

Cryo-EM of human P-glycoprotein reveals an intermediate occluded conformation during active drug transport

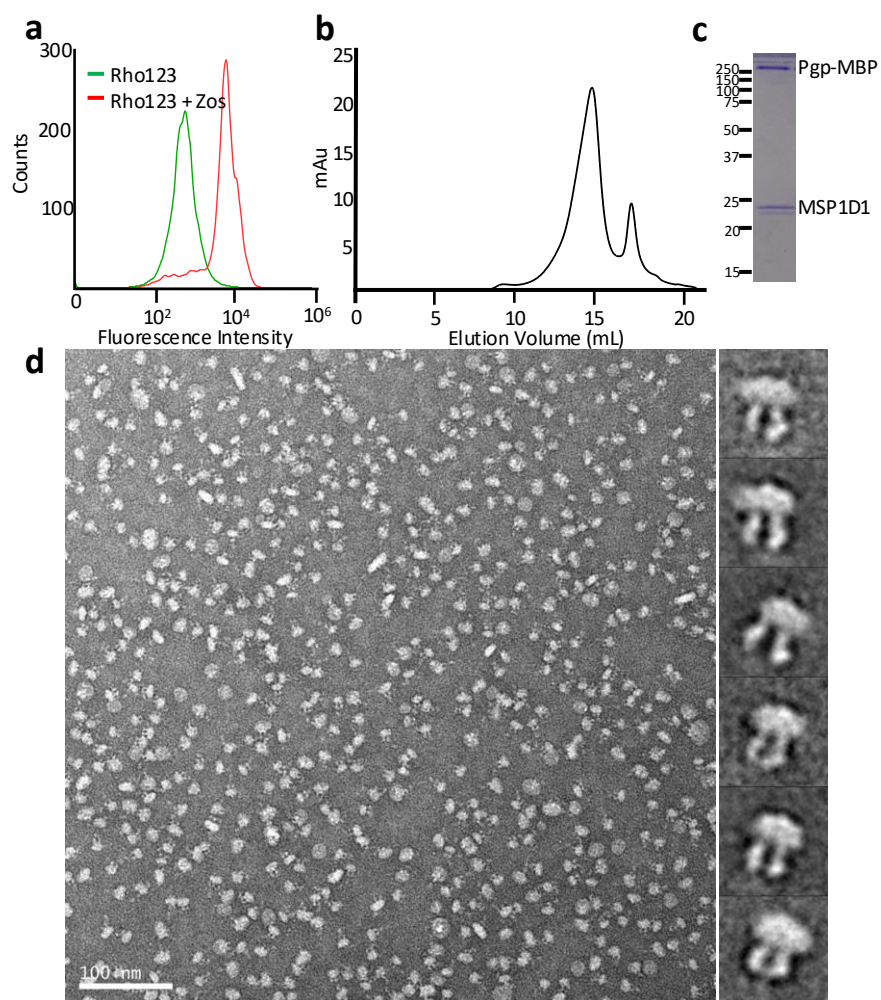
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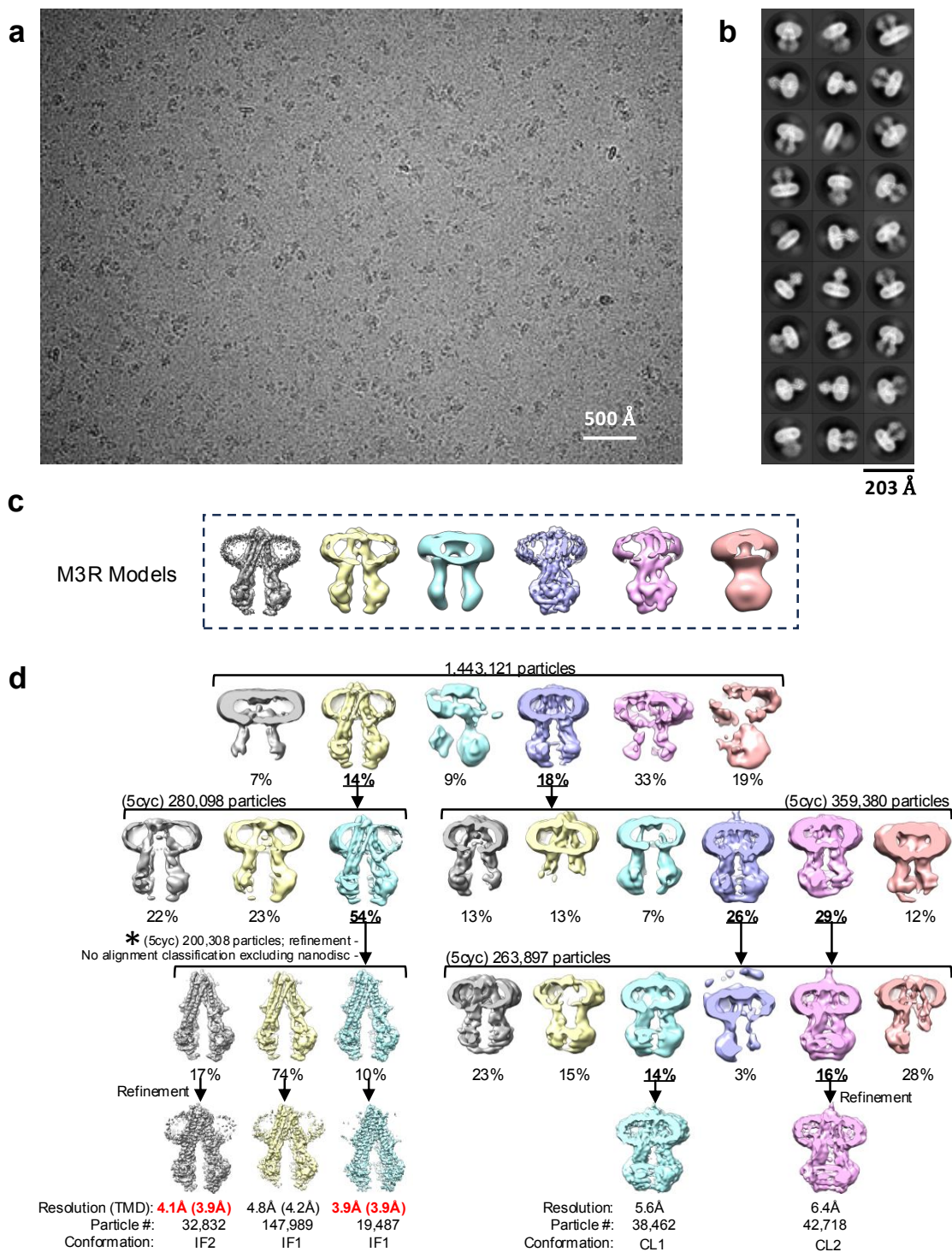
Supplementary Information

