Long-range dynamic correlations regulate the catalytic activity of the bacterial tyrosine kinase Wzc

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BY-kinases represent a highly conserved family of protein tyrosine kinases unique to bacteria without eukaryotic orthologs. BY-kinases are regulated by oligomerization-enabled transphosphorylation on a C-terminal tyrosine cluster through a process with sparse mechanistic detail. Using the catalytic domain (CD) of the archetypal BY-kinase, Escherichia coli Wzc, and enhanced-sampling molecular dynamics simulations, isothermal titration calorimetry and nuclear magnetic resonance measurements, we propose a mechanism for its activation and nucleotide exchange. We find that the monomeric Wzc CD preferentially populates states characterized by distortions at its oligomerization interfaces and by catalytic element conformations that allow high-affinity interactions with ADP but not with ATP-Mg2+. We propose that oligomer formation stabilizes the intermonomer interfaces and results in catalytic element conformations suitable for optimally engaging ATP-Mg2+, facilitating exchange with bound ADP. This sequence of events, oligomerization, i.e., substrate binding, before engaging ATP-Mg2+, facilitates optimal autophosphorylation by preventing a futile cycle of ATP hydrolysis.

INTRODUCTION

BY-kinases (1, 2) comprise a unique family of protein tyrosine kinases (PTKs) that are highly conserved in both Gram-negative and Gram-positive bacteria (3). BY-kinase activity has been shown to be critical in many physiological processes, most notably in the synthesis and export of polysaccharides (fig. S1A). BY-kinases function as part of a multiprotein complex that spans both the inner and outer membranes (TM1 and TM2), and a cytoplasmic catalytic domain (CD), all encoded on a single polypeptide chain (fig. S1A). BY-kinases function as part of a multiprotein complex that spans both the inner and outer membranes (5). BY-kinases have no known eukaryotic orthologs, and the structures of the CDs of the Escherichia coli BY-kinases Etk (EtkCD) (6) and Wzc (WzcCD) (7), together with that of Staphylococcus aureus CapB (CapB) (8), illustrate their notable differences with eukaryotic PTKs (ePTKs) marked by the absence of conserved sequence motifs (9) and the classic two-lobed fold that are the signatures of the latter. BY-kinase CDs closely resemble P-loop adenosine triphosphatases (ATPases), notably those of the MinD family (10), suggesting evolution from a common ancestor (J) in orthogonal fashion to ePTKs (11). Similar to the P-loop ATPases, the BY-kinase CDs contain nucleotide triphosphate–binding Walker-A (A/GX5GK[S/T], X is any residue) and Walker-B (ϕDX2P, ϕ is a hydrophobic residue) motifs (fig. S1B), together with an additional Walker-B–like motif, called Walker-A′ (ϕDXDXR) (3). In addition, BY-kinases contain a loop that is rich in arginine and lysine residues (RK-cluster) that has been shown to be important for autophosphorylation (7). A defining feature of BY-kinases is a C-terminal cluster of five to seven tyrosine residues (Y-cluster) whose intermolecular autophosphorylation (transphosphorylation) drives function (12). The number and precise location of individual tyrosine residues in the Y-cluster are poorly conserved, and the removal of any individual tyrosine has no substantial effect on function. However, replacement of multiple tyrosines from the Y-cluster has deleterious functional consequences (4, 13), suggesting that the overall level of Y-cluster phosphorylation rather than the phosphorylation state of any particular tyrosine residue/s therein relates to function contrasting many ePTKs, e.g., members of the Src family (14).

As in the case of ePTKs, a necessary condition for intermolecular autophosphorylation in BY-kinases is the formation of CD oligomers (fig. S1C). Transphosphorylation between subunits is then achieved by the insertion of a Y-cluster tyrosine of the substrate-acting (S-acting) CD into the catalytic site of an adjoining enzyme-acting (E-acting) CD (fig. S1, B and C). CapBCD (8) and WzcCD (7) both crystallize as front-to-back octamers (fig. S1B). Biochemical studies suggest that two specific features are required for oligomer formation by the isolated BY-kinase CD: (i) a dephosphorylated Y-cluster (or that containing very low levels of phosphorylation) and (ii) a conserved EX2RX2R oligomerization motif (fig. S1B) motif housed on a helical segment (α2) seen in the structures of the CapBCD (8) and WzcCD (7). While the high/low phosphorylation levels of the Y-cluster represent the primary drivers of the oligomerization state of the CD in solution, and likely in the membrane-bound full-length enzyme (fig. S2), the importance of the oligomerization motif is underscored by the fact that the replacement of the conserved residues of this motif by alanine (E508A/R511A/R514A) in WzcCD prevents oligomer formation and leads to diminished levels of Y-cluster phosphorylation in the isolated CD while almost completely abolishing it in the context of the full-length enzyme. E. coli K12 cells expressing the mutant protein are functionally compromised and produce altered levels of the exopolysaccharide colanic acid (7).

While crystallographic snapshots of BY-kinase CDs and supporting biochemical studies have provided major insight into the properties of these unique enzymes (1, 8), the precise mechanism/s of activation and regulation of BY-kinases are still poorly understood. A key open question is about the nature of the coupling between oligomerization and nucleotide exchange: the former, to bring the S-acting and E-acting CDs in close proximity to enable transphosphorylation (fig. S1C), and the latter, to engage adenosine triphosphate (ATP) before chemistry and to release the adenosine diphosphate (ADP) product after the chemical step to prime the catalytic
site for the next phosphorylation event. Given their evolutionary origin, BY-kinases are, not unexpectedly, highly efficient ATPases (15). Thus, the binding of ATP to a monomeric CD before oligomerization, i.e., substrate binding, is likely to result in a futile cycle of hydrolysis. Oligomerization, i.e., formation of the complex between the E-acting and S-acting CDs, would have to precede ATP binding to enable efficient autophosphorylation. One can then reasonably expect that this process is mediated by an allosteric pathway within the kinase core linking the oligomerization interfaces (involving the α2 helix and the α7/α9 helices on the complimentary surface; fig. S1B) to the catalytic site. To test this hypothesis, we used replica exchange molecular dynamics validated by isothermal titration calorimetry (ITC) and solution nuclear magnetic resonance (NMR) measurements using a construct (WzcCDAC) (16) of E. coli (K12) WzcCD that lacks its highly dynamic C-terminal tail and represents its structural core. Our results illustrate the coupling between the catalytic elements and the oligomerization interfaces and help define a mechanism for oligomerization and ATP binding to enable efficient autophosphorylation in BY-kinases.

RESULTS
Monomeric apo-WzcCDAC assumes an open state with key catalytic elements in inactive conformations
We performed replica exchange with solute tempering (REST2) (17) simulations on the unliganded state of WzcCDAC (see Materials and Methods for details). An initial visual inspection of the structures in the REST2-generated ensemble suggested the presence of two dominant global fluctuation modes. These include the following: (i) a downward displacement of α4, loop Lα5 together with additional elements connecting it to α4, with respect to α3, and (ii) coupled outward rotations of α2 and α3 with respect to the protein core (indicated by the red arrows on the left panel of Fig. 1A). These global modes can be efficiently analyzed in a cylindrical coordinate system defined by a rise (|h|) and a polar angle (θ) (Fig. 1A, right; also see the corresponding section in Materials and Methods for additional details). |h| defines the extent of the downward displacement of the center of mass (COM) of α4. The polar angle θ measures the degree of outward rotation of α2 and α3 with respect to the protein core. The probability density function (PDF) of the sampled conformations projected onto this coordinate system reveals a single dominant state with average |h| and θ values of ~4.5 Å and ~145°, respectively (Fig. 1B). This state is significantly more open [referred to as an open state (OS) from here on forward] compared to that represented by the crystal structure for which |h| and θ take values of 3 Å and 131° (indicated by the green dot in Fig. 1B), respectively.

