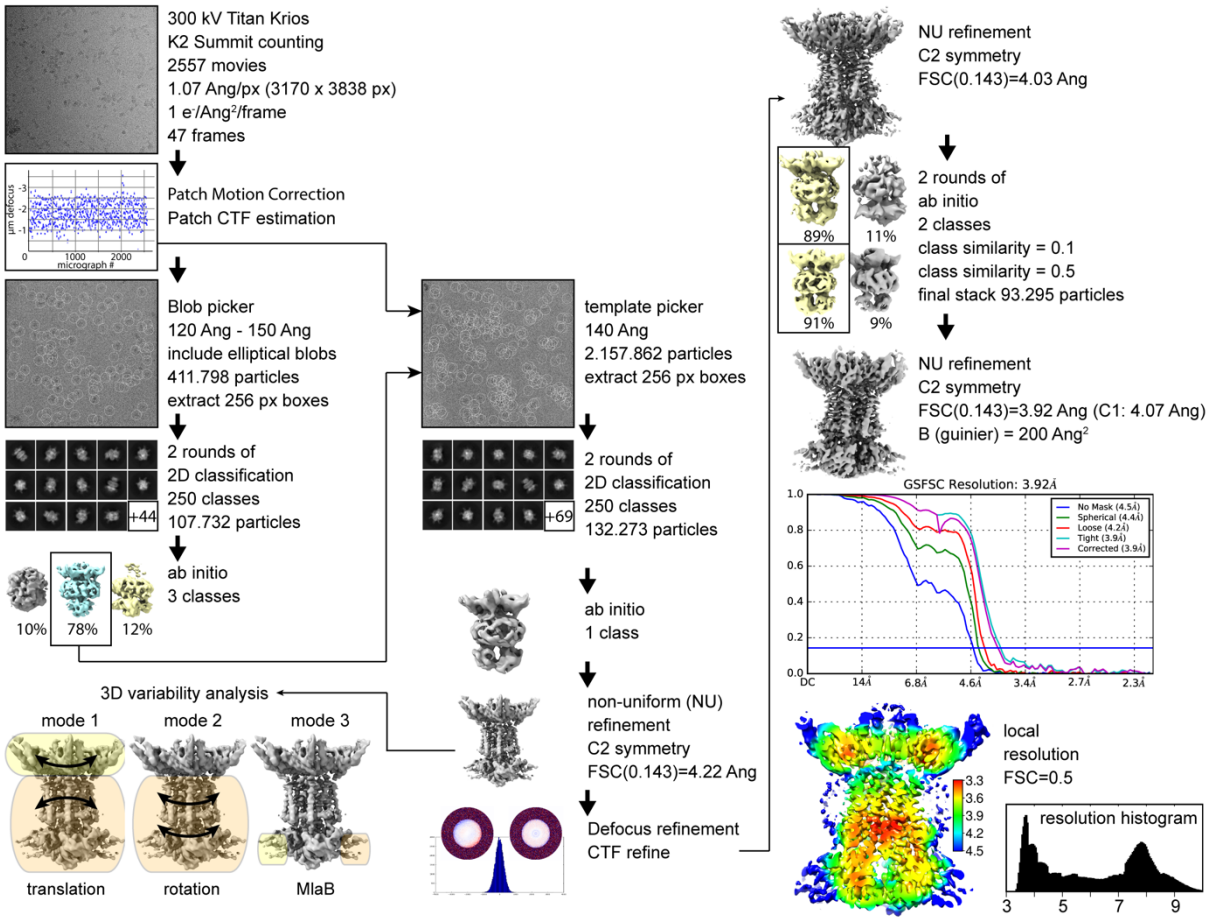
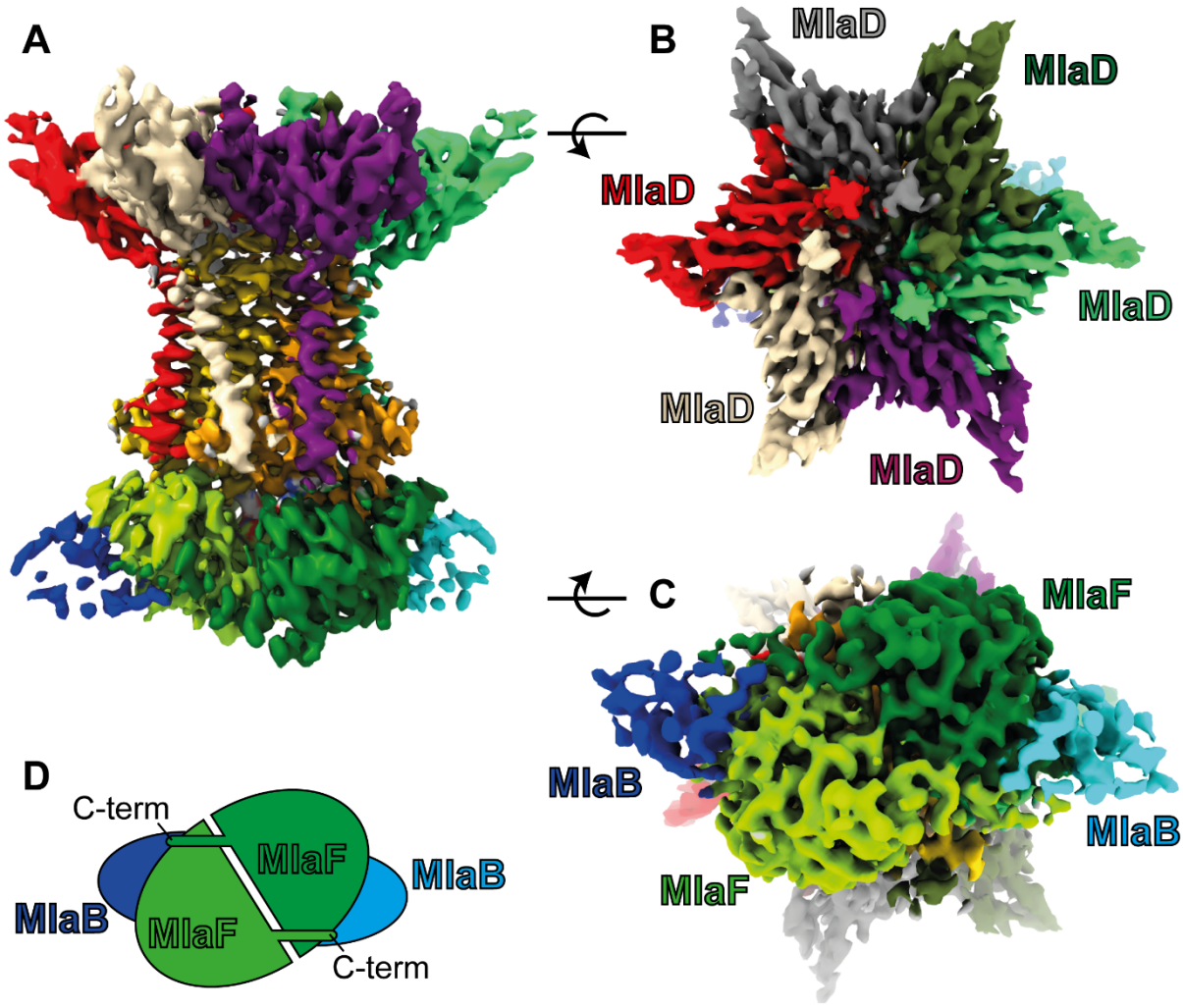