Supplementary Figures 1-20

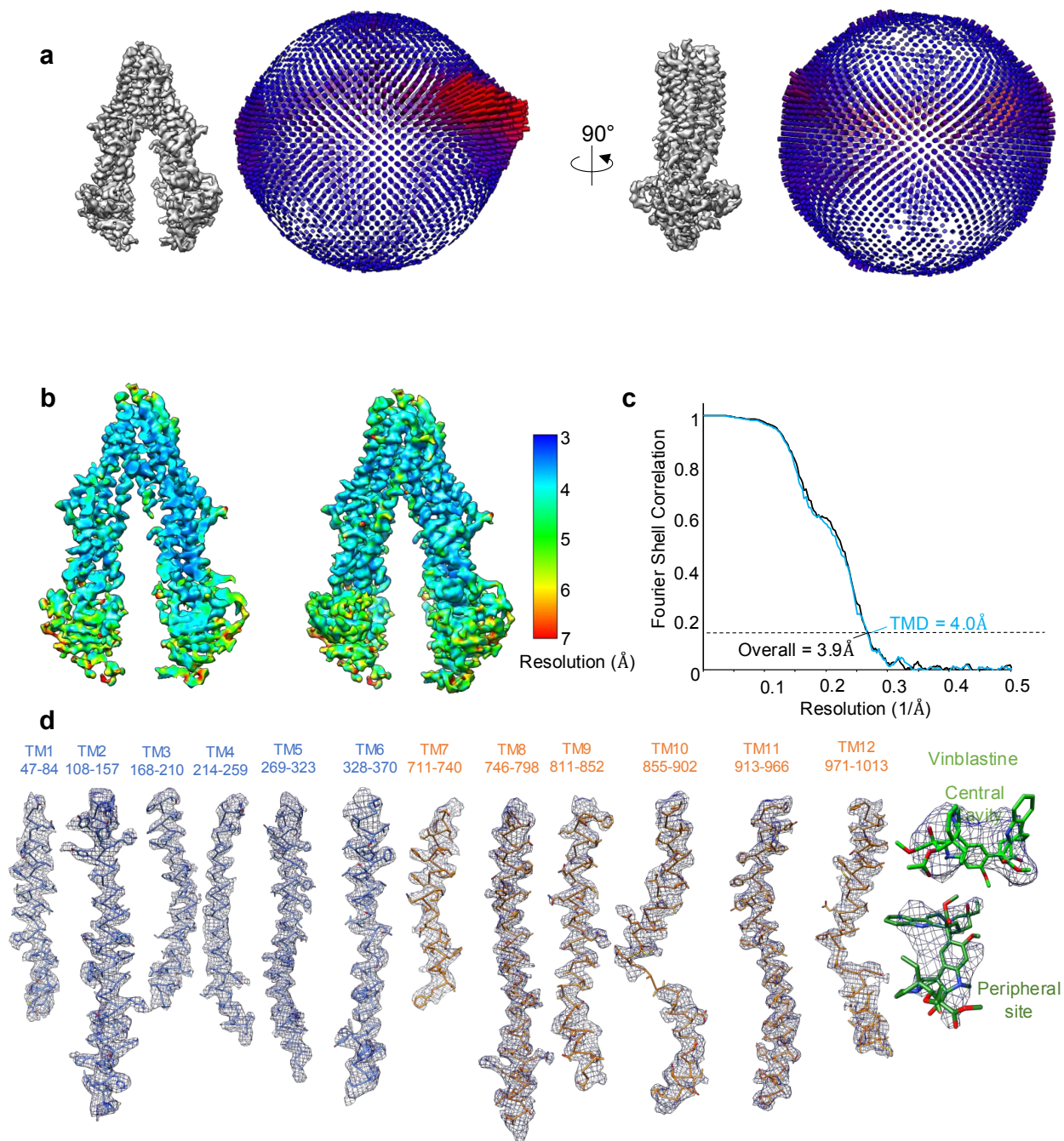
Supplementary Table 1



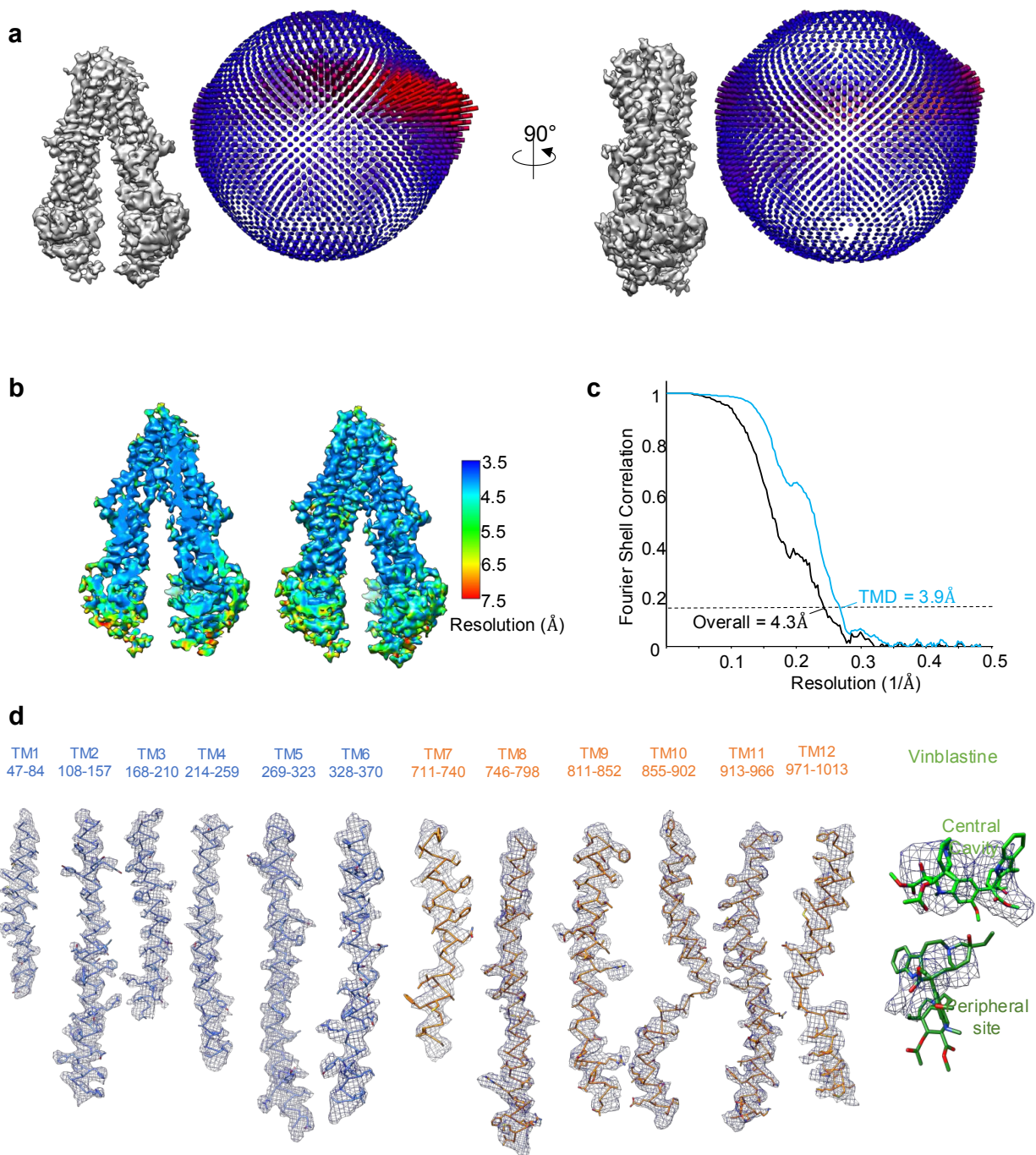
Supplementary Figure 1. Biochemical and negative stain EM characterization of Pgp in nanodiscs. (a) In-cell transport assay of Pgp with the fluorescent drug rhodamine 123 (green) or with rhodamine 123 and zosuquidar, a Pgp inhibitor (red). (b) Gel filtration profile on superose 6 column of purified Pgp in nanodiscs. (c) SDS-PAGE gel of purified Pgp in nanodiscs. (d) Negative stain electron microscopy of purified Pgp in nanodiscs with representative 2D averages (right). Source data are provided as a Source Data file.



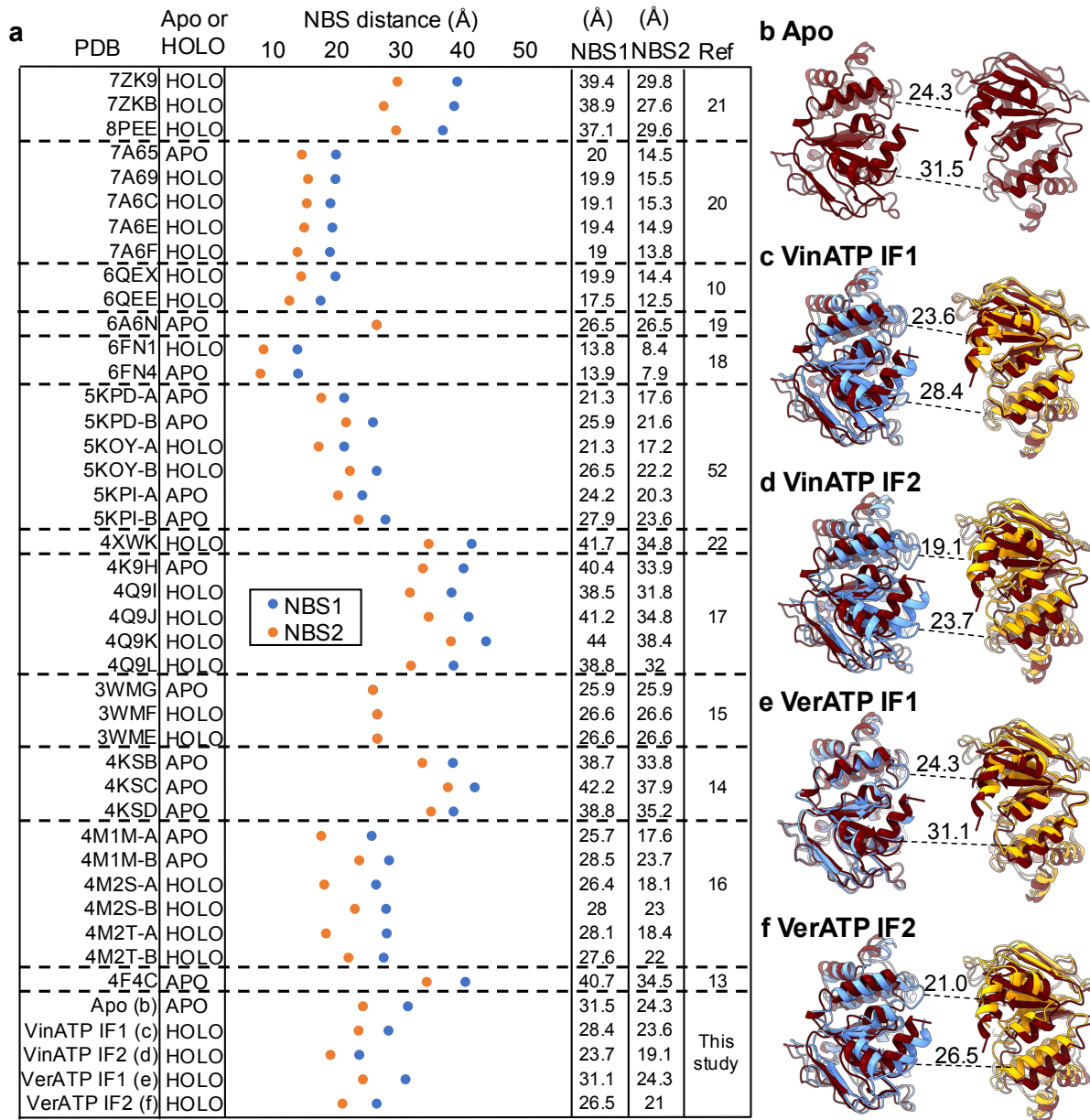
Supplementary Figure 2. Cryo-EM Data Processing of Vinblastine-ATP. (a) Representative electron micrograph. (b) Representative 2D class averages. (c) Six initial models used for 3D classification in the multiple models at multiple resolutions (M3R) strategy. (d) Flowchart showing 3D classification and refinement scheme for data processing. Asterisk represents particles used for 3D variability analysis as described in Supplemental Fig. 15.



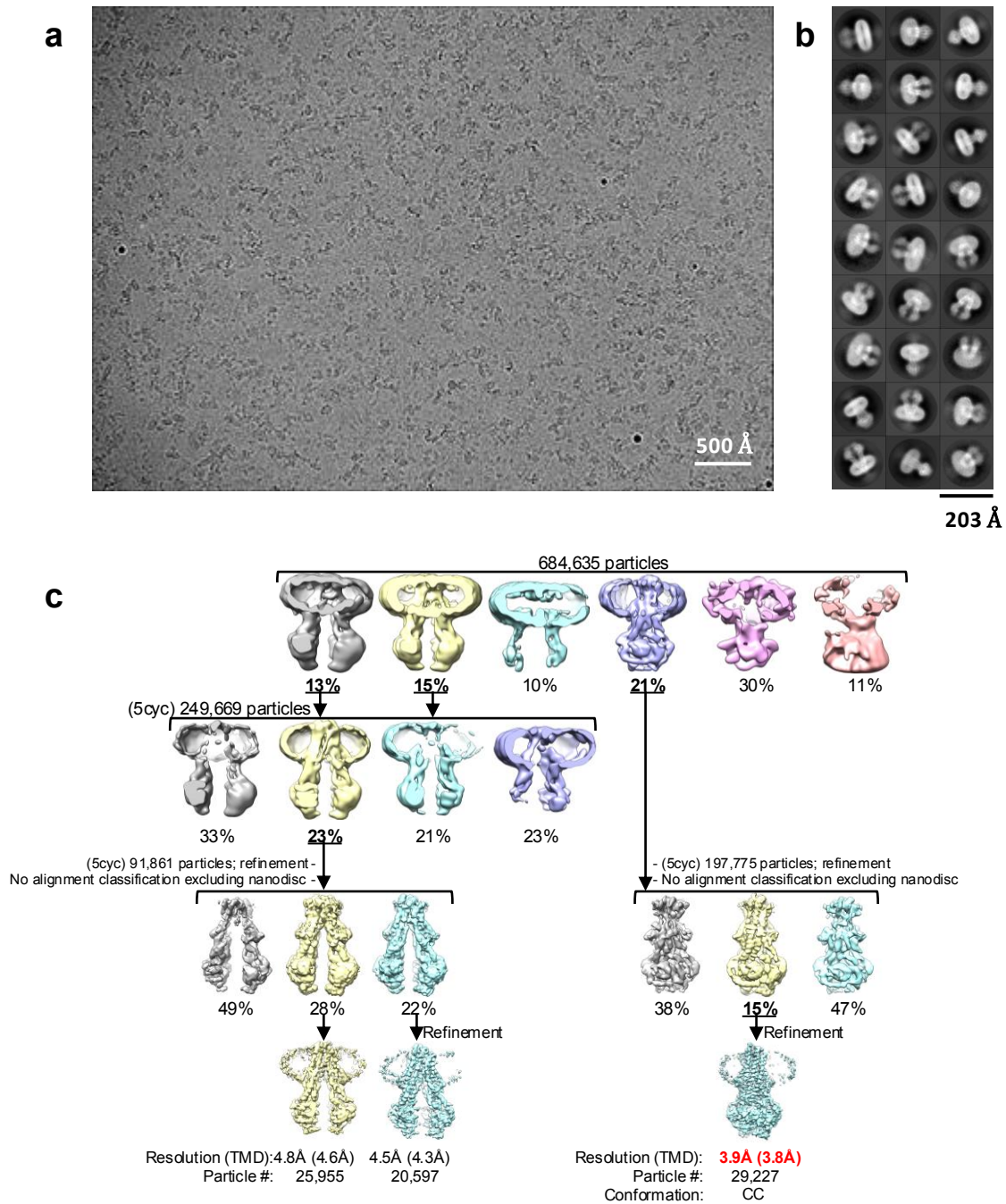
Supplementary Figure 3. Cryo-EM data analysis of vinblastine-ATP inward facing 1 conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. Left map shows cut-through and right map shows surface of Pgp (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black), and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices and ligands with refined model.