Analyses of representative structures drawn from the OS suggests significant evolution of the conformations of key catalytic elements of WzcCDAC with respect to the crystal structure (Fig. 1C), in addition to the differences in the global conformation mentioned earlier. In the crystal structure of WzcCD (7), the side chains of T541 (Walker-A) and D642 (Walker-B) form a hydrogen bond (Fig. 1D) generating an orientation that is critical for the proper coordination of Mg2+. Further, the catalytic lysine on Walker-A (K540, a methionine in the crystal structure) adopts an orientation that allows it to contact the β- and γ-phosphates of ATP in a fashion similar to that seen in the structure of the homologous P-loop ATPase, MinD (Fig. 1D) (10). This conformation of the catalytic lysine represents a key structural feature in facilitating the bond breaking step during ATP hydrolysis in typical ATPases (18–21). However, for the OS seen in our simulations, an outward rotation of α3 alters these sets of interactions (Fig. 1C) in a manner reminiscent of the structure of an inactive mutant (K15M) of shikimate kinase (22), a member of the P-loop kinase family (Fig. 1E) (23). On the basis of these observations, we suspect that the OS seen in our simulations represents an inactive state of WzcCDAC that is incompatible with Mg2+ and, consequently, with ATP coordination. Further, the rotation of α2 (which houses the conserved EX2RXR motif) alters the orientation of the first oligomerization interface (I1) resulting in a more extended geometry in the OS relative to that in the crystal structure, making it incapable of forming the closed octameric ring seen in crystallo (Fig. S3) (7).

The complex of monomeric WzcCDAC with ATP-Mg2+ is incompatible with progress toward efficient transphosphorylation
Next, we performed REST2 simulations on the WzcCDAC-ATP-Mg2+ complex (see Materials and Methods for details). The PDF of the resulting conformations projected onto the cylindrical coordinate frame described above produces three distinct elliptoidal clusters that lie along the diagonal of the coordinate system (Fig. 2A) reflecting the tendency of the system to fluctuate (indicated by the magenta arrows in Fig. 2A) between an OS (~28%; similar to the one described above), a closed state (CS; ~45%; with similarities to the crystal
structure), and a state that is significantly more closed compared to the crystal structure ([|h| and θ values of ~1.9 Å, ~128°, respectively) that we term a hypercompact state (HS; ~11%; Fig. 2A). Inspection of representative structures reveals that, as in our apo simulations, the OS displays the proximity between the side chains K540 and D642 and key ATP-contacting residues (D480 and Y569) have moved away from ATP (green stick representation). In addition, the adenosine moiety of ATP is displaced from its binding pocket relative to that in the HS (tan). (C) The HS (left) shows an ordered RK-cluster; however, helices α7 and α9 of the second interaction interface (I2) are partially unfolded (green ellipses). In contrast, the OS (right) is characterized by an intact I2 but a disordered RK-cluster. (D) The two largest clusters (CS2 and CS5) obtained through an EVA-MS decomposition of the CS (also see fig. S5) are characterized by mutually exclusive distortions at the oligomerization interface: bending of α2 in CS2 (I1, red arrows) or partial unfolding of helices α7 and α9 in CS5 (I2, green ellipses). The EVA-MS approach identifies structural evolution in different parts of the protein and their correlations, if any. Briefly, the method determines the variation in the local environment of each residue (as defined by its local structural contacts) by defining a measure of its specific normalized measure of variability across the ensemble, \( V_i(t) \). A multidimensional PDF can be defined as a function of the \( V_i(t) \) values of the most highly varying residues. Specific conformational modes within this PDF are then identified using the unsupervised mean shift algorithm (see fig. S4 for a simple illustration of the approach). For the CS, the most varying residues were found to be D480 (αB of the RK-cluster), S512 (α2), F519 (α2), S617 (α7), and G656 (α9; fig. S5A). Application of EVA-MS to the five-dimensional PDF comprising the \( V_i(t) \) values for these residues yields several clusters of which four (CS1, CS2, CS4, and CS5) account for ~94% of the structures within the CS (see fig. S5B). Of these, CS2, which accounts for two-thirds of the CS, is characterized by the formation of a bend at the center of α2 (I1; Fig. 2D and fig. S5E). In the second most populated cluster (CS5), while α2 is largely intact, α7 and α9, which comprise the second oligomerization interface (I2), are partially unfolded (Fig. 2D and Fig. S5E). Together, these results suggest that the WzcCDAC-ATP-Mg\(^{2+}\) ensemble consists of structures that display features that are not compatible with efficient
transphosphorylation (we refer to these as “distortions” for simplicity). The features include the following: (i) a significant bend in $\alpha_2$ thereby destabilizing I$_1$ (in CS2), (ii) partial unfolding of $\alpha_7$ and $\alpha_9$ thereby destabilizing I$_2$ (in CS5), or (iii) the displacement of ATP from its binding pocket (in the OS). These results suggest that in the monomeric state, Wzc CD$_{\Delta C}$ cannot simultaneously engage ATP·Mg$^{2+}$ and present a stable oligomerization interface. The destabilization of I$_1$ and I$_2$ comes with the loss of several hydrogen bonds with a substantial associated energetic cost. It is thus reasonable to expect that oligomerization, which provides a means to stabilize the I$_1$/I$_2$ interface, may be necessary for stable ATP·Mg$^{2+}$ binding.

**Characteristics of the HS of the Wzc CD$_{\Delta C}$·ATP·Mg$^{2+}$ complex suggests a catalytic role for the RK-cluster**

The HS (Fig. 2A), which is sampled the least of the three major states in the Wzc CD$_{\Delta C}$·ATP·Mg$^{2+}$ simulations, shows some remarkable structural features. Most notably, the RK-cluster, which is disordered in the crystal structure (7) with the exception of a small helical region ($\alpha B$), shows a high degree of order and makes extensive contacts with ATP (Fig. 3A). The aliphatic segment of the K492 side chain stacks against Y569 (that contacts the $\alpha$-phosphate of ATP) and makes a salt bridge with E572 on $\alpha_4$ (Fig. 3A). The Walker-A K540 and the Walker-B D642 are in their appropriate orientations for catalysis. In addition, R490 is inserted into the active site where it contacts the $\gamma$-phosphate of ATP. The coordination of ATP is very similar to the active, i.e., ATP hydrolysis capable states of MinD (Fig. 3B) (10) and F$_1$-ATPase (Fig. 3C) (24). This observation appears to suggest a critical role for the RK-cluster residue R490. MinD has been shown to dimerize along the active site where its so-called deviant Walker-A lysine inserts into the active site of the neighboring protomer and contacts the $\gamma$-phosphate of ATP, thus activating hydrolysis (Fig. 3B) (10). The arginine finger (Fig. 3C) plays a similar role in the context of the F$_1$-ATPase oligomer. It has now been established that the presence of a positively charged residue proximal to the $\gamma$-phosphate of ATP that serves to stabilize the negative charge buildup during nucleophilic attack by water (18) is a key initiator of hydrolysis in ATPases (20, 21). In the HS of Wzc CD$_{\Delta C}$, R490 appears to adopt a conformation that is similar to the arginine finger in many guanosine triphosphatases and in AAA$^+$ family of ATPases (25) and the signature lysine of the deviant Walker-A in MinD (26). This suggests that R490 likely plays a similar role in activating ATP hydrolysis in Wzc.
An R490A variant of WzcCD shows drastically reduced levels of auto-phosphorylation without significant reduction in nucleotide affinity (7).