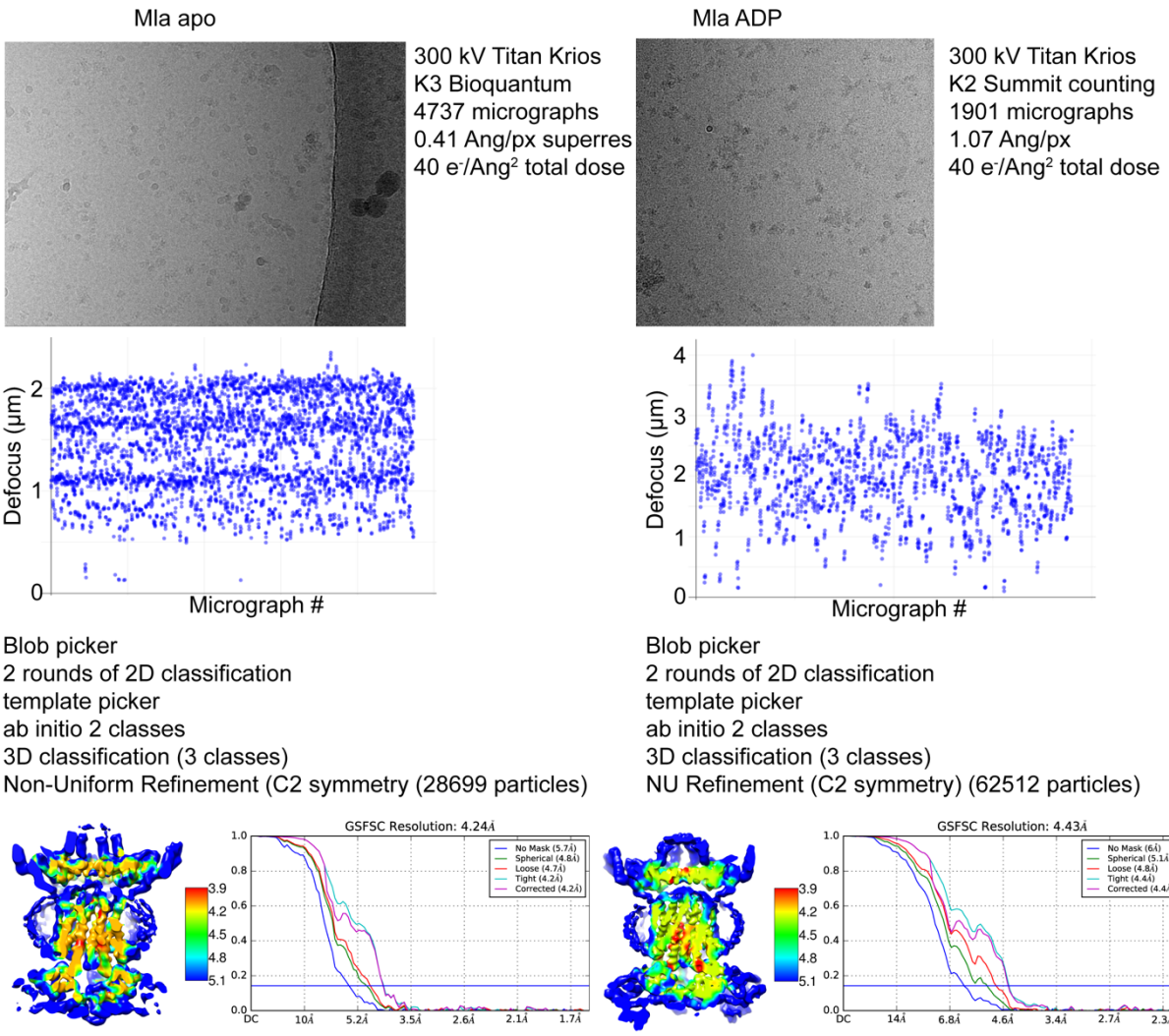