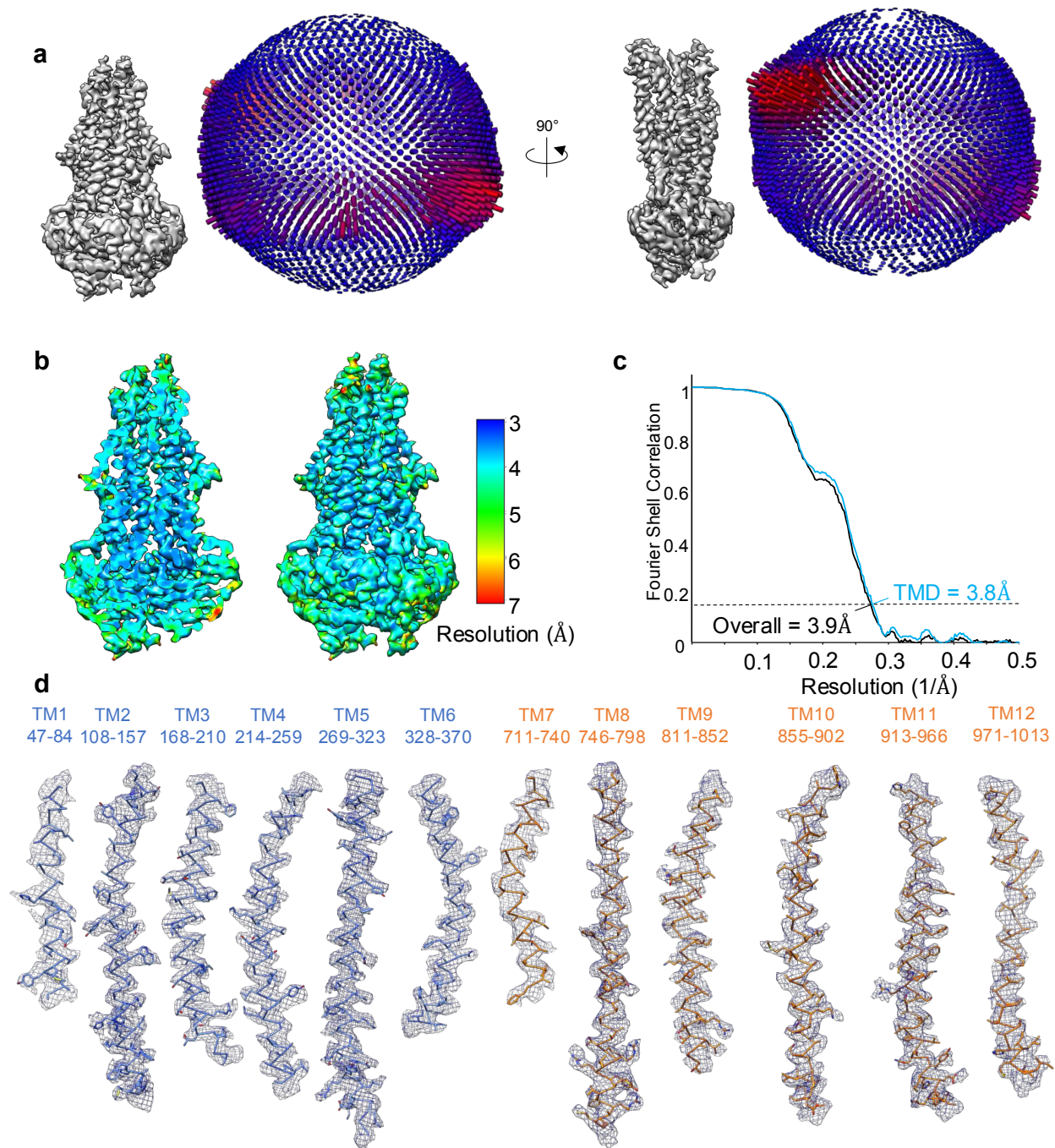
Supplementary Figure 4. Cryo-EM data analysis of vinblastine-ATP inward facing 2 conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. Left map shows cut-through and right map shows surface of Pgp (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black), and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices and ligands with refined model.



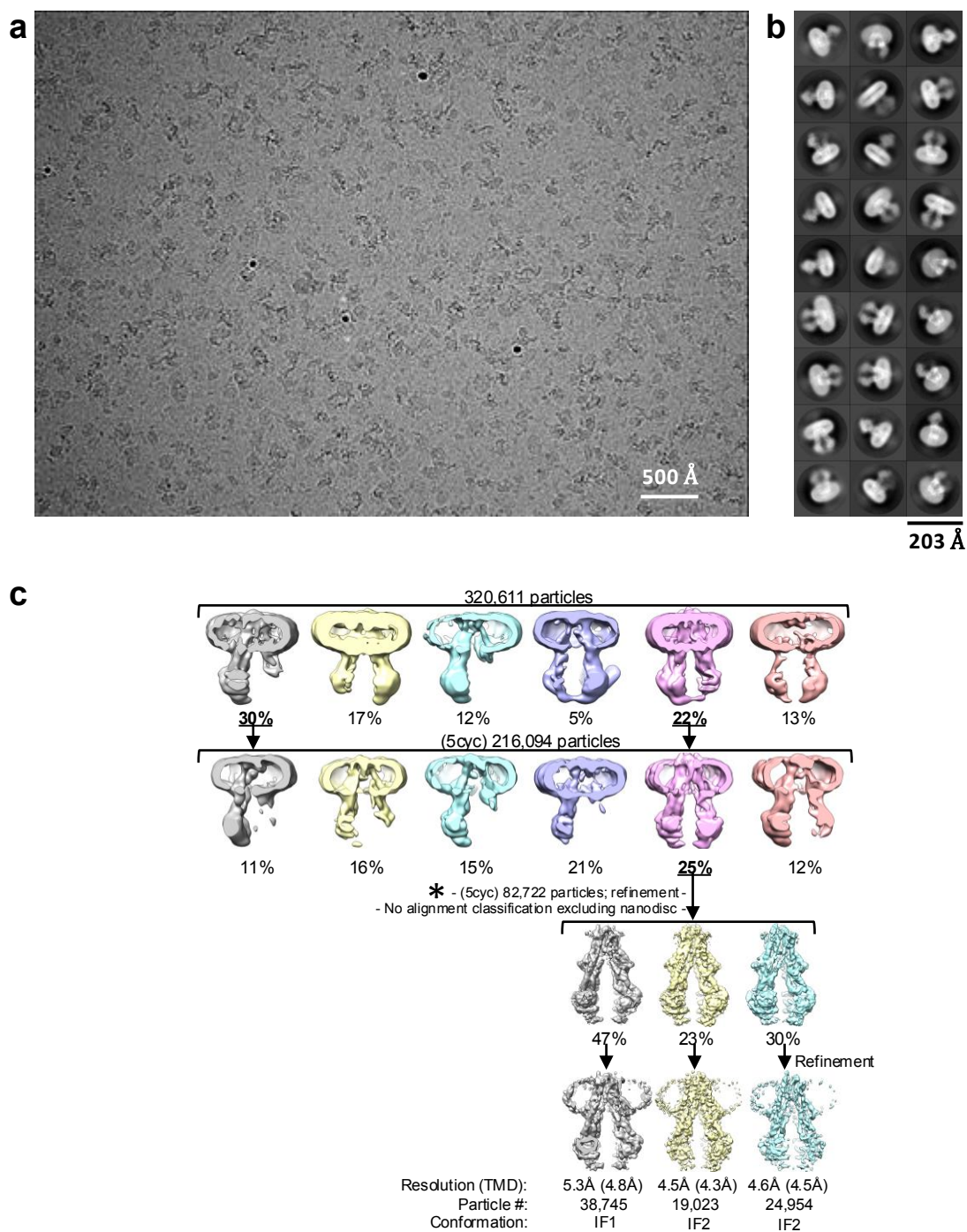
Supplementary Figure 5. Comparison of nucleotide binding site distances of all final cryo-EM maps with inward facing (IF) conformation and all previous Pgp structures. Distance from nucleotide binding site (NBS) were measures from α -carbon from S429 and S1177 for NBS1 or S532 and S1072 for NBS2 for human genes or the corresponding serine in orthologous proteins (S425, S1173, S518 and S1068 for mouse; S453, S1219, S556, and S1114 for *C. elegans*; S480 and S586 for *C. merolae*). **(a)** Distances of NBSs for all previous Pgp structures including this study. From left to right in (a) shows PDB ID, graph comparing NBS distances, numerical number of NBS1 and NBS2, and reference of structure. **(b)** Distances from NBSs for Apo model (red). **(c-d)** VinATP IF 1 (C) and IF2 (D). **(e-f)** VerATP IF1 (E) and IF2 (F). Red model in c-f represents apo structure for comparison.