**Monomeric WzcCD is hindered in its ability to efficiently coordinate Mg^{2+}**

In the apo simulations discussed above, we noted that the coupled outward rotation of α2 and α3 to form the OS leads to a disengagement of T541 and D642, thereby disrupting the ability to coordinate Mg^{2+}. The displacement in α3 appears to represent a response to a dynamic coupling across the oligomerization interface involving α2 (I1) and α7/α9 (I2). The presence of Mg^{2+} in the WzcCD-ATP-Mg^{2+} simulations partially counteracts this effect by reinforcing the T541-D642 interaction, populating the CS that has alternative modes of destabilization, e.g., through distortions in I1 or I2 (as in CS2 or CS5 illustrated in Fig. 2D). It is therefore reasonable to expect that this effect would be modulated by the presence or absence of Mg^{2+}. To test this hypothesis, we performed two additional sets of REST2 simulations on the WzcCD-ADP and WzcCD-ADP-Mg^{2+} complexes using similar protocols as before (see Materials and Methods for details).

For the WzcCD-ADP-Mg^{2+} simulations, the system samples both the CS and OS with frequencies of ~60 and ~34%, respectively (Fig. 4A, left). Application of the EVA-MS procedure on the CS identified the following residues with the highest degree of variation: Q475 and D480 (RK-cluster), S512 (α2), K567 (LαL), and S617 (α7; fig. S6A). Most of the resulting structures can be accounted for by six clusters (fig. S6B). Similar to the WzcCD-ATP-Mg^{2+} simulations, the most populated clusters within the CS were found to be characterized by a bending of α2 (I1; CS1 in fig. S6, D and E) or an unfolding of α7 (I2; CS2 in fig. S6, D and E). In addition, the CS also samples structures where α2 is bent, α7 is unfolded, the RK-cluster is disordered, and the adenosine ring of ADP is displaced from its binding pocket CS3 in fig. S6, D and E). These results suggest that the presence of Mg^{2+} leads to a CS in which the contact between Y569 of α4 and the α-phosphate of ADP persists (fig. 4A, middle). However, there are significant distortions in I1 and I2 together with enhanced disorder within the RK-cluster and, in some cases, breakage of key contacts between D480 and the ribose O3′ and O2′, resulting in the displacement of ADP from its binding pocket. Alternatively, both Y569 and D480 are displaced from ADP, and the system assumes the OS (fig. S6E and the right panel in Fig. 4A). Together, the system evolves either by distortion of the interaction interfaces or by breaking key contacts with the nucleotide (ADP in this case) in a similar fashion to the ATP-Mg^{2+} simulations described above.

In contrast to the WzcCD-ADP-Mg^{2+} simulations, where discrete, well-defined states are seen, projection of the WzcCD-ADP ensemble onto the cylindrical coordinate frame suggests the sampling of a relatively diffuse range of states (Fig. 4B, left). In the absence of well-defined

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Fig. 4. REST2 simulations of WzcCD-ADP-Mg^{2+} and WzcCD-ADP complexes. (A) θ|h| space projection of the WzcCD-ADP-Mg^{2+} simulations. The CS and OS are highlighted by green ellipses (left); the red dot indicates crystal structure values. Close-up (middle) of the active sites of major EVA-MS decomposed CS clusters (CS1, CS2, and CS3; also see fig. S6). ADP (cyan ellipse) and key nucleotide-coordinating residues, D480 and Y569 (green ellipse), are colored according to their constituent clusters. The right panel illustrates a representative OS structure. (B) States sampled in the WzcCD-ADP simulations; the tricolor arrow indicates regions in θ|h| space. Conformations of α2 (bending indicated by red arrows), α3, and α8 of the major EVA-MS-decomposed clusters (C1, C2, and C4; also see fig. S7) are shown in the middle panel. Y569, whose position varies between the three clusters, is also indicated. Collective displacements of secondary structural elements are indicated by tricolor arrows representing specific regions in θ|h| space. Active site configurations for C1 of WzcCD-ADP (left) and CS1 of WzcCD-ADP-Mg^{2+} (right) are compared. (D) Normalized distribution of D480, Cγ-ADP, and O3′ distances (3.6 ± 0.3 Å over 16 chains in crystallo) in the WzcCD-ADP-Mg^{2+} (blue) and WzcCD-ADP (red) simulations.
boundaries between states, we performed EVA-MS analysis on the entire ensemble to define local features in the constituent structures (fig. S7A). The most highly varying residues in this simulation were found to be D480 (αB of the RK-cluster), S512 (α2), Y569 (α4), I592 (I45), and E618 (α7), resulting in six major clusters within the ensemble (fig. S7B). The three most populated clusters, C1, C2, and C4, are all characterized by the evolution on S512 (fig. S7D) in the form of bending of α2 (fig. S7E and the middle panel of Fig. 4B) and differ slightly in the evolution of D480 (RK-cluster), Y569 (α4), and I592 (I45) for each specific case. Given the very small differences observed in the projection of these clusters onto their constituent residues (fig. S7D), we wondered whether each cluster could represent different conformations of αB (RK-cluster), α4, and I45, given that the latter two elements adopt distinct configurations in the CS and the OS described above. A total of 500 structures were randomly selected from each of the C1, C2, and C4 clusters and projected onto the cylindrical coordinate system. These clusters sorted into distinct regions, populating states that, on average, varied from being somewhat closed (in black in fig. S7E; black part of the arrow in Fig. 4B), with occurrence of ~21%, to being more open (blue in fig. S7E; blue part of the arrow in Fig. 4B), with an occurrence of ~23%, and to a state that resembles the OS (red in fig. S7E; red part of the arrow in Fig. 4B), with an occurrence of ~18%. A comparison of representative structures from each cluster aligned on α3 shows an almost perfect alignment on α2 and α3 (Fig. 4B, middle), differing only in the degree of displacement of Y569 relative to ADP, with the difference being a little more pronounced between C1/C4 (black/blue) and C2 (red). In addition, α4 and I45, show different degrees of displacement with respect to α3 illustrated in the right panel of Fig. 4B). This displacement appears to be coupled to larger, more global displacements across α7, α8, and α9 (Fig. 4B, right). Note that despite the relatively small variations in $V(t)$ values across the clusters (fig. S7D), the MS procedure is able to distinguish these clusters from each other. These clusters are characterized by multiple small local variations together with large global rearrangements that are reflected in the $|\Delta \theta|$ space. This shows that a subset of residues that show small variations in their local environment but together give rise to larger global rearrangements can be parsed using the EVA-MS approach. A close-up view of the active site in the partially closed configuration, C1, shows that, despite a higher degree of closure, the active site conformation resembles that in the OS (defined by the presence of a D642-K540 contact; Fig. 4C, left). This contrasts the CS seen for WzCDAC-ADP-Mg$^{2+}$ in which T541 is proximal to D642 (Fig. 4C, right). Together, these data suggest that in the absence of Mg$^{2+}$, a stable interaction with ADP can occur despite the fact that the T541-D642 interaction is broken. This stable engagement of ADP is enabled by the presence of α4 and I45 elements to freely sample the open and partially CSs with similar probability without perturbing key ADP-coordinating residues. On the other hand, the presence of Mg$^{2+}$ appears to force the formation of the CS, thereby disturbing the orientation of the ADP-coordinating residues (notably D480; also see CS3 in fig. S6, D and E) and occasionally displacing the nucleotide from its binding pocket (Fig. 4D). Overall, these findings support our model where formation of OS lies in direct opposition to Mg$^{2+}$ binding, and presence of the divalent cation disrupts nucleotide coordination and destabilizes the oligomerization interface.