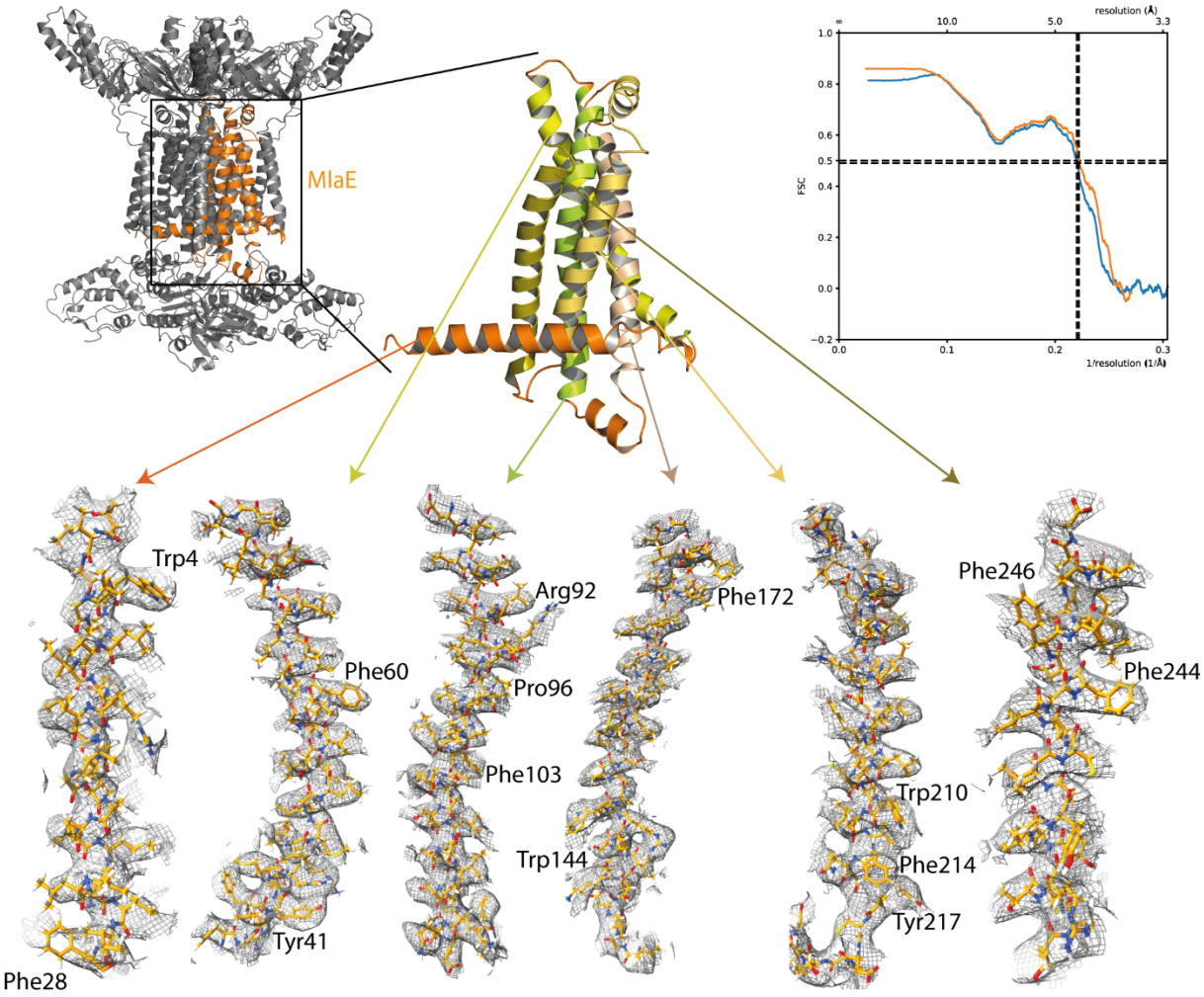
Supplemental Figure 1: processing of the MlaBDEF-AppNHp dataset. Patch motion correction resulted in a detected defocus range of -1 to -2.5 µm. Initial blob picking with a diameter range from 120-150 Å including elliptical shapes were extracted and 2D classified two times into each 250 classes. Ab initio 3D models were generated and the best class was used to re-pick particles template-based. After two rounds of 2D classification an ab initio 3D model was generated and refined using CryoSPARCs Non-Uniform (NU) refinement procedure with C2 symmetry, followed by global and local CTF refinement and two rounds of ab initio model generation with class similarity values of 0.1 and 0.5, respectively. The final particle stack contained 93.295 particles and was NU refined to 3.92 Å with C2 symmetry (4.07 Å with C1 symmetry). The map was sharpened with the Guinier plot B-factor of -200 Å ^{2}. Fourier Shell Correlation plot is indicated as well as local resolutions at FSC=0.5 projected on the final map. A histogram with the full local resolution range is also indicated. The first high resolution 3D structure was used as an input for CryoSPARCs 3D variability jobtype with 6 modes. Only the first three modes showed global changes; firstly, translation of MlaD against MlaBEF (Supplemental Movie 1), secondly, rotation of the MlaBEF part against MlaD (Supplemental Movie 2) and thirdly, alternating appearance of MlaB, indicating lower occupancy of this part compared to the MlaDEF part.



