Negative Stain Single-particle EM of the Maltose Transporter in Nanodiscs Reveals Asymmetric Closure of MalK₂ and Catalytic Roles of ATP, MalE, and Maltose

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The Escherichia coli MalE-MalFGK₂ complex is one of the best characterized members of the large and ubiquitous family of ATP-binding cassette (ABC) transporters. It is composed of a membrane-spanning heterodimer, MalF-MalG; a homodimeric ATPase, MalK₂; and a periplasmic maltose receptor, MalE. Opening and closure of MalK₂ is coupled to conformational changes in MalF-MalG and the alternate exposition of the substrate-binding site to either side of the membrane. To further define this alternate access mechanism and the impact of ATP, MalE, and maltose on the conformation of the transporter during the transport cycle, we have reconstituted MalFGK₂ in nanodiscs and analyzed its conformations under 10 different biochemical conditions using negative stain single-particle EM. EM map results (at 15–25 Å resolution) indicate that binding of ATP to MalK₂ promotes an asymmetric, semi-closed conformation in accordance with the low ATPase activity of MalFGK₂. In the presence of MalE, the MalK dimer becomes fully closed, gaining the ability to hydrolyze ATP. In the presence of ADP or maltose, MalE-MalFGK₂ remains essentially in a semi-closed symmetric conformation, indicating that release of these ligands is required for the return to the initial state. Taken together, this structural information provides a rationale for the stimulation of MalK ATPase activity by MalE as well as by maltose.

The Escherichia coli maltose transporter MalFGK₂ is a prototype for ATP-binding cassette (ABC) transporters, an evolutionarily conserved family of membrane proteins that use ATP to mediate substrate translocation across membranes (1, 2). ABC transporter malfunctions are associated with various diseases including cystic fibrosis, lipid transport defects, and multi-drug resistance (3). ABC transporters have a common architecture with two cytosolic nucleotide-binding domains (NBDs) connected to two transmembrane domains (TMDs) via a pair of coupling helices (4). The binding and subsequent hydrolysis of ATP by the NBDs promote the alternation of the TMDs between the outward facing and inward facing conformations, effectuating substrate transport (4). Although this basic mechanism is conserved between exporters and importers, bacterial importers require additional substrate-binding proteins. The maltose transporter is comprised of the two TMD subunits, MalF and MalG; the NBD MalK₂; and the maltose-binding protein MalE (5).

X-ray crystallography structures of MalFGK₂ in different conformations have provided crucial information regarding the chemistry of ATP binding and hydrolysis (6–11). It is now established that the open, nucleotide-free MalK₂ is associated with the inward facing arrangement of MalF-MalG, whereas the ATP-bound closed MalK₂ is associated with the outward facing conformation. Substrate binding, transport, and release create additional intermediary steps that have not been solved by X-ray crystallography. Moreover, protein crystallization is performed in destabilizing detergent micelles, an environment in which the ATPase activity of MalK₂ is uncoupled from MalE and maltose (5, 12).

Biochemical (12–18) and biophysical (19, 20) studies have greatly advanced our understanding of the transport cycle; however, conflicting data concerning the role of ATP, MalE, and maltose remain to be resolved (17, 19, 20). To do so will require methods to isolate and characterize new intermediate conformations along the transport pathway. In this regard, nanodisc and EM are excellent tools well suited for the biochemical and structural study of membrane proteins. The nanodisc system allows for the stabilization of membrane proteins in a soluble and monodisperse state (21), permitting the determination of the 3D structure using single-particle EM.
Importantly, in nanodiscs (as in proteoliposomes), the ATPase activity of MalFGK2 is coupled to MalE and maltose (12). In this study, we have determined the role of nucleotide, MalE, and maltose on the closure and reopening of MalK2 in the context of the full transporter. In doing so, we have characterized the conformations of MalFGK2 in nanodiscs under 10 different biochemical conditions using single-particle EM combined with a systematic analysis of the conformational heterogeneity in any given states (for a complete summary, see Table 1). Results show that ATP induces the partial asymmetric closure of MalK2. Furthermore, whereas MalK2 can dynamically sample the closed state, complete stabilization in that conformation requires binding of open state MalE. In the presence of ADP or maltose, most of MalE:MalFGK2 remains in a semi-closed conformation, indicating that release ADP and maltose contributes to the return of the transporter to the resting state.

