenization (PYREX, Corning).
      iii. The cell lysate was centrifuged at 20,000 rcf for 30 min to remove cell debris.
iv. The supernatant was centrifugated at 200,000 rcf in a Type 45 Ti rotor (Beckman) for 1 h to obtain the membrane pellet.

b. Purification of V-ATPase by anti-Flag Resin

i. The membrane pellet was resuspended in the buffer E containing 150 mM NaCl, 1 mM TCEP, 1% lauryl maltose neopentyl glycol (LMNG), 0.1% cholesteryl hemisuccinate (CHS), protease inhibitor cocktail (Sigma-Aldrich), and 50 mM HEPES at pH 7.4 and homogenized using a hand-driven Dounce homogenizer (PYREX, Corning).

ii. The homogenized membrane solution was mixed with 0.1 mg flag-tagged SidK protein and incubated for 4 h at 4°C.

iii. After incubation, the mixture was centrifuged at 20,000 rcf for 30 min to remove insoluble material.

iv. Supernatant containing solubilized V-ATPase/SidK complex was then incubated with 1 mL anti-Flag resin for 1 h at 4°C.

v. The resin was collected and washed with 10 mL buffer F containing 150 mM NaCl, 1 mM TCEP, 0.1% lauryl maltose neopentyl glycol (LMNG), 0.01% cholesteryl hemisuccinate (CHS), and 50 mM HEPES at pH 7.4.

vi. The V-ATPase/SidK complex was eluted using 5 mL wash buffer F plus 100 μg/mL 3X FLAG peptide.

c. Further Purification of V-ATPase by Glycerol Gradient Ultracentrifugation
i. Prepare 1 mL each of 15%, 30%, 45%, and 60% glycerol solutions in buffer G plus 0.001% lauryl maltose neopentyl glycol (LMNG) and 0.0001% cholesteryl hemisuccinate CHS.

ii. Prepare glycerol gradient by gently injecting 1 mL 15%, 30%, 45%, and 60% glycerol solutions to the bottom of a polyclear centrifuge tube using a 1 mL syringe.

iii. Slowly load between 100 μL and 500 μL V-ATPase fraction from previous step on top of the glycerol gradient.

iv. Balance the two tubes before loading them into the swing-out rotor.

v. Centrifuge at 171,520 rcf in a Beckman MLS-50 rotor and Beckman Optima MAX ultracentrifuge for 20 h at 4°C.

vi. After centrifugation, manually fractionate by poking a hole at the bottom of the centrifuge tube and collect the first 16 fractions of 250 μL each.

vii. Analyze each fraction by SDS-PAGE to select the fractions with intact V-ATPase (Figure 4B). The V-ATPase concentration was quantified by NanoDrop Spectrophotometer (Thermo Fisher Scientific).

Note: The Freestyle 293 medium needs to be pre-warmed to 37°C before use.

Note: Make sure to add sufficient amount of protease inhibitor to avoid protein degradation by endogenous protease during cell lysis and protein purification processes.

△ CRITICAL: When you load the glycerol into the centrifuge tube, be careful to avoid air bubbles and not to disturb the interface.

△ CRITICAL: Avoid boiling samples for SDS-PAGE, otherwise the protein complex will form large aggregates that are difficult to enter the gel.

Pause point: The harvested cells in step 3 or the isolated membrane pellets in step 4a may be stored in −80°C freezer for up to a month before moving on to the next steps.

Grids preparation and cryo-EM data collection

Timing: 2–4 days

Prepare cryo-EM grids of human V-ATPase and collect cryo-EM data for structure reconstruction (Figure 5).

5. Grid Preparation
   a. Copper grids with less than 3 nm continuous ultrathin carbon and lacey carbon support film was glow-discharged for 1 min at the current of 30 mA.
   b. A 3 μL drop of purified human V-ATPase sample was applied to the glow-discharged grids and incubated at 4°C for 10 min.
   c. The grids were washed for three times using 5 μL buffer G containing 20 mM HEPES at pH 7.5 and 150 mM NaCl to remove the glycerol as much as possible.
   d. The grids were blotted using standard vitrobot filter paper (Ted Pella) for 4 s with force of 5 in 100% humidity at 4°C, and plunged into liquid ethane using an FEI Vitrobot Mark IV.