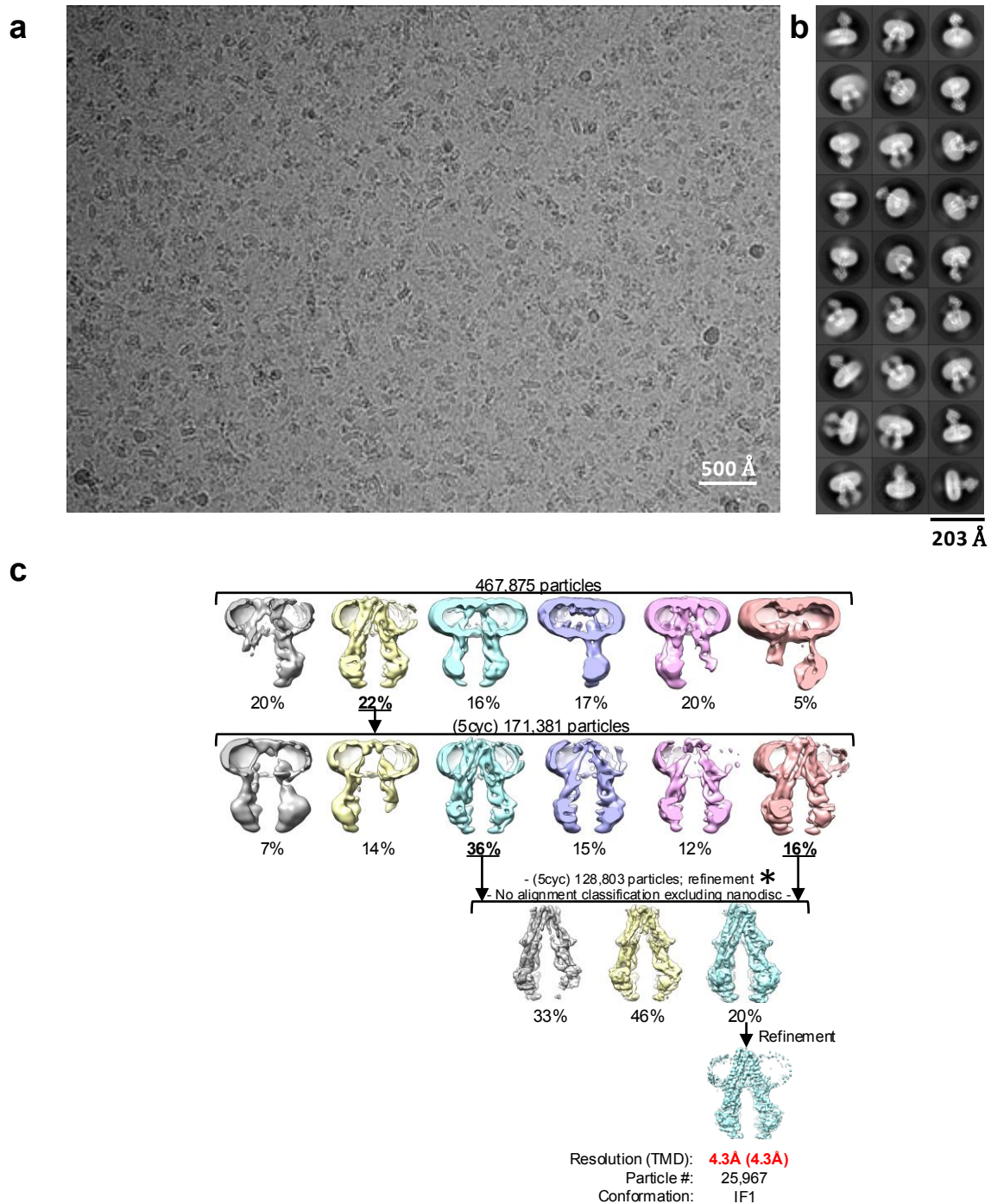
Supplementary Figure 6. Cryo-EM Data Processing of Vinblastine-ATP-vanadate. (a) Representative electron micrograph. **(b)** Representative 2D class averages. **(c)** Flowchart showing 3D classification and refinement scheme for data processing.



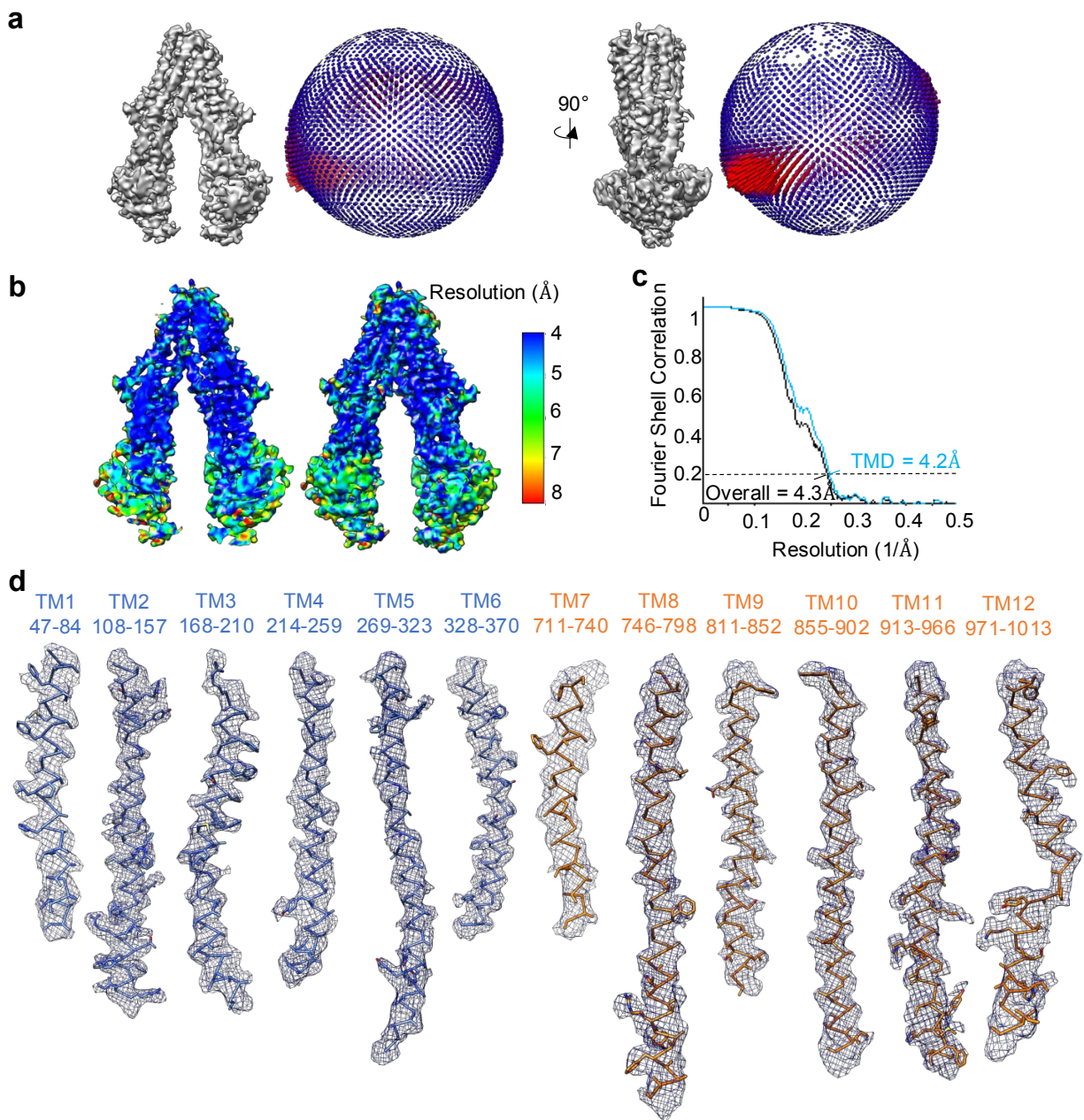
Supplementary Figure 7. Cryo-EM data analysis of vinblastine-ADP-Vi collapsed closed conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. Left map shows cut-through and right map shows surface of Pgp (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black), and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices with refined model.



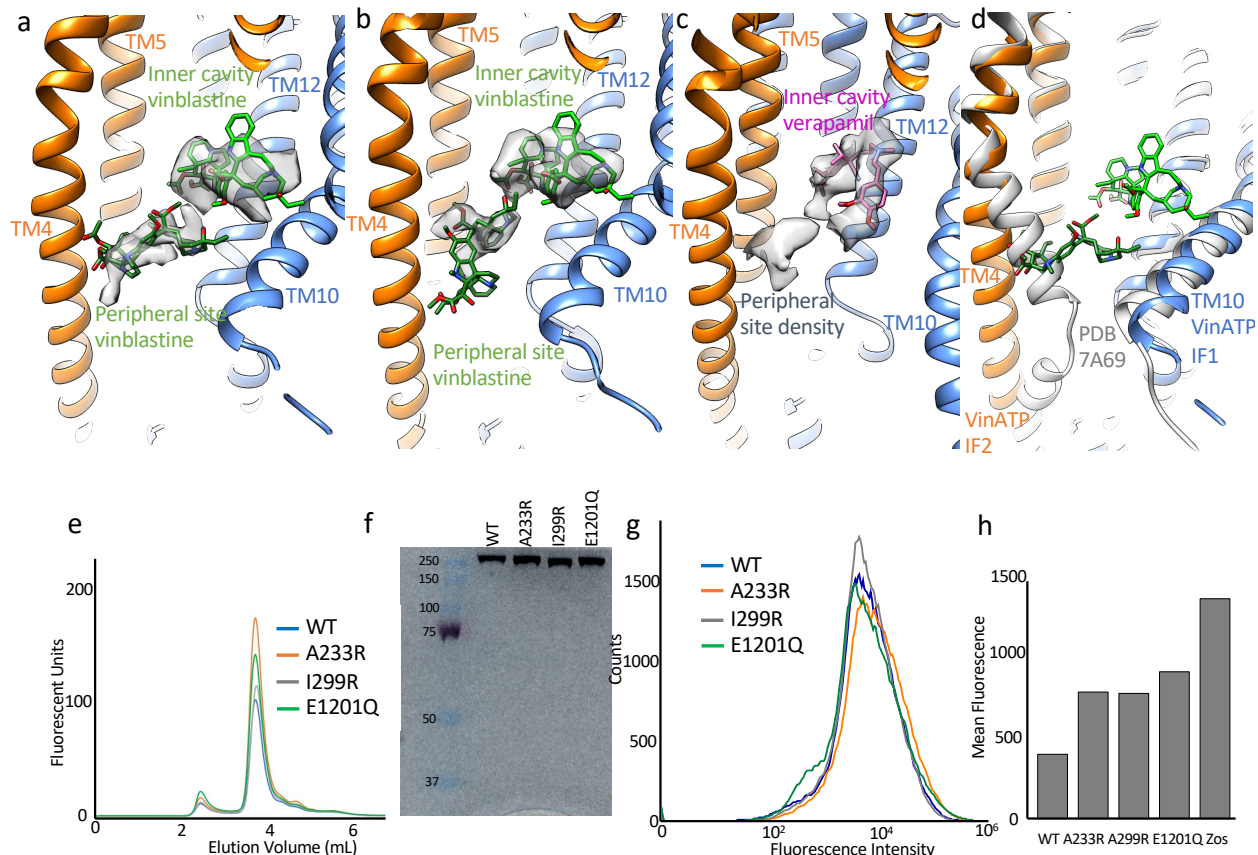
Supplementary Figure 8. Cryo-EM Data Processing of Vinblastine. (a) Representative electron micrograph. (b) Representative 2D class averages. (c) Flowchart showing 3D classification and refinement scheme for data processing. Asterisk represents particles used for 3D variability analysis as described in Supplemental Fig. 15.



