ONLINE METHODS

Purification and crystallization of soluble E–G complexes. Crystals containing the full-length G subunit diffracted poorly, and N-terminal sequencing and MS of the intact *T. thermophilus* A-ATPase suggested subunit G was N-terminally truncated by 17 residues[11,13]. Furthermore, as the truncated EG construct contained only one methionine, residues Leu134, Leu171 and Leu178 in subunit E were mutated to methionine residues to improve the phasing power.

DNA encoding the mutated subunit E (Emmm) and truncated subunit G from *T. thermophilus* were cloned into the bicistronic pETDuet-1 vector (Novagen), with subunit G carrying an N-terminal six-histidine tag followed by a TEV protease cleavage site, then were overexpressed in *E. coli* B834 (DE3) cells (Novagen) with selenomethionine-containing media[48] and purified as described[12], except that 10 mM β-mercaptoethanol was added to all buffers. Overexpressing cells were lysed by multiple cycles of freezing and thawing with liquid N_2, and the supernatant was purified on a Ni^{2+}-NTA column (GE Healthcare). Material that bound to the resin was eluted with 500 mM imidazole and this salt was subsequently removed with a desalting column. The mixture containing the truncated Emmm–G complex was incubated with a double histidine-tagged TEV protease for 16 h at 4 °C, and then was passed over the Ni^{2+}-NTA column a second time. Unbound material was collected, concentrated and passed over a 16/60 Superdex 200 column equilibrated in 20 mM Tris, pH 7.5, 100 mM NaCl and 10 mM β-mercaptoethanol. Peak fractions were pooled and concentrated to 7.5 mg ml^{-1}. For crystallization, sitting drops were prepared by the mixture of 2 μl protein with 2 μl mother liquor (40% (v/v) 2-methyl-2,4-pentanediol, 0.1 M cacodylate, pH 6.4, 5% (w/v) PEG 8000) in 24-well Cryschem plates (Hampton Research), followed by incubation at 18 °C.

Data collection, phasing, model building and refinement. Data used for phasing, model building and refinement were from two separate crystals that were merged together. MAD datasets (wavelengths: 0.97971 Å, 0.97957 Å and 0.95372 Å) of the two crystals (space group $P2_1$ twinned with apparent space group $C222_1$) were collected at APS beamline 14ID-B at 100 K, but only peak wavelength data (0.97957 Å) were used for SAD phasing. The data were processed with MOSFLM[49], scaled and merged with SCALA[50], and phased with SHELX[51] and SHARP[52]. An atomic model was built into the resulting density using Coot[53] and was refined with REFMAC5[54] and Phenix[55] with incorporation of the twinning operator (l -k h) with a twinning fraction of 46.4% and no use of non-crystallographic symmetry restraints on the two non-crystallographic symmetry–related EG complexes in the asymmetric unit. The r.m.s. deviation between the two non-crystallographic symmetry–related complexes is 1.2 Å. Structure validation was performed with MolProbity[56], which gave Ramachandran statistics with 97.1% of residues in favored or allowed regions and 2.9% outliers. Data collection and refinement statistics are in **Table 1**.

The refined model was manually fitted into the 23-Å EM reconstruction with PyMOL and figures were prepared with PyMOL (http://pymol.sourceforge.net).

Determination of the absolute handedness of three-dimensional EM reconstruction from *T. thermophilus*. Experimental efforts to determine the handedness by the random conical tilt method failed to provide an unambiguous solution. Instead, the handedness of EM reconstructions of A-ATPases have previously been derived by fitting of the asymmetric structure of the homologous bovine F_1-ATPase coordinates into the EM density[11,24]. However, the recently published structure

of the A_1 domain revealed gross structural differences between mammalian F-type ATP synthases and A-ATPases[10,35]. Thus, we repeated the analysis of the handedness of the EM density from *T. thermophilus* using the A-ATPase A_1 domain from the same organism. By matching the asymmetric features of the A_1 structure to the EM density we were able to confidently determine the absolute handedness as follows. The most striking asymmetric feature is at the base of the V_1 complex where subunit F bulges away like a foot from the central stalk (subunit D), which itself is asymmetrically positioned. The corresponding asymmetric EM density is a strong feature that is clearly visible even at high contour levels. In the inverted handedness, the shape and size of the foot correlate well with the EM density with over 99% of atoms within the density when contoured at 1.5 σ. In this orientation, each of the A_3B_3 subunits are located precisely in one of the six lobes in the EM density, and the asymmetric features of the catalytic A subunits are clearly matched with asymmetric density in the corresponding lobes. Further, cross-linking studies demonstrate that Lys25, which is situated at the top of subunit B, is in close proximity to subunit E, and docking into the inverted density satisfies this restraint.

In contrast to the inverted density, it is not possible to dock the A_1 crystal structure into the original density in a manner that equally correlates to the EM density or spatial restraints from cross-linking studies. At all contour levels, there are significantly more atoms that are excluded from the EM density in the original hand. As above, we initially docked the A_1 structure via its asymmetric foot. However, the A_3B_3 subunits did not align with the lobes in the EM density and are positioned such that the catalytic A subunits rather than the B subunits are in contact with the E–G peripheral stalk. Hence, in this orientation, the spatial restraints from the Lys25 cross-linking study are not satisfied. In addition, we attempted to fit the A_1 complex by aligning the lobes in the A_3B_3 domain with the corresponding subunits in the crystal structure. However, the asymmetric foot as well as some of the asymmetric features in the catalytic A subunits visibly protrude from the EM density.

In summary, there is a strong correlation between the asymmetric features of the crystal structure of the *T. thermophilus* A_1 domain and the EM density in the inverted handedness that cannot be matched in the original handedness as described[11]. This indicates that the inverted handedness is the absolute handedness of the EM density. This handedness is consistent with the absolute handedness of eukaryotic V-ATPases as determined by the random conical tilt method[20].

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