6. Cryo-EM Data Collection
   a. Grids screening
      i. All the grids were clipped
      ii. The clipped grids were loaded to FEI Talos Arctica microscope for screening.
      iii. The grids with good ice and particle distribution were saved for data collection (Figure 5B).
   b. Data collection
      i. Grids for data collection were loaded to FEI Titan Krios microscope with a K3 detector and energy filter.
Data were collected in counting mode, with 40 total frames per movie in 3.2 s, a total dose of 50 electrons per Å², a physical pixel size of 1.08 Å, and defocus values between 1 nm to 3 nm.

Note: As the protein concentration is relatively low for preparing cryo-grids, we therefore choose carbon-coated grids that help to concentrate protein particles on the grids.

△ CRITICAL: During grids preparation, the elimination of glycerol in samples by washing three times is critical for preparing grids with proper ice.

¶ Pause point: The prepared grids in step 5 may be stored in liquid nitrogen for screening and data collection.
Cryo-EM data processing

- Timing: 2–3 weeks

Process cryo-EM data to obtain cryo-EM density maps of human V-ATPase (Figure 6).

7. Beam-induced motion correction
   Motion correction was done in RELION by the implementation of UCSF MOTIONCOR2 (Zheng et al., 2017, Scheres, 2012).
8. CTF estimation
Contrast transfer function (CTF) estimation was done by CTFFIND4.1 in RELION (Scheres, 2012, Rohou and Grigorieff, 2015).

9. Initial Particle Picking
The particle picking was done using a template-free auto-picking procedure based on a Laplacian-of-Gaussian filter to select an initial set of particles (Wagner et al., 2019).

10. 2D Classification
The selected particles were used in a 2D classification to generate templates for a second round of auto-picking job.

11. Second round particle picking
The particle picking was done by Gautomatch using the templates generated in step 10 as references.

12. 2D Classification
Total 782,583 particles were picked for 2D classification to select good classes for further 3D classification.

13. 3D Classification
The selected particles were 3D-classified into 6 classes and 3 classes out of the 6 are corresponding to the 3 states of the V-ATPase.

14. 3D refinement
The 3 states were selected for non-uniform 3D refinement in cryoSPARC (Punjani et al., 2017). The 3D reconstruction resulted in three maps with resolutions of 3.1 Å, 3.4 Å, and 3.6 Å, respectively.

Note: Regularization parameter Ts for 2D classification (step 12) and 3D classification (step 13) are 2 and 4, respectively.

Model building and refinement

© Timing: 2–4 weeks

Based on the cryo-EM maps we obtained above, we built atomic models of human V-ATPase.

15. Model Building
The initial structure model of human V-ATPase was generated using SWISS-MODEL based on yeast V-ATPase and fitted as rigid bodies into cryo-EM maps using Chimera (Biasini et al., 2014, Pettersen et al., 2004). The structures were further manually adjusted in coot (Emsley and Cowtan, 2004).

16. Refinement
Real-space refinement was performed using PHENIX following the routine protocol and the models were validated by PHENIX (Adams et al., 2010).

EXPECTED OUTCOMES
From 5 L human 293S cells, about 0.05 mg human V-ATPase was purified as estimated by A280 using nanodrop (Figure 4B). The purified protein can be used for biochemical and biophysical studies related to V-ATPase. Furthermore, structures of human V-ATPase can be used for drug design and computational biology (Figure 6).

LIMITATIONS
Due to the low expression level of endogenous V-ATPase, you need large-scale culture of 293S cells for one purification prep.
TROUBLESHOOTING

Problem 1
The particle density on the prepared grids might not be high enough for data collection. (Step 5)

Potential solution
Repeat step 5b once before proceeding to step 5c.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Tian-Min Fu (fu.978@osu.edu).

Materials availability
Plasmids generated in this study are available upon request from the lead contact with a completed Material Transfer Agreement.

Data and code availability
The cryo-EM maps included in this study have been deposited in the Electron Microscopy Data Bank with accession codes: EMD-21844, EMD-21845, EMD-21847, EMD-21848, and EMD-21849. The atomic coordinates have been deposited in the Protein Data Bank with accession codes: 6WM2, 6WM3, 6WM4, 6WLZ, and 6WLW.

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AUTHOR CONTRIBUTIONS
T.-M.F., L.W., and H.W. conceived the project. L.W., T.M.F., and Z.C. performed the experiments. T.-M.F. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS
H.W. is a member of scientific advisory board of Cell. The other authors declare no competing interests.

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