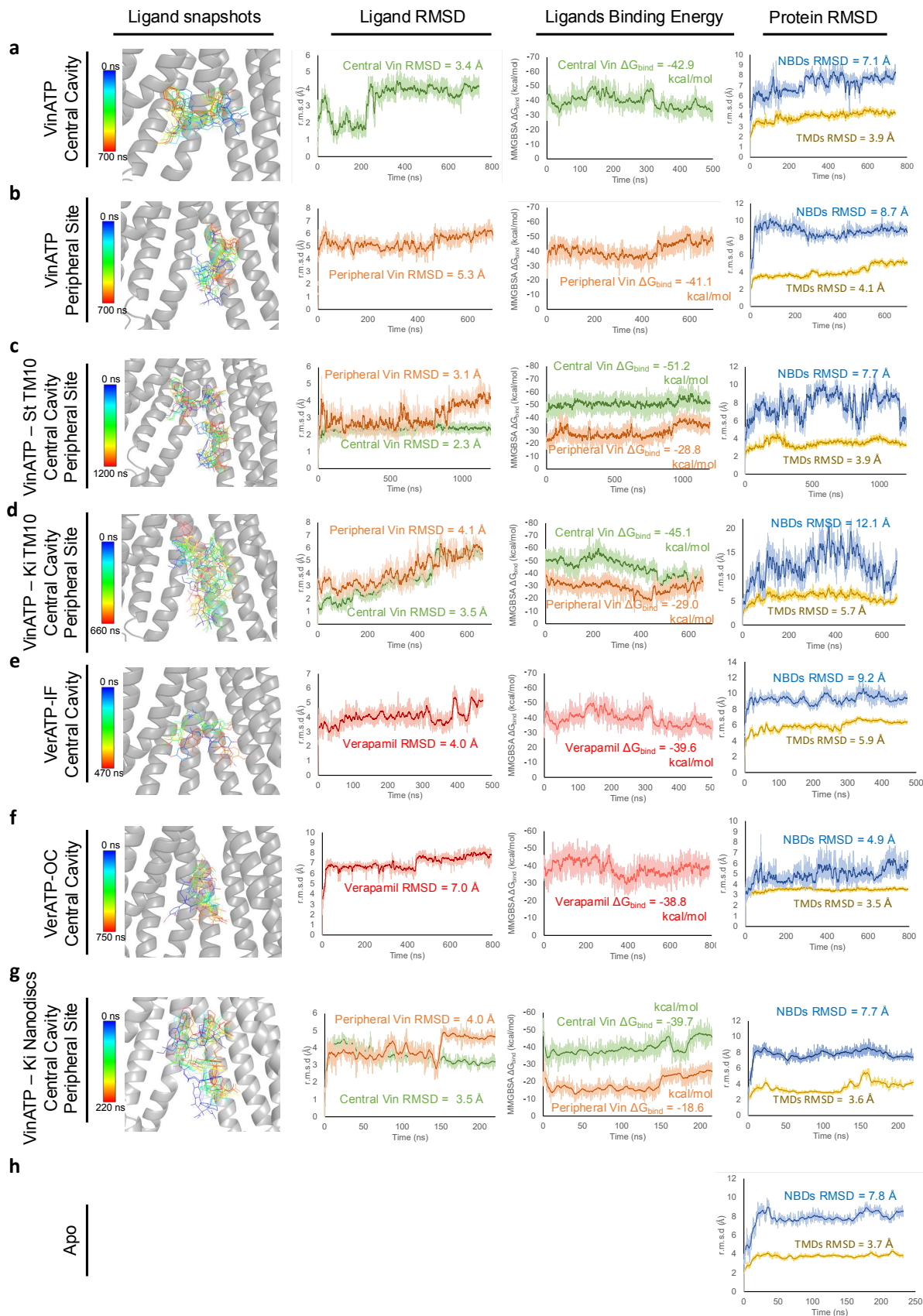
Supplementary Figure 9. Cryo-EM Data Processing of Apo. (a) Representative electron micrograph. (b) Representative 2D class averages. (c) Flowchart showing 3D classification and refinement scheme for data processing. Asterisk represents particles used for 3D variability analysis as described in Supplemental Fig. 15



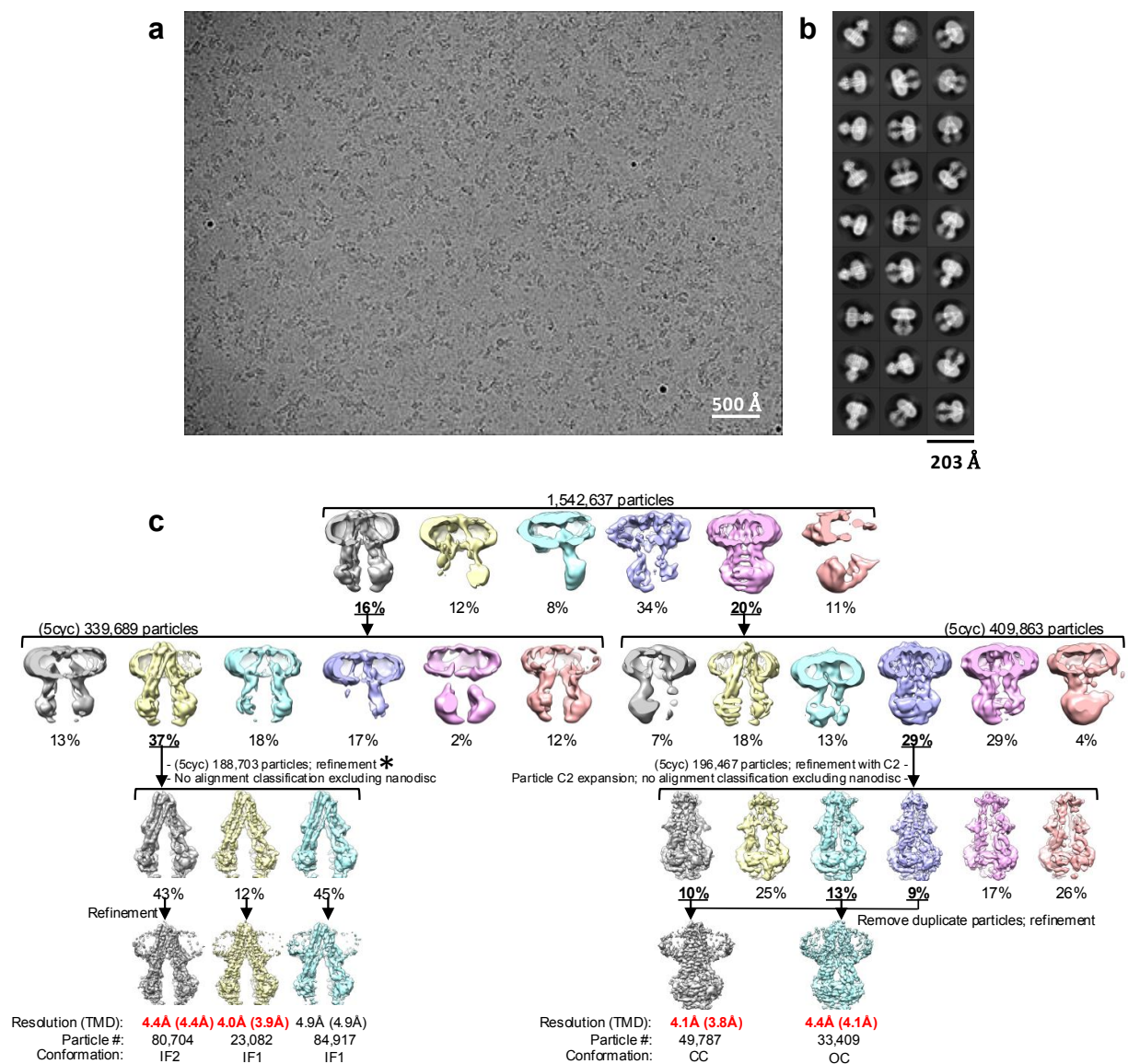
Supplementary Figure 10. Cryo-EM data analysis of apo conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. Left map shows cut-through and right map shows surface of Pgp (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black), and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices with refined model.



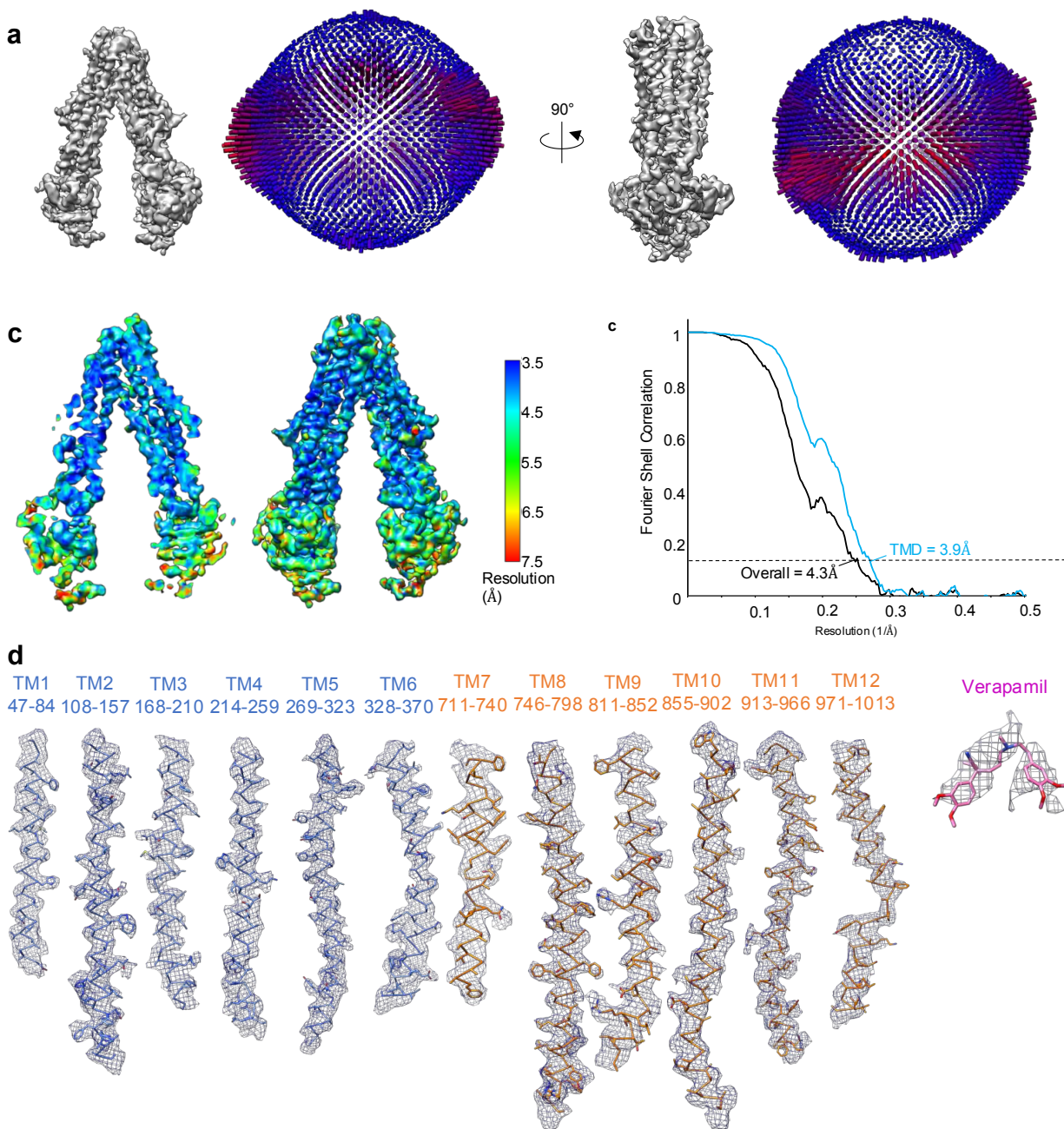
Supplementary Figure 11. Pgp peripheral site and biochemical characterization of Pgp peripheral site mutants. (a-c) Cryo-EM density at the peripheral site in Vin-ATP IF1 (A), Vin-ATP IF2 (B), and Ver-ATP IF2 (C). (d) Overlay of VinATP-IF2 with Pgp bound to vincristine and inhibitory fab (20; PDB 7A69). This shows the steric clash between peripheral vinblastine in VinATP-IF1 with the inward kinking TM4 in PDB 7A69. (e) Fluorescence size exclusion chromatography of crude lysate of WT and mutant Pgp. (f) SDS-PAGE of WT and mutant PGP detecting fluorescent. For (e) and (f), Pgp-GFP mutant or WT expressing cells were solubilized in DDM/CHS and supernatant was used for either FSEC (e) or SDS-PAGE gel with GFP fluorescence detection (f). (g) Flow cytometry of WT and mutant Pgp expressing cells showing expression level using GFP channel. (h) Conversion of flow cytometry histograms data shown in Fig. 2g to mean fluorescence. Source data are provided as a Source Data file. Gating strategy for (g) is provided in the Source Data file.