Supplemental Figure 2: Side (A), top (B) and bottom (C) views of MlaBDEF. The MlaBFFB tetramer is stabilized by a "handshake mechanism" (D). C-terminal regions of MlaF bind the opposing MlaB subunit.



Supplemental Figure 3: processing of the MlaBDEF-ADP and MlaBDEF apo datasets in After blob picking and 2D classification selected 2D classes were used for template picking. After several rounds of ab initio 3D structure generation and 3D classification Non-uniform refinement in C2 symmetry resulted in slightly lower resolutions compared to MlaBDEF-AppNHp. Global and local CTF refinement did not increase the map resolution.



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401 **Supplemental Figure 4:** de novo model building of MlaE (orange). Large side chains that
402 allowed sequence mapping are indicated as well as map to model FSC of the whole
403 MlaBDEF protein complex to the AppNHp map.

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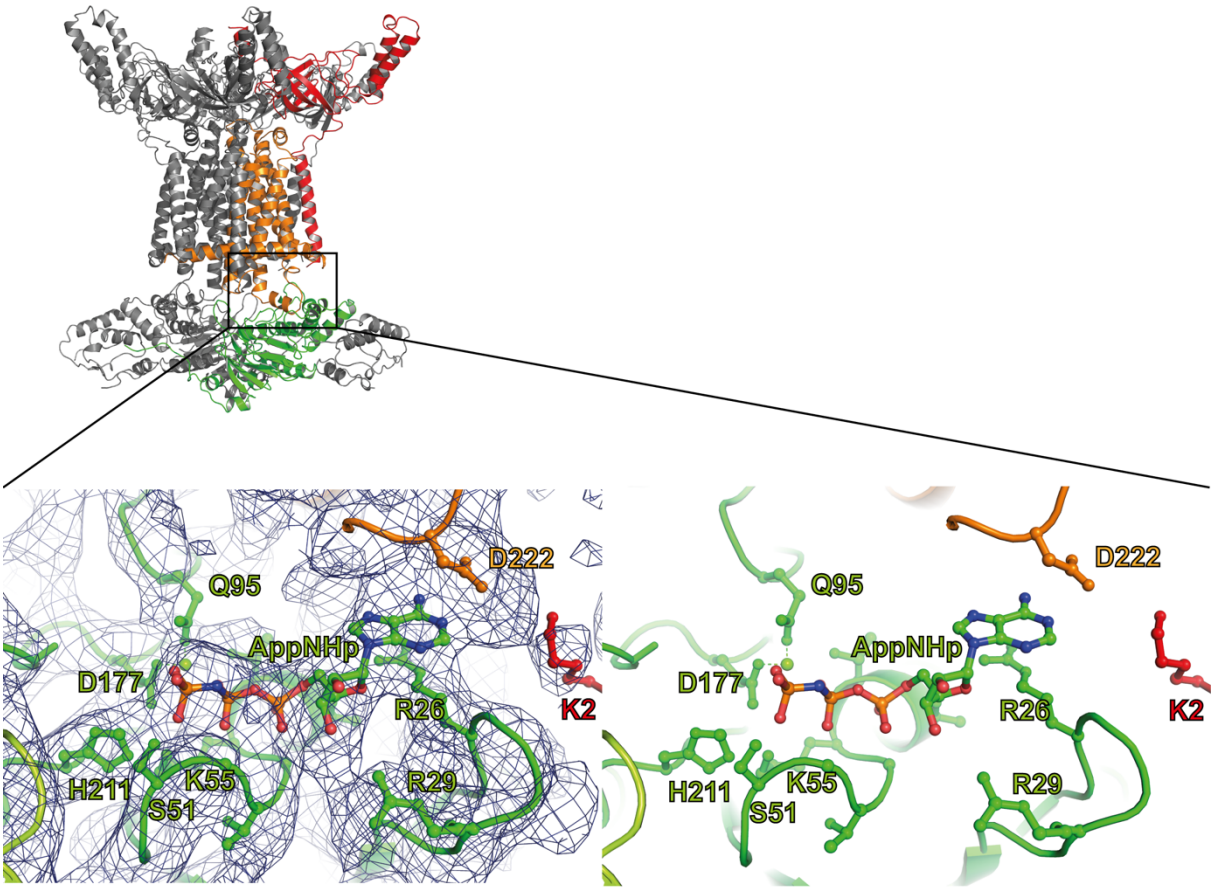
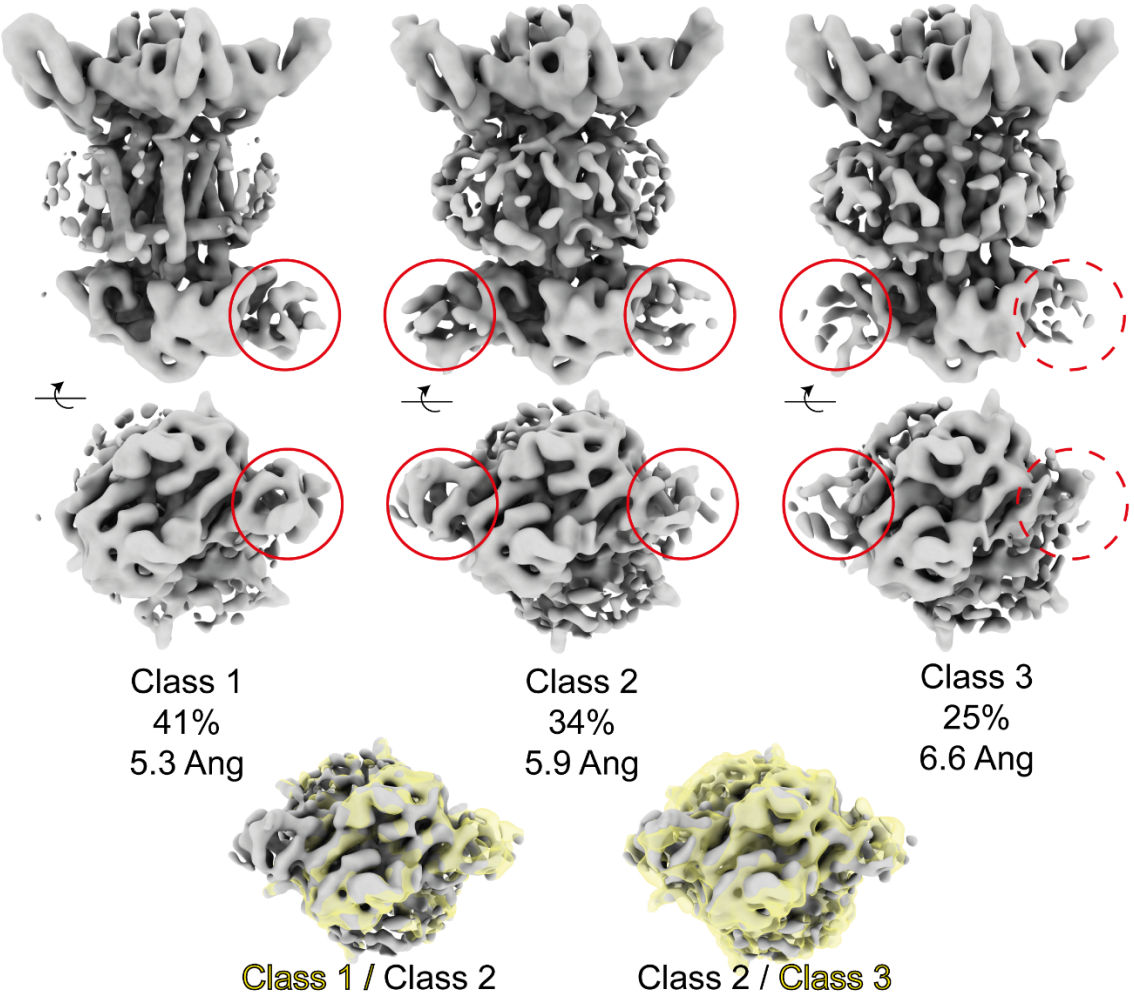
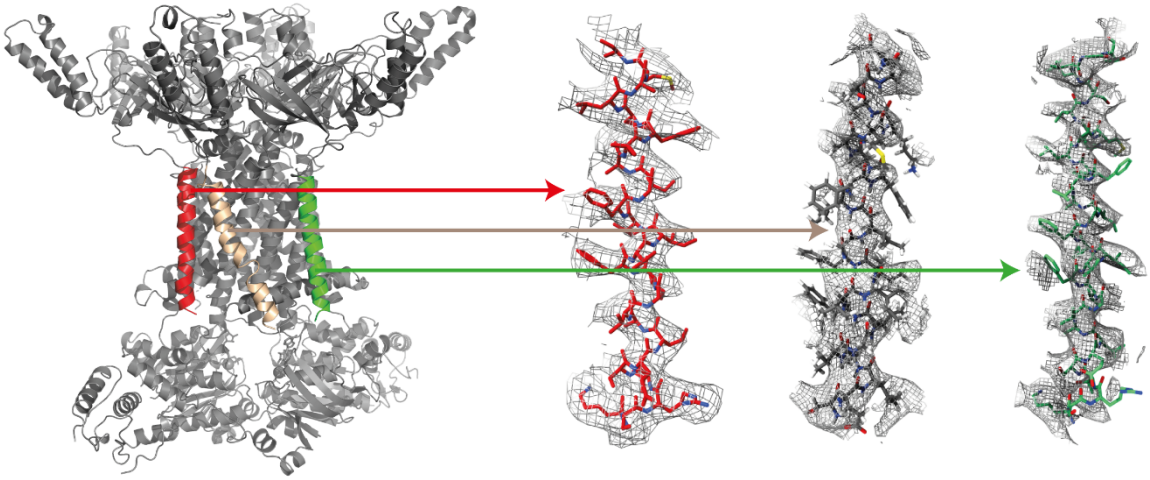