**Results**

ATP Binding Induces Asymmetric Closure of MalK2—We previously reported using cysteine cross-linking with variant MalK$_{S83C}$ that when the transporter was reconstituted in nanodiscs, ATP alone was sufficient to trigger the closure of MalK2 (17). In contrast, spin-labeling EPR studies of MalFGK2 in detergent micelles showed that maltose-bound MalE and ATP are both required for the closure of MalK2 (20). The crystal structures of the MalE:MalFGK2 obtained with ATP (6) or different nucleotides such ATP$\gamma$S and AMP-PNP (8, 9, 22) or ADP plus BeF$_3$, VO$_4^{3-}$, or AlF$_4^-$ (8), all show MalK2 in the closed conformation. However, the crystal structure of the MalFGK$_2$ liganded with ATP/ATP analogs in the absence of MalE is currently not available. We therefore set out to determine the impact of nucleotides on the conformation of MalFGK$_2$ via EM analysis. The quality of the protein complex preparations used in this study has been previously documented (17).

The EM map of MalFGK$_2$ obtained in the absence of nucleotide reveals that MalK2 rests in the open conformation, as expected (Figs. 1 and 2A and supplemental Fig. S1). There was no contact between the two MalK subunits at the level of the nucleotide binding pockets, and the electron densities corresponding to the interface MalK-MalG and MalK-MalF (henceforth called MalKF junction and MalKG junction) were separated from each other by $\sim$58 Å. This open conformation of MalK2 was reported by X-ray crystallography (7).

In the presence of ATP, full closure of MalK2 was obtained; however, it was only when using the MalK$_{S83C}$ variant and the cysteine-reacting cross-linker BMOE (MalFGK$_2$:ATP+BMOE; Fig. 2B and supplemental Fig. S2) (17). In this closed conformation adopted by 100% of the particles, there was extensive contact at the level of the nucleotide binding pockets, and the electron densities corresponding to the interface MalK-MalG and MalK-MalF (henceforth called MalK$_F$ junction and MalK$_G$ junction) were separated from each other by $\sim$58 Å. This open conformation of MalK$_2$ was reported by X-ray crystallography (7).

In the presence of ATP, full closure of MalK$_2$ was obtained; however, it was only when using the MalK$_{S83C}$ variant and the cysteine-reacting cross-linker BMOE (MalFGK$_2$:ATP+BMOE; Fig. 2B and supplemental Fig. S2) (17). In this closed conformation adopted by 100% of the particles, there was extensive contact at the level of the nucleotide binding pockets, and the distance between MalK$_F$ and MalK$_G$ junctions was $\sim$16 Å.

In the presence of the non-hydrolyzable ATP analog (ATP$\gamma$S) and ADP-phosphate mimics (ADP-VO$_4^-$ or ADP-AlF$_4^-$) MalK$_2$ was not stabilized in the closed state. Instead, two different semi-closed conformations were obtained, termed asymmetric and symmetric. In the semi-closed asymmetric
state (adopted by the majority of the particles), the MalK\(_G\) junction was closer to the center of the complex, whereas the MalK\(_F\) junction remained in the open position, similar to the nucleotide-free state (Table 1, Fig. 2C, and supplemental Fig. S3). Electron densities at the level of the nucleotide pockets were well separated, and the distance between MalK\(_F\) and MalK\(_G\) was \(\approx 40\) Å. In the semi-closed symmetric state (31% of the particles incubated with ADP-AlF\(_4\)), the MalK\(_G\) and MalK\(_F\) junctions were both closer to the center of the complex (Fig. 2D and Table 1). The MalK\(_F\) and MalK\(_G\) junctions were separated by only \(\approx 22\) Å, but the contact between the nucleotide pockets in MalK\(_2\) was not as extensive as in the closed conformation (Fig. 3).

Finally, in the presence of ADP, 47% of MalFGK\(_2\) adopted the semi-closed asymmetric conformation (Fig. 2E), whereas the remaining 53% were in the open state (Table 1). ADP was therefore able to affect the conformation of the MalK\(_G\) junction, as demonstrated by the presence of some transporters in the semi-closed asymmetric conformation.