**Experimental measurements validate in silico predictions**

The simulations discussed above suggest that the complex of monomeric WzCDAC with ADP is stable in the absence of Mg$^{2+}$ and that the divalent cation appears to destabilize it. In addition, the ATP-Mg$^{2+}$ complex of monomeric WzCDAC appears to display various distortions, suggesting that binding of ATP-Mg$^{2+}$ in the monomeric state carries with it an energetic cost. Given these observations and the fact that WzCDAC is monomeric in solution (16), we would expect that its affinity for ATP-Mg$^{2+}$ would be significantly lower than that for ADP, with the affinity of the latter also being reduced in the presence of Mg$^{2+}$. The binding affinities measured using ITC indicate the following dissociation constant ($K_d$) values (averaged over three independent measurements): AMPPCP-Mg$^{2+}$, 163 ± 23 μM; ADP-Mg$^{2+}$, 2.56 ± 1.08 μM; and ADP in the absence of Mg$^{2+}$, 0.70 ± 0.38 μM (see Fig. 5A) in line with our predicted trends. This observation is relevant in the cellular context, since ATP is necessarily bound to Mg$^{2+}$ while ADP may not be, given their relative affinities for Mg$^{2+}$ (27).

As shown above, our simulations suggest that apo-WzCDAC exists in the OS (Fig. 1A) and that ADP-bound WzCDAC is capable of populating the OS where ADP is properly coordinated (Fig. 4B). However, in these cases, α2 (I1) is bent, and the second interaction interface (I2) together with α4 and I45 are free to undergo rigid-body oscillations occasionally disrupting the contact between Y569 and the α-phosphate of ADP. Given that ~80% of NMR assignments are available for the amide $^{15}$N, $^1$H resonances of WzCDAC (16), we felt that these resonances were likely to sense the predicted ADP-induced changes and serve to validate the computational results. $^{15}$N, $^1$H transverse relaxation optimized spectroscopy (TROSY) spectra of $^{15}$N, $^1$H-labeled WzCDAC in the presence of substoichiometric amounts of ADP show that the resonances of WzCDAC in the ADP-bound and nucleotide-free states are in the slow-exchange regime, a result that is not unexpected given the submicromolar affinity. Significant chemical shift differences between resonances corresponding to free and ADP-bound states are seen across the two interaction interfaces (I1 and I2; top panels of Fig. 5, B and C). SS12, which forms the hinge in the bending of α2, shows a difference of 0.06 parts per million (ppm) between the free and ADP-bound states. Strong perturbations are also seen on $\alpha7$ (E618, 0.07 ppm; L619, 0.05 ppm; L620, 0.07 ppm) and $\alpha9$ (A652, 0.12 ppm). Additional perturbations are also seen on α4, I45, and on α8 that contacts I45. This analysis suggests the presence of a long-range network, predicted in our simulations, that couples the catalytic site to remote regions, most notably the oligomerization interfaces I1 and I2.

As noted earlier, our simulations suggest that Mg$^{2+}$ destabilizes the ensemble by enforcing the T541-D642 interaction to enable its coordination; the CS is then characterized by a bending (CS1) or distortion of the C terminus of α2 (OS), the destabilization of I2 (CS2) coupled with increased disorder in the RK-cluster leading to the ultimate displacement of ADP (CS3, fig. S6E). Most of these predicted effects are consistent with the perturbations seen by the addition of an excess of Mg$^{2+}$ to ADP-saturated WzCDAC (bottom panels in Fig. 5, B and C). While amide resonance assignments for T541 and D642 are not available, the state of this interaction is reported by C544 that is in spatial proximity to these residues and is therefore an excellent probe for their relative orientation (Fig. 4C). The perturbation seen for C544 (0.03 ppm) is among the largest spectral changes seen in the presence of Mg$^{2+}$ (bottom panels in Fig. 5, B and C). In addition, perturbations exceeding 3 SDs above the mean are also seen on the RK-cluster and on $\alpha2$ (I1) and $\alpha7$/$\alpha9$ (I2), in line with the in silico predictions (bottom panels in Fig. 5, B and C).
Fig. 5. Experimental verification of in silico predictions. (A) Representative ITC thermograms of the interaction of WzcCD with ADP (left), ADP·Mg\(^{2+}\) (middle), and AMPPCP·Mg\(^{2+}\) (right). (B) Top: Amide chemical shift perturbations (\(\Delta \delta\)) induced by ADP on WzcCD in the absence of Mg\(^{2+}\). \(\Delta \delta\) values are shown only for well-resolved resonances corresponding to the apo and ADP-bound species under partial ADP saturation for the spectra that are in slow exchange. Bottom: Amide chemical shift perturbations induced by 200 molar equivalents of Mg\(^{2+}\) on the ADP-saturated spectrum for the spectra that are in slow exchange. Bottom: Amide chemical shift perturbations induced by 200 molar equivalents of Mg\(^{2+}\) on the ADP-saturated spectra of WzcCD·ADP·Mg\(^{2+}\). Red and cyan dashed lines indicate 2- and 3-SD (\(\sigma\)) threshold beyond the mean (\(\langle \Delta \delta \rangle\)), respectively; green bars indicate exchange-broadened resonances. (C) Top: ADP-induced perturbations from the top panel of (B) depicted as red spheres on the structure of WzcCD·ADP·Mg\(^{2+}\) (Fig. 6B). S512, a key probe of the active site conformation of Wzc CD reveals a high-degree disorder at the C-terminal end of \(\alpha_2\) (dotted circle). (C) Ten randomly selected structures superimposed on C\(_\alpha\) atoms are shown. The \(\alpha_2\) configuration resembles that seen in the crystal structure (i.e., no bending is observed) and the introduction of the (G)\(_4\) in the CS is dynamic. However, no other structural evolution (e.g., the unfolding of the \(\alpha_7\) or \(\alpha_9\) helices; i.e., disruption of I\(_2\)) is seen. (D) The key ATP-coordinating elements at the catalytic site are ordered, and ATP remains stably bound.