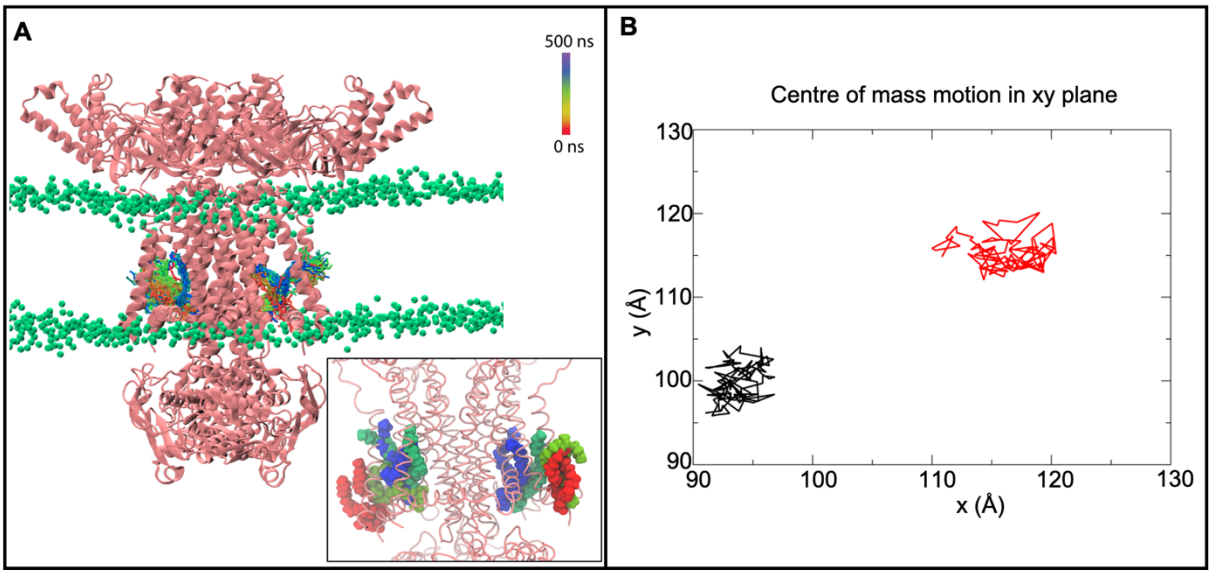
Supplemental Figure 5: AppNHp is bound at the interface of MlaE (orange), MlaD (red) and MlaF (green).