Taken together, these results indicate that binding of ATP and non-hydrolyzable ATP analogs stabilize the asymmetric semi-closed conformation. The transporter is able to transiently sample the closed state because this conformation is captured by the cross-linker BMOE. However, without the cross-linker agent, MalK\(_2\) is most stable in the asymmetric semi-closed conformation.

**MalE Stabilizes the Closure of MalK\(_2\)—**In the crystal structures of MalE-MalFGK\(_2\) bound to ATP/AMP-PNP/ADP-P\(_i\) mimics, MalK\(_2\) was fully closed (6, 8, 9). In the EM maps of MalFGK\(_2\)-ATP\(_\gamma\)S, MalFGK\(_2\)-ADP-VO\(_4\), and MalFGK\(_2\)-ADP-AlF\(_4\) (Fig. 2), MalK\(_2\) was partially closed. MalE therefore plays a critical role for the closure of MalK\(_2\). Confirming this, the EM

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**FIGURE 2. Effect of nucleotide on MalFGK\(_2\) conformations.** EM maps of MalFGK\(_2\) obtained in the absence of nucleotide and presence of various nucleotides as indicated. All maps were obtained from wild type MalF, MalG, and MalK proteins except for the map labeled ATP-BMOE(*). This map was obtained using the variant MalK\(_S\)\(_{83C}\) stabilized in the closed conformation with ATP and the cross-linker BMOE. The maps are shown as isosurface representation at the contours of 350 and 250 Å\(^3\) (first and second rows, respectively). The crystal structure of MalFGK\(_2\) (PDB code 3FH6) docked in the EM map is shown for orientation purposes. The Walker A and LSGGQ motifs are shown in sphere representation in A, and their positions are marked by green and red triangles, respectively, in B–E. The red dashed line indicates the position used for slicing the maps to show the MalK\(_G\) and MalK\(_F\) junctions. The distance between the center of mass of the junctions is indicated. Junction distances less than 20 Å and greater than 50 Å are characteristic of the closed and open conformations respectively. Junction distances between 20 and 40 Å were observed for the semi-closed and asymmetric conformations.
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TABLE 1

Summary of the conformational states adopted by MalFGK2 in 10 different biochemical conditions

| Experimental conditions          | Open | Semi-closed | Closed | MalE detached | Total number of particles analyzed |
|---------------------------------|------|-------------|--------|---------------|-----------------------------------|
|                                | %    | %           | %      | %             |                                   |
| MalFGK2                         | 100  | 0           | 0      | 100           | N/A                               |
| MalFGK2-ATP + BMOE              | 0    | 0           | 0      | 0             | N/A                               |
| MalFGK2-ADP-VO4                 | 0    | 100         | 0      | 0             | N/A                               |
| MalFGK2-ATP-γ                  | 0    | 100         | 0      | 0             | N/A                               |
| MalFGK2-ADP-αf                  | 0    | 69          | 31     | 0             | N/A                               |
| MalE-MalFGK2-ADP                | 53   | 47          | 0      | 100           | 0                                 |
| MalE-x-MalFGK2/ADP              | 21 (24) | 65 (76) | 0      | 14            | 41,719                            |
| MalE-x-MalFGK2                  | 81 (100) | 0       | 0      | 19            | 7046                             |
| MalE-x-MalFGK2-maltose          | 14 (21) | 54 (79) | 0      | 32            | 11,678                            |

FIGURE 3. The open, closed, and semi-closed symmetric conformations of MalK2. EM maps of MalFGK2 maps shown as isosurface representations with the corresponding crystal structure of MalK2 dimer (upper panels), with PDB code 1Q1E, B; PDB code 2AWO) and ATP (C; PDB code 1Q12). Residues of the Walker A and LSGGQ motifs are shown as red and green spheres, respectively. The lower panels represent top view cross-sections after removal of the densities and atoms above the dashed line.

map obtained for MalE-MalFGK2 in the presence of ADP-VO4 revealed that MalK2 was fully closed in 100% of the particles analyzed (Fig. 4A and Table 1). The conformation of our EM map matches that of the equivalent crystal structures (6, 8, 9) (supplemental Fig. S4). The contact at the level of the nucleotide binding pockets was extensive, and the distance between MalF and MalG junction by ~20 Å between the open and closed conformations, 2) adoption of a symmetric or asymmetric conformation of MalK2, and 3) the degree of contact at the level of the nucleotide binding sites. These are persistently visible at