Stabilizing the CS enables the appropriate coordination of ATP-Mg\(^{2+}\) by monomeric WzcCD\(_{ADP}\)

As noted above, in the REST2 simulations on the WzcCD-ATP-Mg\(^{2+}\) complex, the dominant clusters of the CS composed of structures with distorted oligomerization interfaces (I\(_1\) or I\(_2\); Fig. 2D). On the other hand, the OS, while displaying intact, i.e., unbroken, I\(_1\) and I\(_2\) (Fig. 2C), is unable to coordinate ATP-Mg\(^{2+}\) appropriately (Fig. 2B). We asked the question of whether it is possible to rationally engineer a mutation (or a set of mutations) that would stabilize the CS and prevent the displacement of ATP from its binding site. To design these mutations in silico, we focused on the \(\alpha_2\) helix within I\(_1\), since it carries the conserved EX\(_R\)RX\(_R\)R motif that has been confirmed to play a critical role in function (7). Further, while most of the \(\alpha_2\) helix is largely ordered in the crystal structure (for which all the constituent monomers exist in the CS) of WzcCD, its C terminus displays significantly enhanced B-factor values (Fig. 6A) (7), reminiscent of CS4 in the WzcCD-ATP-Mg\(^{2+}\) simulations (fig. S5E). Drawing further inspiration from the crystal structure of S. aureus CapB\(_{CX}\) (8) in which the region corresponding to \(\alpha_2\) in WzcCD is significantly shorter and terminates in a long flexible linker, we generated a WzcCD\(_{ADP}\) mutant where residues 523 to 526 (that includes the last two residues of \(\alpha_2\)) were replaced in silico by glycines. We performed REST2 simulations with this construct, WzcCD\(_{ADP}\)·(G)\(_4\) bound to ATP-Mg\(^{2+}\) using the same protocols described in the previous sections and analyzed the resultant PDF in the cylindrical coordinate system. Only a single major state, the CS, proximal to the crystal structure (Fig. 6B), is found. Inspection of randomly selected structures show that, as expected, the mutated region (C-terminal end of \(\alpha_2\)) forms a flexible loop, and the bending about S512 is never observed (Fig. 6C) nor is the unfolding of the \(\alpha_7\) or \(\alpha_9\) helices; i.e., disruption of I\(_2\)) is seen. (D) The key ATP-coordinating elements at the catalytic site are ordered, and ATP remains stably bound.

Fig. 6. Stabilization of the CS in silico. (A) B-factor values from the crystal structure of WzcCD reveal a high-degree disorder at the C-terminal end of \(\alpha_2\) (dotted circle). (B) Projection of the REST2-generated ensemble of WzcCD·(G)\(_4\)·ATP·Mg\(^{2+}\) onto \(\theta\)–\(\phi\) space reveals the sampling of only a CS (the WzcCD crystal conformation is indicated by the green dot); the OS and HS seen in the WzcCD·ATP-Mg\(^{2+}\) simulations (see Fig. 2A) are no longer seen. (C) Ten randomly selected structures superimposed on C\(_\alpha\) atoms are shown. The \(\alpha_2\) configuration resembles that seen in the crystal structure (i.e., no bending is observed) and the introduction of the (G)\(_4\) introduces flexibility at the C-terminal end of \(\alpha_2\). The RK-cluster [(cyan and magenta) is dynamic. However, no other structural evolution (e.g., the unfolding of the \(\alpha_7\) or \(\alpha_9\) helices; i.e., disruption of I\(_2\)) is seen. (D) The key ATP-coordinating elements at the catalytic site are ordered, and ATP remains stably bound.
of α7/α9. While the RK-cluster is still flexible, no other remarkable features are evident in the structures in which ATP and Mg^{2+} remain in their appropriate locations (Fig. 6D).

Formation of the octameric ring stabilizes the WzcCD_{ΔC}-ATP-Mg^{2+} complex

As noted above, it is possible to stabilize the complex of ATP-Mg^{2+} with monomeric WzcCD_{ΔC} by stabilizing the CS and preventing formation of the OS. It is notable that all the monomers in the crystal structure of WzcCD are in the CS. This suggests that formation of the octameric ring likely constitutes the mechanism by which the CS may be stabilized, enables the stable engagement of ATP-Mg^{2+}, and allows transphosphorylation to proceed. As an initial test of this hypothesis, we performed three sets of classical molecular dynamics (MD) simulations on a reduced system, a trimer. Coarse-grained simulations suggest that it is absolutely necessary to stabilize both

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Fig. 7. Classical MD simulations of WzcCD_{ΔC}-ATP-Mg^{2+} in the monomeric and pseudo-ring states. (A) θ-|h| space representation of a monomer trajectory (T3) with the points color-coded according to their time stamps (in nanoseconds; the black dot indicates crystal structure values); OS-like and CS-like states are observed. (B) Comparison of structures in the early (left; bending of α2 shown by red arrows) and late (right; displacement of Y569 and D480 from ATP indicated by blue arrows) time points from (A). (C) Conformations of the central monomer from a representative pseudo-ring trajectory (T1) plotted in θ-|h| space shows only a CS-like state. (D) The last frame from T1 shows that α2 is intact, and ATP continues to be engaged by Y569 and D480. For the monomer (E) and the central monomer in the pseudo-ring (F) simulations, the evolution of the SS12_Co-TS15_Co (proxy for α2 bending; top) and D480_Cγ-ATP_O3' (proxy for ATP-contact; bottom) distances are shown. The left panels indicate time courses in the three independent trajectories (T1, T2, and T3), and right panels indicate frequencies of the corresponding distances. The green/black arrows highlight the inverse correlation between the distances for the monomer. Also, see fig. S8.
interaction interfaces as in a ring configuration to prevent the dissociation of dangling monomers, e.g., in a minimal open system such as a trimer. Therefore, for all-atom simulations, in lieu of the octamer ring, we used a trimer but imposed position restraints on all Ca atoms of the flanking monomers, while the central monomer was unrestrained. This system, which we call a “pseudo-ring,” maintains the fixed arc length of the octamer ring and is expected to reasonably approximate the effects of the ring on the global transitions between the CS and the OS in the central monomer. Further, since the pseudo-ring simulations are shorter and use classical MD, quantitative comparisons with the REST2 simulations using the WzCDAC monomer would be inappropriate since the latter samples a significantly larger conformational landscape. To establish an appropriate reference for the pseudo-ring simulations, we preformed three additional sets of classical MD simulations of WzCDAC in the monomeric state in complex with ATP-Mg$^{2+}$.

As expected, all three monomer trajectories populate the major states (although not all the constituent clusters are populated given the limited sampling of conformational space) seen in our REST2 simulations. In particular, one trajectory (T3) samples the OS (Fig. 7A), in which the interactions of ATP with Y569 and D480 are broken (Fig. 7B, right), presumably representing a state primed eject to the limited sampling of conformational space) seen in our REST2 states (although not all the constituent clusters are populated given Wzc CD interactions reflect the tendencies noted in the REST2 simulations for the interaction is broken (Fig. 7E, red traces). Overall, these simulations reflect the tendencies noted in the REST2 simulations for the WzCDAC-ATP-Mg$^{2+}$ complex reflecting the fact that the two conditions, a stable oligomeric interface and bound ATP-Mg$^{2+}$, cannot be simultaneously satisfied in the monomeric state. In the pseudo-ring simulations, on the other hand, the system only samples the CS (Fig. 7C); the D480-ATP interaction remains intact (Fig. 7E, black traces). The third trajectory (T2) displays no bend in α2, although the D480-ATP interaction is broken (Fig. 7E, red traces). Overall, these simulations reflect the tendencies noted in the REST2 simulations for the WzCDAC-ATP-Mg$^{2+}$ complex reflecting the fact that the two conditions, a stable oligomeric interface and bound ATP-Mg$^{2+}$, cannot be simultaneously satisfied in the monomeric state. In the pseudo-ring simulations, on the other hand, the system only samples the CS (Fig. 7C); the D480-ATP contact is maintained, and the bending of α2 is not observed (left panel of Fig. 7D and the bottom panel of Fig. 7F).

There is some disorder introduced at the C-terminal end of α2 (fig. S8). The disorder in this region (fig. S8) is similar to that observed in the crystal structure that shows elevated B-factor values (Fig. 6A) (7). This is also the condition exploited to stabilize the CS in the WzCDAC-(G)4·ATP-Mg$^{2+}$ simulations described above (Fig. 6C). One of the pseudo-ring trajectories shows a reintroduction of order in this region and this effect is coupled with a small change in the orientation of D480 from its optimal position with respect to ATP (fig. S8). These classical MD simulations provide further evidence of the intimate coupling of the oligomerization interfaces and the catalytic site of WzCDAC.