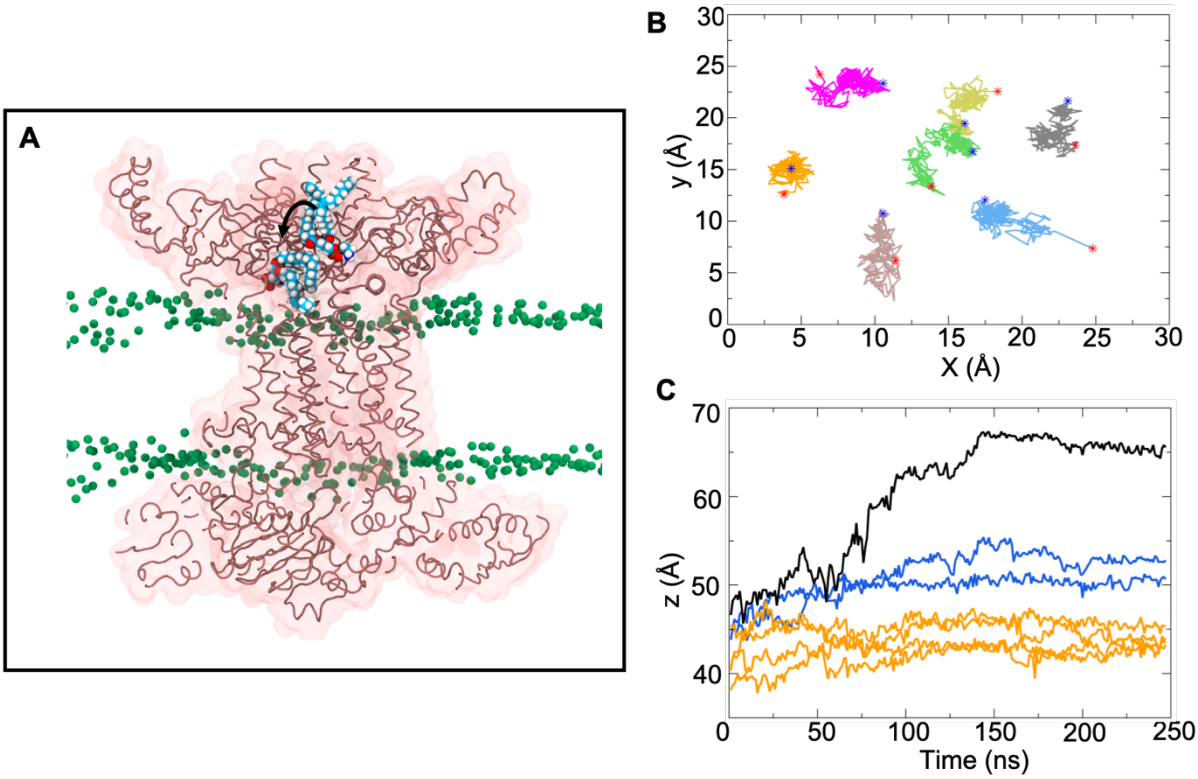
Supplemental Figure 6: MlaB binding (red circles) occurred on both binding sites in about 50% of the particles (classes 2 and 3) and on only one binding site in the other 50% of the particles (class 1). Maps were obtained by Non-Uniform refinement in C1 symmetry after heterogeneous refinement with 3 classes in CryoSPARC. Alignments of Classes 1-3 snow no major structural changes upon MlaB binding.