“bound MalE”) or via its N-lobe only because of the disulfide bridge (referred as “detached MalE”). MalE was detached in 19% of the particles in the absence of nucleotide and 14% of the particles in the presence ADP (Table 1). Interestingly, with ADP, 53% of MalFGK2 had MalK2 in the open conformation, but this number was decreased to 21% in the presence of MalE (Table 1 and supplemental Figs. S3 and S5). The remaining particles adopted the semi-closed asymmetric (for MalFGK2, Fig. 2E) and symmetric conformations (for MalE-x-MalFGK2; Fig. 4B). In the absence of nucleotide, MalK2 was always in the open conformation, even when MalE was bound to MalFGK2 (Table 1 and Fig. 4C). Thus, the tethering of MalE to MalFGK2 causes the motion of the MalK2 junction but only in the presence of nucleotides.

Maltose Induces Detachment of MalE and Prevents Reopening of MalK2—We compared the conformations of MalE-x-MalFGK2 in the presence and absence of maltose (Fig. 4, C–G). The number of particles with detached MalE increased from 19 to 32% upon the addition of the sugar (Table 1), consistent with the fact that closed liganded MalE has little affinity for the transporter (16). Interestingly, when MalE remained bound to the transporter in the presence of maltose, the number of particles with MalK2 in the open conformation decreased from 100 to 21%. The majority of the particles (~79%) were in the semi-closed symmetric conformation (Table 1 and Fig. 4E). The dominance of this conformation suggests that release of maltose from the transporter is necessary to permit return of MalK2 to the open resting state.

Discussion

This single-particle EM analysis has defined the conformational changes of MalK2 in response to the three factors—nucleotide, MalE, and maltose—individually and cooperatively. It has also identified intermediate conformations not previously captured by X-ray crystallography. The resolution of the EM maps was between 15 and 25 Å, which is sufficient for the detection of the large scale conformational changes of MalK2 (supplemental Fig. S5). These include 1) the motion of both MalK2 and MalK2 junction by ~20 Å between the open and closed conformations, 2) adoption of a symmetric or asymmetric conformation of MalK2, and 3) the degree of contact at the level of the nucleotide binding sites. These are persistently visible at
different contour levels. The differences observed between the EM maps are consistent with the differences between simulated maps of MalK₂ in the open, closed, and semi-closed conformations (Fig. S6).

Together, our results combined with those in the literature have allowed us to refine the model of transport (Fig. 6). In the resting apo-state, the MalK₂ dimer exists in an open conformation. The binding of ATP drives the closure of MalK₂; however, this conformation is unstable, and MalK₂ reverts to the asymmetrical state (Fig. 6A). The presence of MalE stabilizes the closed conformation of MalK₂ induced by ATP (Fig. 6B), which is in accordance with our previous report that ATP cleavage is stimulated by MalE (5, 23). MalE further increases the rate of ATP hydrolysis by increasing the rate of ADP and Pi release from the nucleotide binding pockets (23) (Fig. 6C). This latter point is supported by our results, which show that MalK₂ is primarily in a semi-closed conformation when the transporter is incubated with maltose (Fig. 5, B and E), thus indicating that dissociation of the sugar contributes to the return to the resting state. Additionally, we find that maltose triggers the dissociation of MalE from the transporter, as expected because closed liganded MalE has weak affinity for MalF and MalG (16). Finally, in the absence of nucleotide and maltose, MalK₂ is exclusively in the open conformation, whereas in the presence of either of these factors, it adopts a range of conformations, suggesting that the release of ADP and maltose enhances the return of the transporter to the open resting state.

In the absence of MalE, full closure of MalK₂ is obtained with ATP only when a cross-linking reagent designed to stabilize this conformation is also included during incubation (17). Interestingly, with ATPyS or the ADP-P, mimics ADP-VO₄ and ADP-AlF₄, the majority of the particles remain in the asymmetric conformation in which only the MalF₉ junction has moved toward the center of the complex. This asymmetry indicates that neither ATPyS nor the ADP-P, mimics have the ability to stabilize MalK₂ in the closed state. In the case of ADP-AlF₄, ~30% of the complexes adopt a semi-closed symmetric conformation in which both MalK₉ and MalK₈ junctions have moved toward the center of the complex. This later conformation may be due to the specific or higher number of liaisons that ADP-
AlF₄ establishes at the nucleotide-binding pockets (8). It could also be because the ADP-AlF₄ state is reached by a backward reaction upon addition of ADP and Al³⁺/H⁺ ions, whereas the ADP-VO₄ is reached by a forward reaction that requires ATP binding and hydrolysis.