**DISCUSSION**

We used enhanced sampling methods to probe the conformational landscapes of a variety of complexes involving the CD of the *E. coli* BY-kinase Wzc. Our results (shown schematically in Fig. 8) indicate the existence of a complex dynamic network within the kinase core that links the conformational states of the nucleotide-binding and catalytic elements to those at the oligomerization surfaces. Our studies suggest the presence of discrete global conformations represented by an OS, a CS that is similar to that seen in crystallo (7), and a sparsely populated HS that only forms in the presence of ATP and Mg$^{2+}$ (fig. S9). WzCDAC bereft of any ligands assumes an OS that is characterized by the coupled rotations of helix α2, which harbors a conserved EX2RXXR motif required for oligomerization, and helix α3, which contains the Walker-A motif. This rotation of α3 results in an interconversion between the CS and the OS in the central monomer. Further, since the pseudo-ring simulations are shorter and use classical MD, quantitative comparisons with the REST2 simulations using the WzCDAC monomer would be inappropriate since the latter samples a significantly larger conformational landscape. To establish an appropriate reference for the pseudo-ring simulations, we preformed three additional sets of classical MD simulations of WzCDAC in the monomeric state in complex with ATP-Mg$^{2+}$.

As expected, all three monomer trajectories populate the major states (although not all the constituent clusters are populated given the limited sampling of conformational space) seen in our REST2 simulations. In particular, one trajectory (T3) samples the OS (Fig. 7A), in which the interactions of ATP with Y569 and D480 are broken (Fig. 7B, right), presumably representing a state primed eject to the limited sampling of conformational space) seen in our REST2 states (although not all the constituent clusters are populated given Wzc CD interactions reflect the tendencies noted in the REST2 simulations for the interaction is broken (Fig. 7E, red traces). Overall, these simulations reflect the tendencies noted in the REST2 simulations for the WzCDAC-ATP-Mg$^{2+}$ complex reflecting the fact that the two conditions, a stable oligomeric interface and bound ATP-Mg$^{2+}$, cannot be simultaneously satisfied in the monomeric state. In the pseudo-ring simulations, on the other hand, the system only samples the CS (Fig. 7C); the D480-ATP interaction remains intact (Fig. 7E, black traces). The third trajectory (T2) displays no bend in α2, although the D480-ATP interaction is broken (Fig. 7E, red traces). Overall, these simulations reflect the tendencies noted in the REST2 simulations for the WzCDAC-ATP-Mg$^{2+}$ complex reflecting the fact that the two conditions, a stable oligomeric interface and bound ATP-Mg$^{2+}$, cannot be simultaneously satisfied in the monomeric state. In the pseudo-ring simulations, on the other hand, the system only samples the CS (Fig. 7C); the D480-ATP contact is maintained, and the bending of α2 is not observed (left panel of Fig. 7D and the bottom panel of Fig. 7F).

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**Fig. 8. Schematic representation of key conformational features identified in the REST2 simulations.** The RK-cluster (cyan, with α8 in magenta), α2 (light blue), α7 and α9 (light red), α3 (black), and α4 (dark red) are indicated. Monomeric, unliganded WzCDAC exists in the OS; binding of ATP-Mg$^{2+}$ forces the formation of the CS (red arrows). This state can be qualitatively conceptualized as a strained network (black arc) of connected deformed springs. The CS is composed of several exchanging substates (clusters) with distortions that are incompatible with transphosphorylation. The most populated of these clusters show different sorts of distortions: CS5, partial unfolding of α7 and α9 (light red) CS4, enhanced disorder in the RK-cluster (dotted line) and at the N-terminal end of α2 (red arc); and CS2, bending of α2 (light green arrows). Alternatively, ATP is displaced from the binding site (purple arrow) restoring the OS. These various distortions may be considered to be independent mechanisms by which this mechanical network “releases its internal strain.” A HS (proposed to represent a reactive state) that is also populated, albeit sparsely, when bound to ATP-Mg$^{2+}$, is not shown for simplicity.
The OS, and allow proper ATP-Mg²⁺ coordination by introduction of a polyglycine segment at the C-terminal end of α2 on I₁ (Fig. 6).

Thus, our results taken together suggest a scenario in which ATP-Mg²⁺, in contrast to ADP, would be unable to form a stable complex with WzcCD in its monomeric state since its engagement would lead to structural distortions presumably at an energetic cost through the loss of several hydrogen bonds at the oligomerization interfaces involving α2, α7, and α9. ITC experiments confirm a significantly reduced affinity (by ~200-fold) for ATP-Mg²⁺ compared to ADP alone. We predict, that oligomerization and formation of the octameric ring stabilizes the two interfaces and thereby locks in the CS, enabling the T541-D642 interaction while decoupling the latter from the catalytic K540 and allowing the stable coordination of ATP-Mg²⁺ (Fig. 9). Our preliminary results through classical MD simulations are in line with this prediction. Clearly, a full set of REST2 simulations on the octameric state are needed to fully establish this model. Thus, given the enhanced affinity for ATP-Mg²⁺ in the oligomeric state compared to that of the monomer, the cellular ATP/ADP ratio would then ensure the exchange of ADP, which is bound to the monomer, for ATP-Mg²⁺, upon oligomer formation. The system would then be able to sample the HS state that has an ordered RK-cluster, enabling the insertion R490 into the active site and initiating chemistry.

Our model for WzcCD activation predicts that oligomerization, i.e., substrate docking, must precede ATP-Mg²⁺ binding and the formation of an active conformation. It is notable that this scenario provides a distinct advantage to ensure efficient phosphorylation in that it avoids a futile cycle of ATP hydrolysis that would necessarily occur upon ATP-Mg²⁺ binding before substrate docking due to the high inherent ATPase activity of WzcCD (15). A similar mechanism to prevent a futile hydrolysis cycle has been proposed for ATP-binding cassette (ABC) transporters (28, 29). ATPases such as F₁-ATPase have also developed mechanisms to prevent the wasteful expenditure of ATP (30). This mechanism likely represents another example of the elaborate means evolved by bacteria to avoid these sorts of energy-spilling reactions (31). Further, the formation of a closed ring in the case of WzcCD provides the possibility that all constituent monomers could appropriately engage ATP-Mg²⁺ and be optimally primed for catalysis without the nonproductive consumption of ATP. It is of note that for several canonical, i.e., eukaryotic, protein kinases, there is growing evidence that a fully activated state is achieved only upon substrate docking. A prime example is the mitogen-activated protein kinase p38α, for which it has been shown that docking of the substrate peptide, in addition to dual phosphorylation on its activation loop, is required to achieve a fully active conformation (32). Substrate docking leads to significant enhancement in the affinity of p38α for ATP-Mg²⁺ (33). Thus, many features of the proposed activation mechanism of Wzc, and by inference of other BY-kinases, appear to resemble that seen in several eukaryotic protein kinases despite the deployment of a unique CD. It is possible that the sequence of events suggested by our model, where the substrate is engaged (oligomerization) before binding ATP, allowed BY-kinases to select as their phosphoreceiver, an –OH moiety housed on a tyrosine of the Y-cluster rather than on water, providing bacteria with the ability to phosphorylate on tyrosine deploying an ATPase-like fold rather than a canonical dual-lobe kinase scaffold used by eukaryotic cells (11).