Supplemental Figure 7: Enclosed N-Helix of MlaD is significantly better resolved compared to peripheral MlaD N-helices (grey, green).



Supplemental Figure 8: Panel A shows the location of two POPE lipids during a 500 ns simulation, the colour scheme indicates the movement of the lipids as shown in the legend. In this simulation the lipids moved into this location spontaneously during the equilibration process, as shown in the close-up view in the inset in which the red coloured lipids indicate positions lipid positions at the start of the equilibration process. Panel B shows the motion of the lipids in the xy plane over 500 ns. The lipids are confined within an area of 10 x 10 Å for 500 ns, showing this location is favourable.



Supplemental Figure 9: Panel A shows a cut-away view of the protein with a POPE lipid at two time points during the simulation, time = 0 ns and 150 ns. The Mla$_{7PE}$ simulation was initiated with seven POPE lipids placed at the periplasmic end of the protein corresponding to the density for detergents in the cryo-EM data (Table S2). The lipid which is displaced more towards the cytoplasmic end is from the frame at t = 150 ns. The central hydrophobic 'channel' of the protein is a clear conduit for lipids given the spontaneous movement of POPE into this region in just 150 ns. Panel B shows the center of mass motion of the seven lipids in the xy plane. They are confined to an area of ~5 x 5 Å, indicating this is a high lipid affinity region. Panel C shows the center of mass movement of the seven lipids in the Z dimension as a function of time. The higher values of z correspond to the cytoplasmic end of the protein. The lipid shown in panel A corresponds to the black curve showing a clear movement towards the cytoplasmic end. Three other lipids (blue) move into this channel but to a lesser extent than the aforementioned lipid, whereas three others (orange) remain close to their starting positions. It is important to note that the simulations from a model at the reported resolution do not really tell us about the directionality of the movement, but rather that these regions are conduits for lipids

448 **Table S1: cryo-EM data collection, refinement and validation statistics**

	AppNHp (EMDB-11082) (PDB: 6Z5U)	**Apo** (EMDB-11083)	**ADP** (EMDB-11084)
Data Collection and Processing			
Microscope	Titan Krios	Titan Krios	Titan Krios
Voltage (kV)	300	300	300
Camera	K2 summit	K3 bioquantum	K2 summit
Pixel size (Å)	1.07	0.41	1.07
Defocus range (μm)	-1 to -2.5	-0.8 to -2.0	-1 to -2.5
Total dose (eÅ^{-2})	47	40	40
Number of micrographs	2557	4737	1901
Total particles used	93,295	28,699	62,512
Map Resolution (Å)	3.92	4.24	4.43
Refinement			
Model composition			
Non-hydrogen atoms	17,892		
Protein Residues	2334		
Ligand atoms	4		
B factors (Å^{-1})			
Protein	145.24		
Ligand	170.31		
R.m.s. deviations			
Bond lengths (Å)	0.008		
Bond angles (°)	1.384		
Validation			
MolProbity score	3.43		
Clashscore	35.15		
Poor rotamers (%)	10.94		
Ramachandran plot			
Favoured (%)	87.45		
Allowed (%)	10.94		
Disallowed (%)	0.43		

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Table S2: Equilibrium Protocol for MD simulations

Step	Time Step (fs)	Total Time (ns)	Protein Backbone Restraints (kJ mol^{-1} nm^{-2})	Protein Sidechain Restraints (kJ mol^{-1} nm^{-2})	Ensemble
1	1	0.125	4000	2000	NVT
2	1	0.125	2000	1000	NVT
3	1	0.125	1000	500	NPT
4	2	0.5	500	200	NPT
5	2	0.5	200	50	NPT
6	2	20	50	0	NPT

Table S3: Summary of the equilibrium MD simulation systems

System	Additional lipids	Temperature (K)	Simulation Length (ns)
Mla	-	310	500 (× 2)
Mla	-	323	500 (× 2)
$Mla_{\_7PE}$	7 POPE	310	250 (× 2)