Importantly, the semi-closed asymmetric conformation of MalK₂ has never been reported by X-ray crystallography. All crystal structures of MalFGK₂ and MalE-MalFGK₂ show nearly identical positioning of the MalKF and MalKG junctions, which has led to the proposal that MalK₂ adopts a symmetrical “tweezers-like” motion during the transport cycle (6–11). Our results now reveal that binding of the nucleotide results solely in the stabilization of the movement of the MalKG junction but not that of the MalKF junction. We therefore propose that closure of MalK₂ occurs in a sequential and asymmetric manner, in agreement with recent molecular dynamics simulations (24) and earlier cross-linking experiments (14, 15). Because only the MalK₉₇ junction moves toward the center of the complex during asymmetrical closure, this motion may also drive the asymmetric movement of MalG and MalF during transport.

In the presence MalE and ADP-VO₄, we find that MalK₂ is converted to the closed conformation. This dependence on MalE for the closure of MalK₂ explains why the cleavage of ATP relies on MalE, resulting in ~4-fold increase in the overall ATP turnover rate (5, 12). It also explains why binding of vanadate becomes irreversible upon addition of MalE (25) and why EPR studies have reported that ATP alone is insufficient to support the full closure of MalK₂ (20, 26). We find that in the presence MalE and ADP, there was a reduction in the number of particles with MalK₂ in the open conformation, as well as a conversion of the semi-closed conformation of MalK₂ from asymmetric to symmetric. In the absence of nucleotide, MalK₂ is in the open conformation independently of the presence of MalE. To-

FIGURE 6. Model of the maltose transport cycle. A, ATP induces closure of MalK₂ (EM map MalFGK₂-ATP+BMOE), but this conformation is unstable in the absence of MalE (EM maps MalFGK₂-non-hydrolyzable ATP and ADP-Pₗ analogs) and reverts to a semi-closed symmetric conformation. B, MalE stabilizes the ATP-induced closed conformation (EM map MalE-MalFGK₂-ADP-VO₄), and therefore ATP cleavage occurs. C, maltose enhances the return of the transporter to inward facing conformation, thereby increasing the rate of ADP and Pₗ release and consequently increasing the ATP hydrolytic cycle. MalE alone does not modify the open conformation of MalK₂. The ATPase activity indicated is that previously reported (5, 28).
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gether, these results imply that MalE allosteric regulation is nucleotide-dependent.

Finally, in the presence of maltose the majority of the particles MalE-x-MalFGK₂ (~79%) switch to the semi-closed symmetric conformation in lieu of remaining in the open state. This semi-closed symmetric conformation is very similar to the conformation of MalE-MalFGK₉-maltose reported by X-ray crystallography (PDB code 3PV0) (9). The X-ray structure shows that one molecule of maltose is trapped in the MalF-MalG transport cavity (9). It is therefore possible that the semi-closed symmetric conformation of MalE-x-MalFGK₂ is caused by the presence of this maltose molecule in the TMDs. This conformation may also be caused by the closure of MalE upon maltose binding. The current resolution of our maps does not allow us to differentiate between these two possibilities. Regardless, the fact that 100% of the particles MalE-x-MalFGK₂ exist in the open conformation without maltose versus 21% with maltose strongly suggests that release of the sugar precedes the return of the transporter to the initial inward facing state.

In conclusion, negative stain single-particle EM combined with 3D classification negative stain single-particle EM allows to assess protein conformations and their equilibrium in many different biochemical conditions. Here, the EM study of MalFGK₂ in nanodiscs, by assessing how three factors modified the conformations of MalK₂, has enabled us to refine the model of maltose transport.

Experimental Procedures

Proteins Preparation—MalFGK₂ and MalE proteins were overexpressed and purified as before (18, 27). Nanodiscs were prepared at a molar ratio MalFGK₂:MSP:lipids of 1:3:60 as described previously (12, 28). The membrane scaffold MSP1D1 was obtained from the Sligar laboratory (29). Phospholipids (lipid 1, 2-dioleoyl-sn-glycero-3-phosphocholine; Avanti Polar lipids) were dissolved in chloroform and dried under a steam of nitrogen. The lipids were resuspended in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂ at room temperature for 20 min, time enough for trapping to occur (supplemental Fig. S2). The complexes were then purified by size exclusion chromatography in reaction buffer plus 1 mM ATP and 10 μM vanadate.