**MATERIALS AND METHODS**

**Computational details**

**Structure preparation**

Chain A (average root mean square deviation over the all chains based on Ca atoms, 0.12 ± 0.04 Å) was randomly selected from the crystal structure [Protein Data Bank (PDB): 3LA6] of the CD of the K540M mutant of Wzc (WzcCD) (7). The missing segment of the RK-cluster was built using the Rosetta kinematic closure (KIC) protocol (34) using a single round of modeling without subsequent energy minimization (we refer to this as the crystal structure from here on). In all cases, the bound Ca²⁺ ion present in the crystal structure (which already contains bound ADP) was replaced by a Mg²⁺ ion. M540 on the Walker-A motif was mutated back to the native lysine using the mutagenesis tool in PyMOL. In addition, all residues spanning the dynamic Y-cluster at the C-terminal tail (705 to 720) were removed to generate the WzcCD kinase core construct that was used in all simulations. A series of classical MD simulations (using the setup described below) were performed using the structure generated above containing an intact RK-cluster with ADP-Mg²⁺ bound to the active site to generate starting structures for all subsequent simulations. The structure was selected on the basis of the presence of all ADP contacts that are observed in the crystal structure after equilibration of the conformation of the RK-cluster in the MD runs. In the case of the WzcCD-ADP-Mg²⁺ simulations, this structure was used as the starting structure, without further processing. For the WzcCD-ADP simulations, the Mg²⁺ ion was removed from the starting structure in WzcCD-ADP-Mg²⁺ to initiate the run. For the WzcCD-ATP-Mg²⁺ simulations, the ATP-Mg²⁺ configuration was obtained from the crystal structure of

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**Fig. 9. Model for WzcCD activation and nucleotide exchange.** In the monomeric state, WzcCD is stably bound to ADP in the absence of Mg²⁺. Although α2 is bent (green arrows), the absence of Mg²⁺ allows a coupled rigid body motion (red arrows; no unfolding occurs) of α7 and α9 (I2) together with α4 allowing for the proper coordination of ADP. Oligomerization and formation of the octameric ring (only three constituent monomers are represented) stabilize I₁ and I₂ (light green arrows) and allows formation of the CS. This state also carries some “strain” that is “released” by introducing disorder at the C terminus of α2 (red arcs), a segment that does not participate in oligomerization. In this oligomeric state, ATP-Mg²⁺ can be stably engaged with an affinity that is similar to (or possibly exceeds that) of ADP. Consequently, the cellular ATP/ADP ratio allows nucleotide exchange and the formation of an active conformation with ATP-Mg²⁺ correctly coordinated, the catalytic elements properly oriented, and the RK-cluster well ordered (thick lines) and appropriately engaged to enable catalysis.
the Wzc homolog MinD (PDB: 3Q9L) (10) and used to replace ADP-Mg\(^{2+}\) moiety. A short classical MD simulation using the setup described below was performed using Wzc\(_{CD}\) -ATP-Mg\(^{2+}\) at 400 K to achieve proper solvation of the Mg\(^{2+}\) ion. The appropriately solvated Wzc\(_{CD}\) -ATP-Mg\(^{2+}\) configuration was then used as the starting structure for further simulations. The starting Wzc\(_{CD}\) -(G)\(_4\) -ATP-Mg\(^{2+}\) structure used in the REST2 simulations was generated using the Wzc\(_{CD}\) -ATP-Mg\(^{2+}\) structure by replacing residues 523 to 526 by glycines using the mutagenesis tool in PyMOL. Following this, the system was prepared in the same manner as in other cases.

All simulations were performed using the GROMACS 2019 software suite (35, 36); the CHARMM36m (37) force field was used to parameterize the system. The system was solvated using TIP3P waters in a cubic box with a 1.5-nm edge distance from the solute. In all cases, charge-neutralizing ions were added to the system. Energy minimization with gradient descent was performed with a 0.1-fs time step until energy convergence was obtained. Before the production runs, the system was equilibrated, first in the NVT and then in the NPT ensemble, for 100 ps each. NVT and NPT equilibrations were conducted with position restraints on all heavy atoms using a 2-fs integration step. The production run was performed in the NPT ensemble using the Berendsen (38) and the Parrinello-Rahman (39) thermostat and barostat, respectively. Classical MD simulations were carried out at 300 K with a 2-fs integration step. The particle-mesh Ewald summation procedure with a cutoff of 1.0 nm was used to compute long-range electrostatic interactions, and periodic boundary conditions were used to handle edge effects. In all cases, coordinates were stored every 10 ps.

**REST2 simulations**

Structures were prepared as described above and used after NVT and NPT equilibration to initiate the REST2 simulations (17). Simulations were performed using the PLUMED 2 (40) plugin–patched GROMACS 2019 suite. The "hot" region in our simulation encompassed all solute atoms including the nucleotide and Mg\(^{2+}\) (where present). A total of 14 replicas were used, where the lowest temperature (\(T_{\text{min}}\)) was set to 300 K. A series of short 2-ns REST2 simulations were performed by varying the temperature of the highest temperature replica (\(T_{\text{max}}\)) until the average replica exchange probability fell in the acceptable range. The desired exchange probability was achieved with a \(T_{\text{max}}\) of 400 K using the 14 replicas with exchange probabilities of 23.6 ± 0.8% for apo, 23.0 ± 1.0% for Wzc\(_{CD}\) -ATP-Mg\(^{2+}\), 23.2 ± 0.9% for Wzc\(_{CD}\) -ADP-Mg\(^{2+}\), 23.4 ± 0.5% for Wzc\(_{CD}\) -ADP, and 21.0 ± 6.0% for Wzc\(_{CD}\) -(G)\(_4\) -ATP-Mg\(^{2+}\). In all cases, the temperature difference between replicas was obtained using the following expression

\[
T_n = T_{\text{min}} * e^{\frac{T_{\text{max}} - T_{\text{min}}}{n - 1}}
\]

where \(n\) is the \(n\)th replica, \(T_n\) is its corresponding temperature, and \(T_{\text{min}}\) and \(T_{\text{max}}\) are the minimum and maximum temperatures 300 and 400 K, respectively. The factor \(\lambda\), which describes the scaling of the solute intramolecular potentials, was defined by the \(T_{\text{min}}/T_n\) ratio, which then was used to reparameterize the system for each replica \(n\). Production runs in all cases were carried out for 200 ns per replica, for an effective 28,000-ns sampling time; exchange attempts were made every 20 ps. Only the final 160 ns was used in the analyses in each case. The final temperatures and scaling factors \(\lambda\) for each replica were as follows: (1) 300.0, 1.000; (2) 306.7, 0.978; (3) 313.6, 0.957; (4) 320.6, 0.936; (5) 327.8, 0.915; (6) 335.1, 0.895; (7) 342.6, 0.875; (8) 350.3, 0.856; (9) 358.1, 0.838; (10) 366.1, 0.819; (11) 374.3, 0.801; (12) 382.7, 0.784; (13) 391.3, 0.767; and (14) 400.0, 0.750.