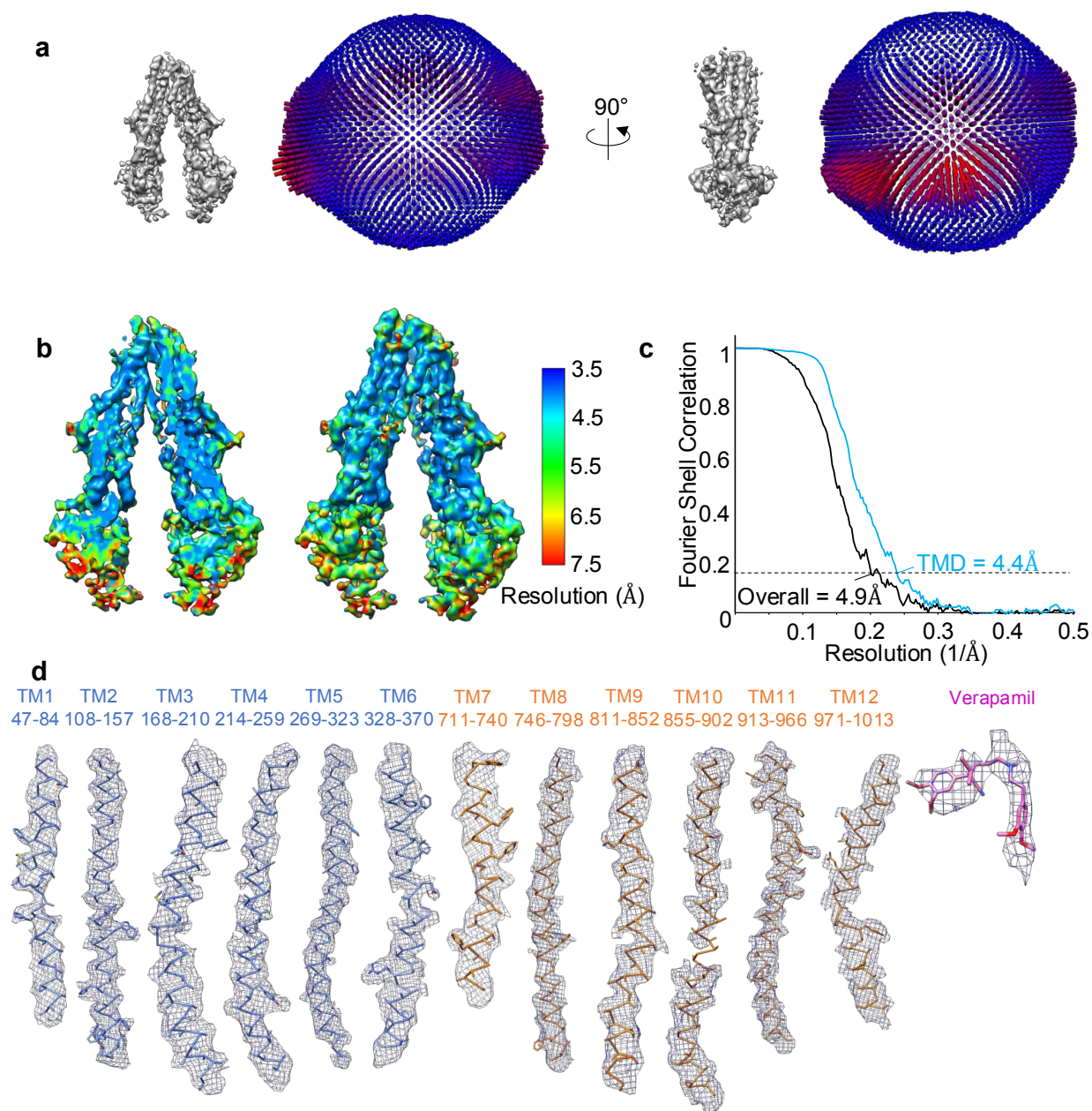
Supplementary Figure 12. Molecular dynamics of Pgp bound to vinblastine and verapamil. Molecular dynamics simulations of various states of Pgp. All root mean square deviation (RMSD) calculations are based on the initial structure after alignment of the TMD region. For each panel, the left row shows various ligand poses, the middle left row shows ligand RMSD, the middle right shows ligand binding energy, and the right row shows protein RMSD. For RMSD and binding energy graphs, the colors are central vinblastine (green), peripheral vinblastine (orange), verapamil (red), NBDs (blue) and TMD (yellow). Left model images show various ligand poses are colored according to simulation time with initial state in blue and final state in red. Simulations include **(a)** Vin-ATP IF1 state with only central vinblastine molecule (Supplementary Data 1). **(b)** Vin-ATP IF1 state with only the peripheral vinblastine molecule and straight TM10 (Supplementary Data 2). **(c)** Vin-ATP IF1 state with both central and peripheral vinblastine molecules and straight TM10 (Supplementary Data 3). **(d)** Vin-ATP IF1 state with both central and peripheral vinblastine molecules and kinked TM10 (Supplementary Data 4). **(e)** Ver-ATP IF2 state (Supplementary Data 5). **(f)** Ver-ATP occluded state (Supplementary Data 6). **(g)** Vin-ATP IF1 state with both central and peripheral vinblastine molecules and kinked TM10 in POPG nanodisc (Supplementary Data 7). **(h)** Apo state (Supplementary Data 8). Source data are provided as a Source Data file.



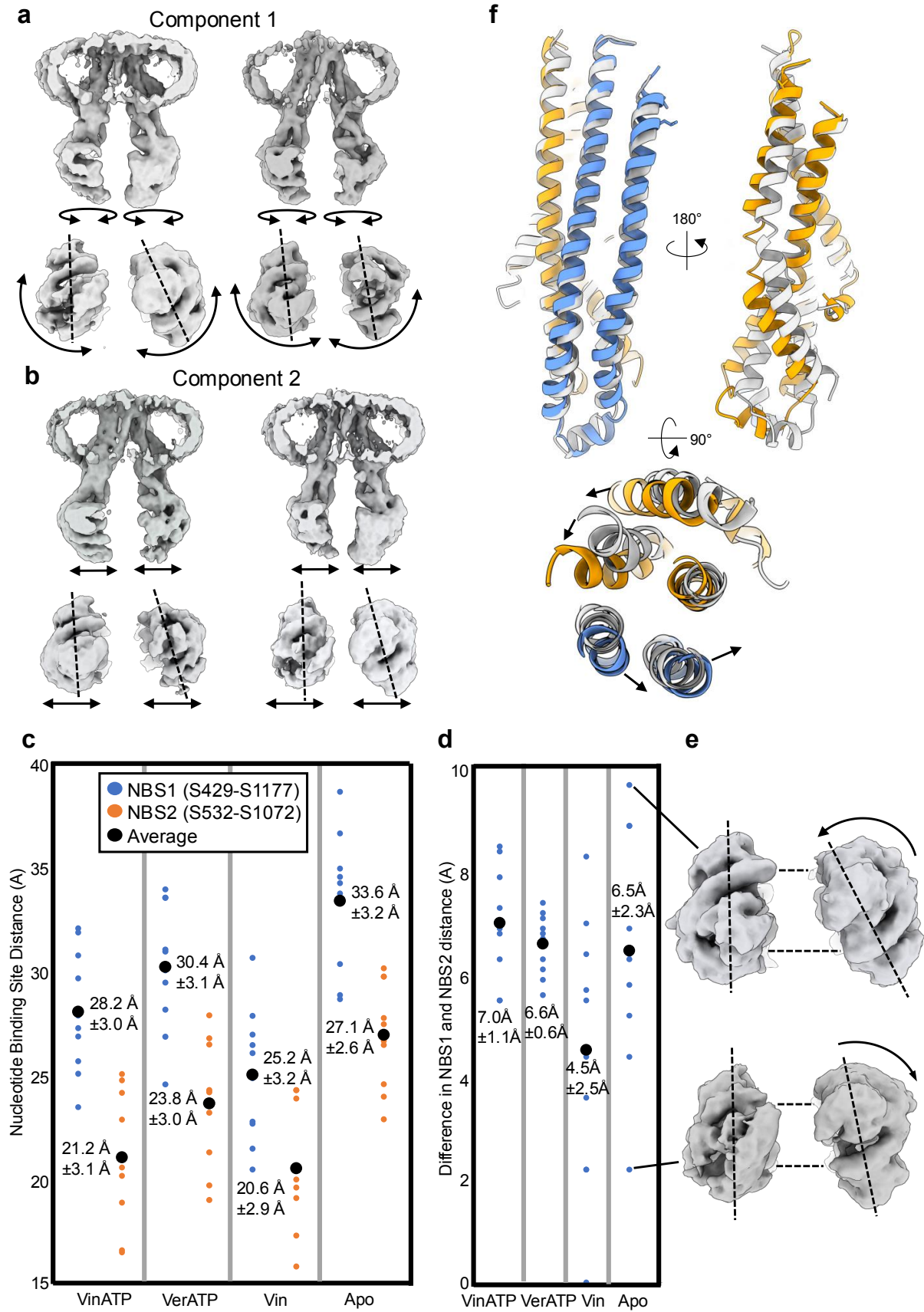
Supplementary Figure 13. Cryo-EM Data Processing of Verapamil-ATP. (a) Representative electron micrograph. **(b)** Representative 2D class averages. **(c)** Flowchart showing 3D classification and refinement scheme for data processing. Asterisk represents particles used for 3D variability analysis as described in Supplemental Fig. 15.