For the ADP-AlFx state, the protein in the ADP buffer was incubated for ~4 h at 4 °C with 2 mM ADP, 8 mM NaF, and 2 mM AlCl₃ in TEM buffer.

EM Sample Preparation and Image Acquisition—MalFGK₂ and derivates samples (each 5 μl diluted to 50 μg/ml) were applied onto negatively glow-discharged carbon-coated grids (400 mesh, copper grid) for 1 min, and excess liquid was removed by blotting with filter paper. Freshly prepared 1.5% uranyl formate (pH 5) was added (5 μl) for 1 min and then blotted. Digital micrographs were collected using a FEI Tecnai G2 F20 microscope operated at 200 kV and equipped with a Gatan Ultrascan 4k × 4k Digital CCD Camera. The images were recorded at defocus between 0.7 and 1.4 μm at a magnification of 134,010× at the camera and a pixel size of 1.12 Å.

EM Data Processing and Image Analysis—Contrast transfer function parameters were determined using CTFFIND3 (30), and micrographs were phase flipped using XMIPP 2.3 software (31). Protein particles were boxed using e2boxer from the EMAN2 software suite (32). After extraction, images of the particles were binned twice for a final box size of 128 × 128 pixels and a resulting pixel size of 2.24 Å at specimen level. False positives (images that do not contain particles), images of empty nanodiscs, and images that show particles too closed to one another, were eliminated after 2D classification using the K-means ascendant classification (with 2, 4, 8, 16, 32, and 64 classes successively) of the SPARX software suite (33). The reference volume was obtained by EMAN2 common line algorithm using the resting state particles. This same reference volume was used for 3D reconstructions of all the data sets 3D analysis and was low pass filtered to 60 Å at the start of the analysis procedure of each data set. The 3D analysis was conducted using the RELION-1.2 software suite (34) was used for analyzing each data set. The first step consisted of a maximum likelihood 3D classification with 4–7 seeds. This classification allowed for the identification of stable dominant conformations and the elimination of poorly defined particles. The second step was a refinement of each stable conformation. When similar classes were obtained as for the MalFGK₂-only particles (supplemental Fig. S1) belonging to the best defined class were further analyzed in a refinement conducted using Relion (34). When different classes were obtained, all were refined separately. For the MalFGK₂-ATP+BMOE and MalE-MalFGK₂-ADP-VO₄ data sets, an additional refinement step using SPARX was performed to further improve the maps. For the refinement, the initial volume was low pass filtered to 50 Å, and refinement was performed for 10 iterations with an angular sampling of 10 degrees, followed by 10 iterations with an angular sampling of 5° degrees. Resolution was estimated using forward scatter and gold standard forward scatter with the criteria of 0.5 and 0.143 (supplemental Table S1 and Fig. S5). The 3D-EM density maps of the complex without MalE were visualized using Chimera (35). All docking shown in this study was performed using Chimera rigid body docking tools.
Accession Codes—The negative EM maps have been deposited in the Electron Microscopy Data Bank under access codes EMD-8524 (MalFGK2, Apo), EMD-8525 (MalFGK2, ATP-BMOE), EMD-8526 (MalFGK2,ADP-VO4), EMD-8527 (MalFGK2,ADP-AIF4), EMD-8529 (MalFGK2,ADP), EMD-8530 (MalFGK2,ADP-VO4), EMD-8531 (MalFGK2,ADP), EMD-8533 (MalFGK2, Apo), EMD-8534 (MalE-MalFGK2 maltose, open conformation), EMD-8535 (MalE-MalFGK2 maltose, semi-closed symmetric conformation), and EMD-8536 (MalE-MalFGK2 maltose, MalE detached).

Author Contributions—L. F. conducted most of the EM experiments and image analysis. H. B. purified and reconstituted MalE and MalFGK2 in nanodiscs. I. J. conducted the EM studies. L. F., H. B., I. R., and F. D. conceived the idea for the project, analyzed and interpreted the results, and wrote the paper.

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