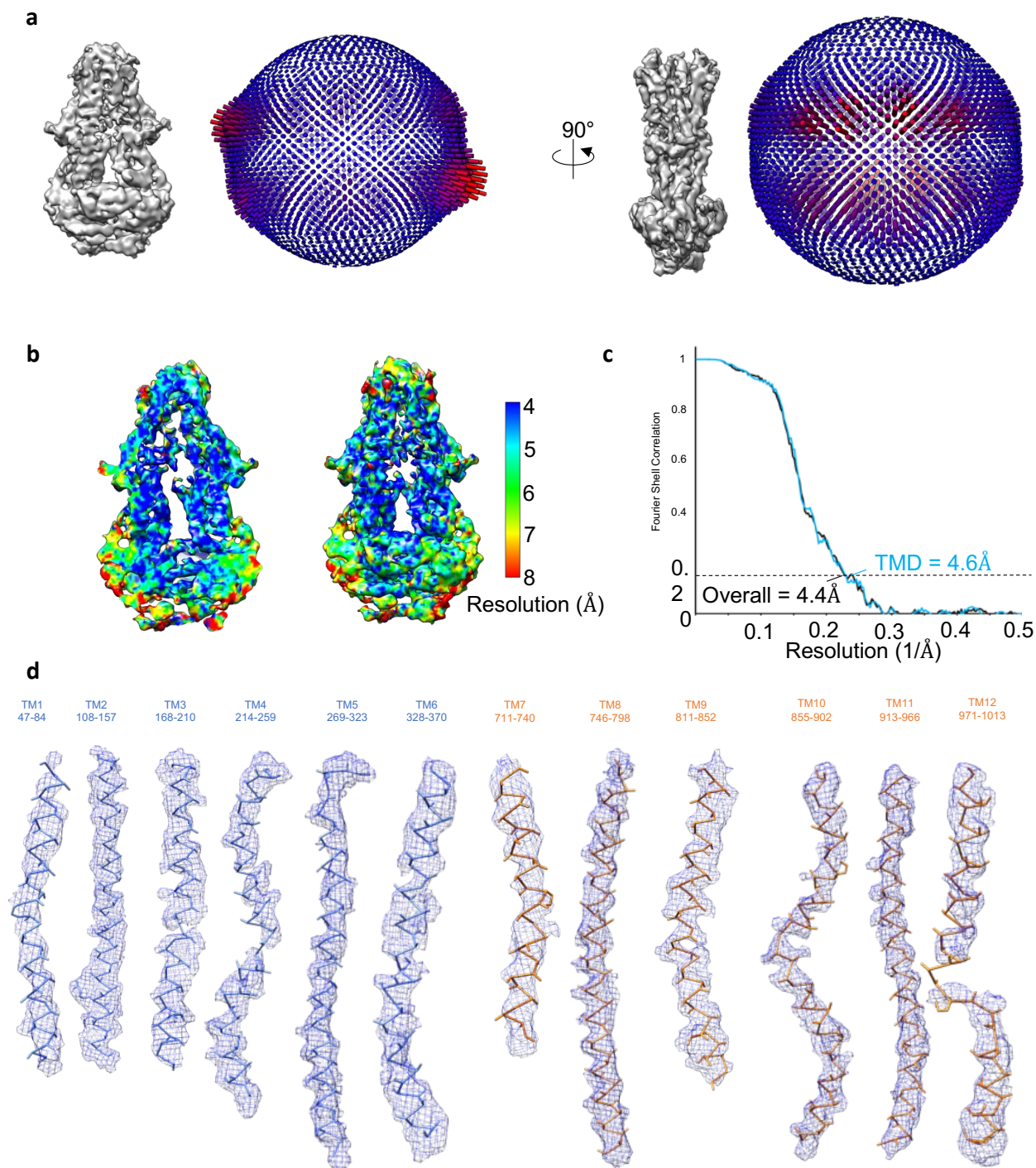
Supplementary Figure 14. Cryo-EM data analysis of verapamil-ATP inward facing 1 conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black), and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices with refined model.



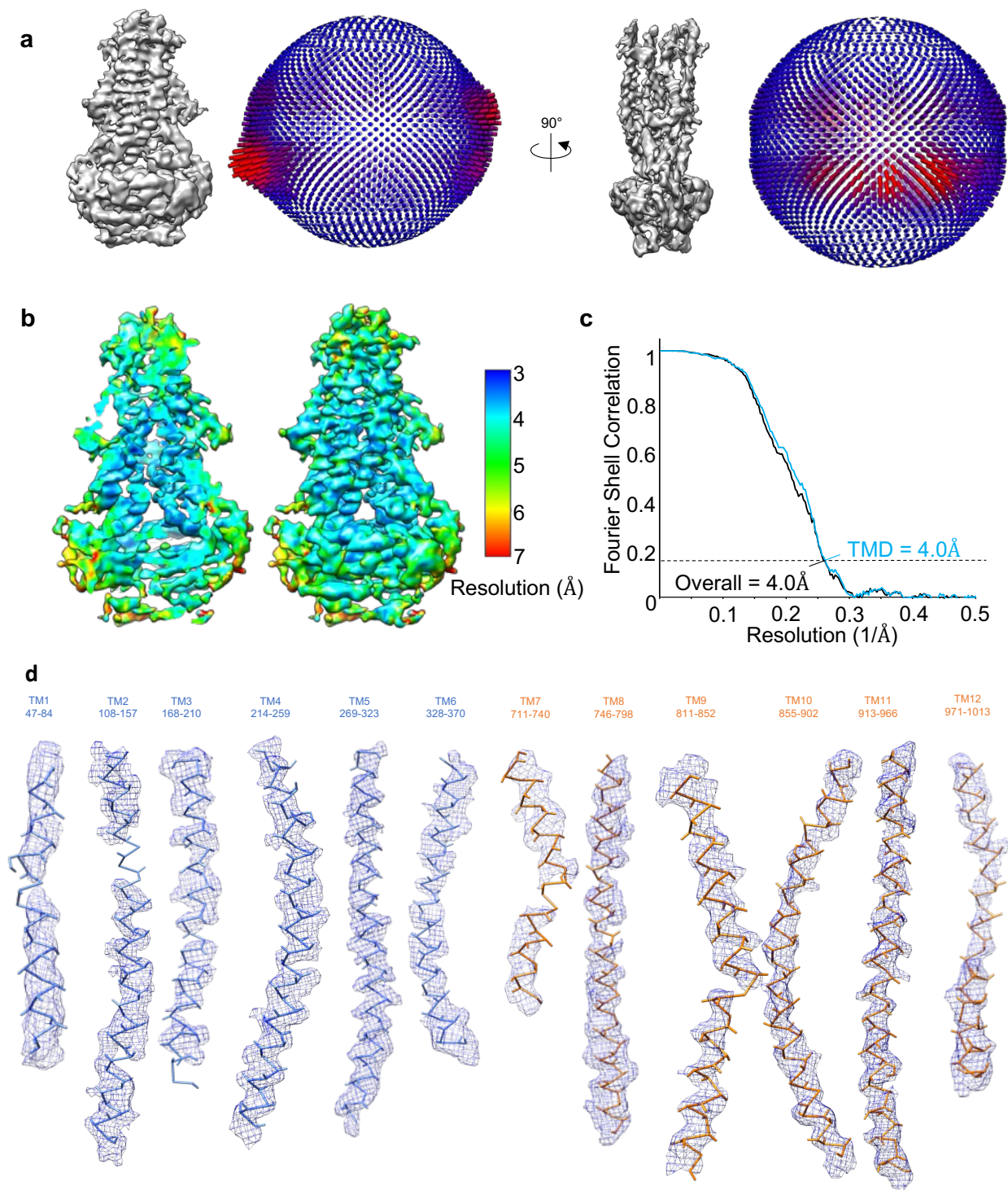
Supplementary Figure 15. Cryo-EM data analysis of verapamil-ATP inward facing 2 conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black) and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices with refined model.



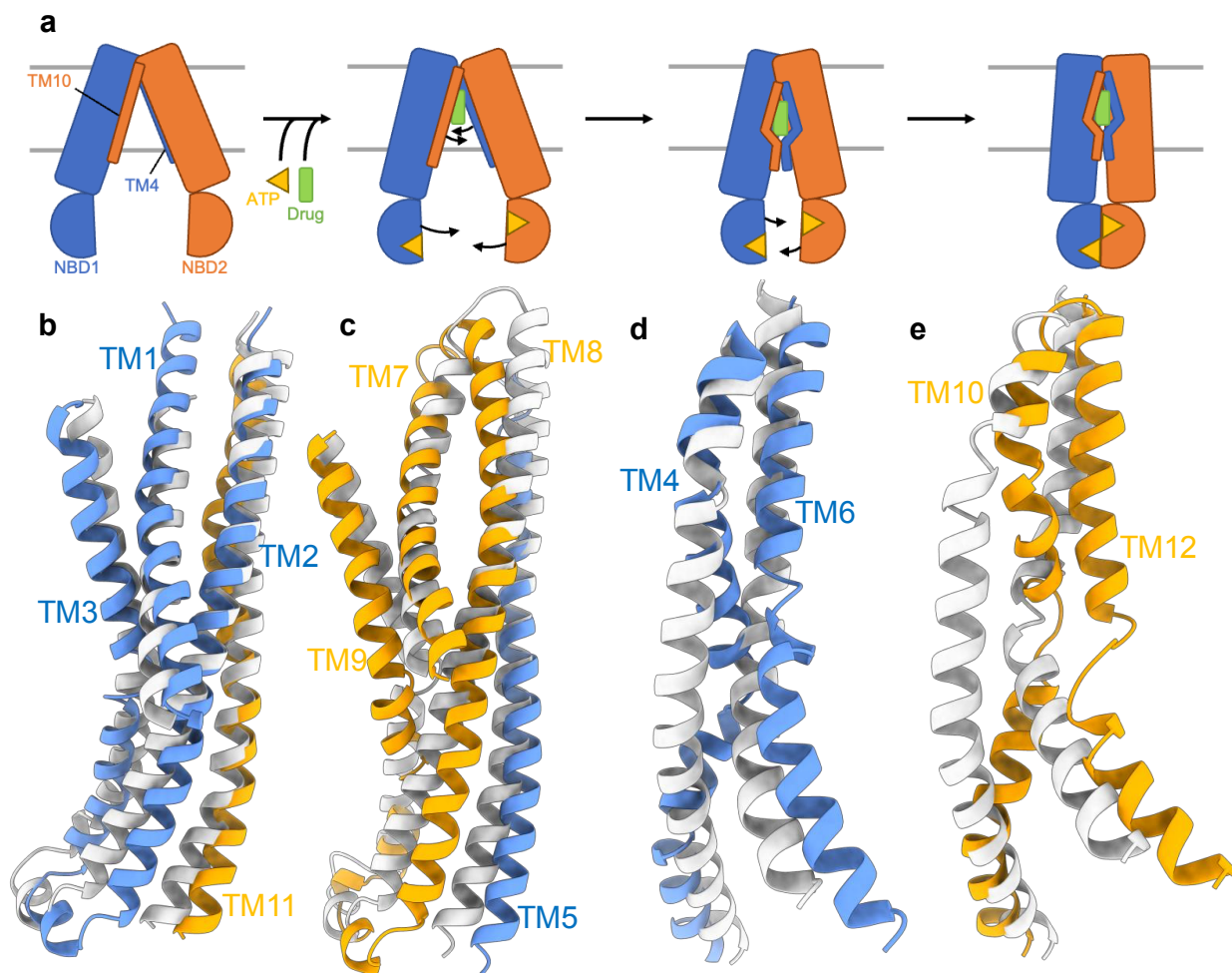
Supplementary Figure 16. Comparison of nucleotide binding site (NBS) distances. Cryosparc 3D variability analysis was performed using data after initial global refinement for each dataset. Analysis was done using three eigenvectors with “intermediate” viewing mode (**a-b**) and cluster viewing mode (**c-e**) with 10 total clusters. This analysis separated the various particles with different NBD translational distances and NBD rotation. For distance measurements, nucleotide binding domain were “fit to map” using UCSF chimera into NBD density using Ver-ATP IF2 NBDs. Measurements were from Walker A motif serine (S532 and S1177) and signature motif serine (S429 and S1072) (**a-b**) Cryo-EM maps of two extremes of Apo dataset showing component with NBD rotation (a) and NBD opening-and-closing (b). (**c**) Distances of NBS1 (blue; S429 to S1177) and NBS2 (orange; S532 to S1072) for each dataset. (**d**) NBD asymmetry of the difference in distance of NBS1 and NBS2 for each individual cluster. Dark black points are average for each set of data. Numbers in c and d represent the average and standard deviation for each set of data. (**e**) Examples of the two extremes in NBD asymmetry from the Apo dataset showing different degrees of NBD asymmetry. (**f**) Alignment of the two halves of the Pgp apo structure. White models represent half 1 (TM1-6) and yellow/blue models represent half 2 (TM7-12).



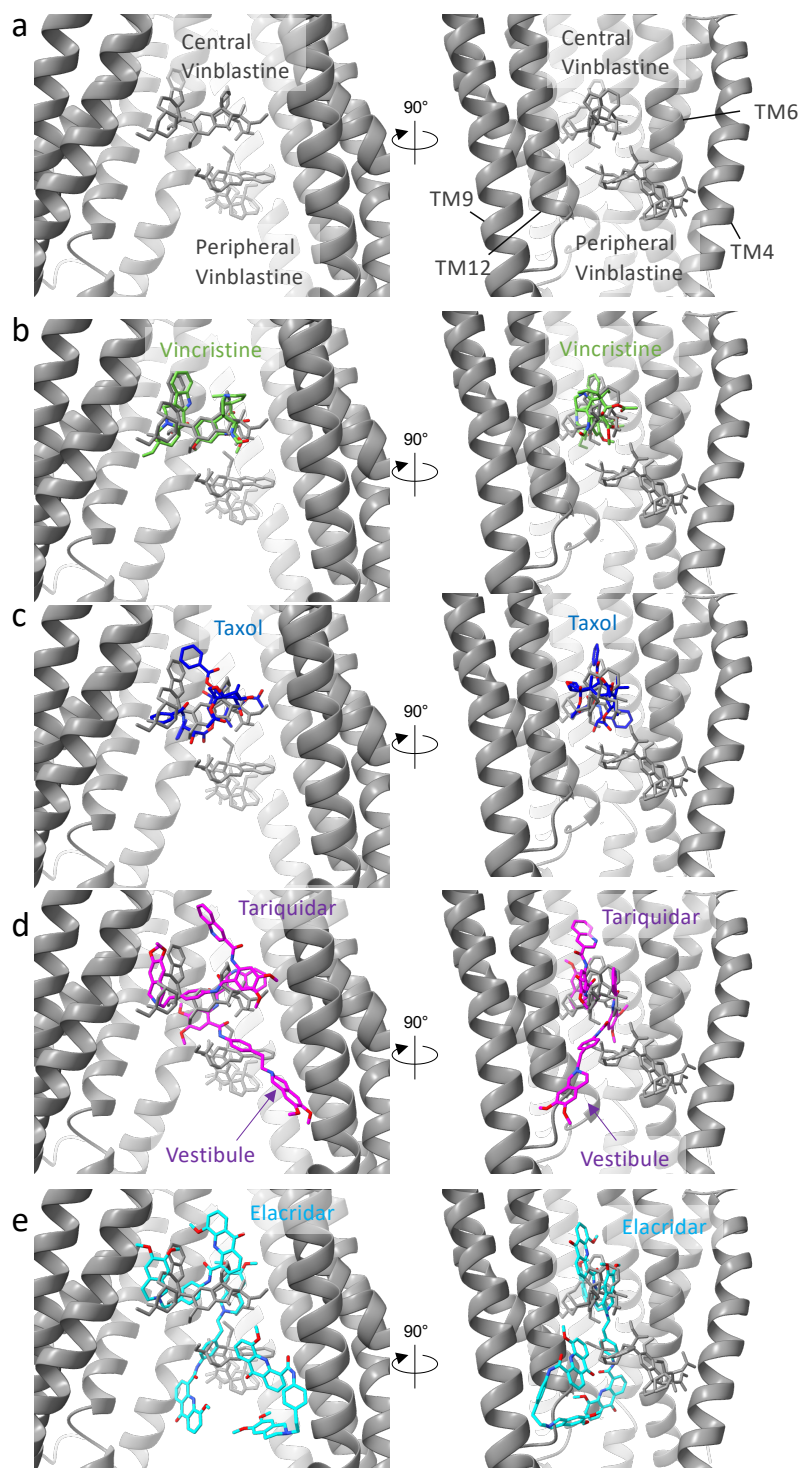
Supplementary Figure 17. Cryo-EM data analysis of verapamil-ATP occluded closed conformation. (a) Angular distribution of particle images included in the final 3D reconstruction. (b) Local resolution of the final cryo-EM map. (c) Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black) and with mask around only TMDs (blue). (d) Cryo-EM map for TM helices with refined model.



Supplementary Figure 18. Cryo-EM data analysis of verapamil-ATP collapsed closed. (a) Angular distribution of particle images included in the final 3D reconstruction. **(b)** Local resolution of the final cryo-EM map. **(c)** Fourier shell correlation (FSC) curves: gold standard FSC curve between the two half maps with mask around whole Pgp molecule with indicated resolution at FSC = 0.143 (black) and with mask around only TMDs (blue). **(d)** Cryo-EM map for TM helices with refined model.



Supplementary Figure 19. Structural alignment of Verapamil-ATP in the occluded closed and collapsed closed state. (a) Model for coupling between TM kinking and NBD closure. Substrate binding induces inward kinking of TM4 and TM10. These helices are connected to coupling helices and their inward movement induces NBD inward movement. (b-e) Structural alignment was done in TM region. Occluded closed state is colored and collapsed closed state is grey. For clarity, only selected TMs are shown including (b) TM1-TM3 and TM11, (c) TM7-9 and TM5, (d) TM4 and TM6, and (e) TM10 and TM12.



Supplementary Figure 20. Comparison of VinATP-IF1 with previously published Pgp structures. (a) VinATP-IF1 with no overlaid structures. (b-d) VinATP-IF1 (grey) with overlaid ligand binding positions including (b) vincristine (green; PDB 7A69), (c) taxol (blue; PDB 6QEX), (d) tariquidar (magenta PDB 7A6E), and (e) elacridar (cyan; PDB 8Y6I). Structural overlay was done by aligning the TM regions. Left image has TM10 and TM12 removed for clarity. Right image is rotated by 90 degree and has TM7, TM8, and TM5 removed for clarity.

Supplementary Table 1. Statistics of Cryo-EM structures

Dataset	Apo		Vin		VinATP				VerATP				ADP-Vi	
State	IF		IF1	IF2	IF1	IF2	CL1	CL2	IF1	IF2	OC	CC	IF	CC
PDB	8GMG				8SB9	8SBA			8SB7	8SA1	8SA0	8GMJ		8SB8
EMDB	40226		40340	40341	40294	40295	40342	40343	40292	40259	40258	40227	40326	40293

Data collection and processing

Micrographs	4996/3987		3064/3274		3357/6015				3075/8816				3792	
Microscope	Krios		Krios		Krios				Krios				Krios	
Magnification	81,000		81,000		81,000/105,000				81,000				81,000	
Voltage (kV)	300		300		300				300				300	
Electron Exposure (e-/Å²)	52.66/50.4		55.43/49.79		57.75/46.06				53.0/51.86				48.46	
Defocus Range (um)	-1 to -2.5		-1 to -2.5		-1 to -2.5				-1 to -2.5				-1 to -2.5	
Pixel size (Å)	1.06		1.06		1.06/0.83				1.06				1.06	
Initial particles images (#)	467,875		320,611		1,443,121				1,542,637				684,635	
Final Particle images	25,967	38,745	19,023	19,487	32,832	38,462	42,718	23,082	80,704	49,787	33,409	20,597	29,227	
Map resolution	4.3	5.3	4.5	3.9	4.1	5.6	6.4	4.0	4.4	4.1	4.4	4.5	3.9	
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	
Map sharpening B factor	-150	-200	-150	-150	-100	-200	-250	-100	-200	-150	-100	-150	-120	

Refinement

Model													
Composition													
Non-hydrogen atoms	6328			7381	7150			6886	6138	5832	5766		7340
Protein residues	1158			1159	1156			1156	1156	1151	1145		1146
Ligands	0			4	4			3	3	3	2		2
Mg	0			2	2			2	2	2	2		2
B-factors													
Protein	72.23			83.3	61.33			76.80	68.9	94.92	159.45		57.9
Ligands	N/A			80.28	57.41			89.45	87.11	50.7	127.1		52.6
RMS deviations													
Bond length (Å)	0.003			0.003	0.002			0.002	0.002	0.003	0.007		0.003
Bond angles (degree)	0.446			0.525	0.459			0.472	0.483	0.493	0.824		0.46
Validation													
Mol probity score	1.42			1.74	1.45			1.74	1.61	1.3	1.84		1.46
Clashscore	5.44			9.41	6.3			8.32	5.83	3.4	10.65		6.7
Poor rotamers (%)	0			0.23	0			0	0	0	0		0
Ramachandran													
Favored (%)	97.3			96.36	97.48			95.8	95.8	97.4	95.8		97.5
Allowed (%)	2.69			3.64	2.52			4.2	4.2	2.5	4.1		2.5
Outliers (%)	0			0	0			0	0	0.1	0.2		0

References:

52. L. Esser, F. Zhou, K. M. Pluchino, J. Shiloach, J. Ma, W. K. Tang, C. Gutierrez, A. Zhang, S. Shukla, J.P. Madigan, T. Zhou, P.D. Kwong, S.V. Ambudkar, M.M. Gottesman, D. Xia, Structures of the Multidrug Transporter P-glycoprotein Reveal Asymmetric ATP Binding and the Mechanism of Polyspecificity. *The Journal of biological chemistry* **292**, 446-461 (2017).