**Definition of the cylindrical frame of reference**

A cylindrical coordinate system with an angle, \(\theta\), and a rise, \(|h|\), was defined to characterize the conformations of the structures generated in the REST2 simulations (see Fig. 1A). To define this frame, four reference points (labeled P1, P2, P3, and P4 in Fig. 1A) were selected: the COM of the Ca atoms of a segment comprising residues 541 to 547 (the first two turns of the N-terminal end of helix \(\alpha3\); the COM is indicated by a yellow sphere and labeled P1 in the left panel of Fig. 1A), the COM of the Ca atoms of residues 548 to 552 (the penultimate turn from the C-terminal end of helix \(\alpha3\), indicated by the magenta sphere and labeled P2 in the left panel of Fig. 1A), the COM of the Ca atoms of residues 569 to 573 (\(\alpha4\), indicated by the red sphere and labeled P3 in the left panel of Fig. 1A), and the COM of the Ca atoms of residues 505 to 509 (the first turn of the N-terminal end of helix \(\alpha2\), indicated by the green sphere and labeled P4 in the left panel of Fig. 1A). Alignment of structures was then performed as follows: All structures were aligned such that points P1 and P2 were oriented along the \(z\) axis. Next, the structures were rotated about the \(z\) axis such that P3 was placed on the \(x\) axis in the positive direction. The rise \(|h|\) (these, as defined, were negative in all cases; hence, we use the absolute value for simplicity) was then the \(z\) coordinate of P1, and the angle \(\theta\) was defined as the angle between the \(x\) axis and the \(xy\) projection of the vector pointing from P1 to P4 (see Fig. 1A). These manipulations were performed using appropriate rotation matrices using an in-house code that used the C++ programming language. The PTEROS (41) library was used for trajectory file handling.

**Classical MD simulations on the monomeric and trimeric (pseudo-ring) states of Wzc\(_{CD}\)**

A single structure from cluster CS1 (that is almost in the same as the conformation seen in the crystal structure; see fig. S5D) of our Wzc\(_{CD}\) -ATP-Mg\(^{2+}\) REST2 simulations was randomly selected and, in the case of the monomer simulations, served as the starting structure. In the case of the trimer simulations, chains A, B, and C were selected from the Wzc\(_{CD}\) crystal structure, and the CS1 structure was aligned to chain B via Ca atoms. Chain B was subsequently removed from the configuration. In addition, the C-terminal tails (705 to 720) were also removed from chains A and C, while the ADP-Mg\(^{2+}\) was kept in place. The simulation setup was the same as described above. In the case of the trimer (pseudo-ring) simulations during the production runs, harmonic position restraints were imposed on all Ca atoms of chains A and C with a force constant of 1000 J mol\(^{-1}\) nm\(^2\) acting along the \(x\), \(y\), and \(z\) directions. Simulations (100 ns) were performed in triplicate, and in each case, the initial velocities were obtained from a Maxwell distribution at 300 K using a random seed. In the case of the trimer, the structural evolution was only analyzed for the central (unrestrained) monomer.

**Expression and purification of Wzc\(_{CD}\) and Wzc\(_{DD}\)**

A pET15b vector containing either His\(_6\) -tagged Wzc\(_{CD}\) (447 to 704) or Wzc\(_{DD}\) (447 to 720), was used to transform BL21 DE3 (Thermo Fisher Scientific) cells for protein expression. A single colony was used to inoculate 20 ml of Luria-Bertani (LB) media in the presence of ampicillin (0.1 mg/ml), grown at 37°C overnight, and used to inoculate 500 ml of LB medium in the presence of ampicillin (0.1 mg/ml).
The culture was grown at 37°C until an optical density at 600 nm of 1 was reached at which time expression was induced by the addition of 400 μM isopropyl β-D-thiogalactopyranoside. Expression was conducted at 37°C for 4 hours, after which the cells were harvested via centrifugation at 4000 rpm for 30 min using a Fiberlite F12-6 × 500 LEX rotor (Thermo Fisher Scientific). All subsequent steps were carried out at 4°C. The cells were lysed via sonication (3 min, pulse on for 0.3 s, pulse off for 0.7 s with a power amplitude of 30%) using a Sonic Dismembrator Model 500 (Thermo Fisher Scientific) in lysis buffer containing 25 mM Pipes (pH 6.5), 300 mM NaCl, 30% glycerol, 5 mM β-mercaptoethanol (BME), and 1:500 lysozyme and supplemented with half a Roche protease inhibitor tablet. The cell lysate was centrifuged at 10,000 rpm for 40 min to remove cell debris using a Fiberlite F21-8x50y rotor. The soluble lysate was then added to 5 ml of Ni-NTA affinity beads (GoldBio) and incubated for 1 hour with gentle rotation. Following this, the slurry was poured over a chromatography column, and the flow through was discarded. The bound protein was washed with 50 ml of wash buffer (lysis buffer containing 20 mM imidazole) and then eluted with 50 ml of elution buffer (lysis buffer containing 250 mM imidazole). The expressed protein in all cases was concentrated and further purified through gel filtration using a Superdex 200 10/300 (GE Healthcare Biosciences) column. WzcCDAC and WzcCD samples were dialyzed in dialysis buffer containing 25 mM tris-HCl (pH 7.5), 300 mM NaCl, 30% glycerol, and 5 mM BME. A portion of the WzcCD sample was also treated with ~0.25 nmol of λ-phosphatase (prepared in house) and ~0.35 nmol of AMPPCP (disodium salt, Sigma-Aldrich) was dissolved in the Mg²⁺-containing buffer, and the pH was subsequently adjusted to 6.5 using sodium hydroxide (NaOH). ITC measurements were carried out at 37°C using a MicroCal ITC200 isothermal titration calorimeter (Malvern). All measurements were performed in triplicate. The protein/titrant concentrations used were 40 μM/400 μM and 40 μM/1.6 mM for the ADP·Mg²⁺ and AMPPCP·Mg²⁺ titrations, respectively. Separate affinity measurements for ADP in the absence of Mg²⁺ were done using 40 μM/400 μM, 20 μM/300 μM, and 15 μM/180 μM protein/titrant concentrations. All titrations were performed with a rotation speed of 500 rpm. A total of 16 injections were made in each case where the first one consisted of 0.4 μl with a duration of 0.8 s and the rest consisted of 2.4 μl with a duration of 4.8 s and filter periods of 5 s. The spacing between injections used was 240 s. In all cases, the same experiment was performed by titrating the titrant into buffer alone and used to normalize the data. The normalized data were fitted to the one-site binding model using Origin (OriginLab).

**Analysis of the oligomerization state of WzcCD**

Nucleotide-free WzcCDC or nucleotide-free, λ-phosphatase–treated WzcCDC were each concentrated to 200 μM and then passed through a Superdex 200 10/300 (GE Healthcare Biosciences) gel filtration column pre-equilibrated with buffer containing 25 mM Pipes (pH 6.5), 300 mM NaCl, 30% glycerol, and 5 mM BME to assess their respective oligomerization states. Fractions suspected to contain WzcCD were analyzed by SDS–polyacrylamide gel electrophoresis.

**Chemical shift perturbation analysis**

Uniformly ¹H,¹⁵N-labeled WzcCDAC was expressed and purified using previously described protocols (16) with the following minor modifications. After elution from the Ni²⁺-affinity column, the protein was incubated overnight with an excess of ATP (~20-fold) and Mg²⁺ (~200-fold). After adding an excess of EDTA, the sample was concentrated and injected on a size exclusion column (Superdex 75, GE Healthcare) pre-equilibrated with NMR buffer containing 50 mM phosphate (pH 6.0), 50 mM NaCl, 25 mM dithiothreitol (DTT), and 200 mM EDTA. Unlike the previously published protocol, no further nucleotides were added at this point. To eliminate bound ADP, the sample was buffer-exchanged (1/10,000 dilution factor) against the NMR buffer using spin columns (10-kDa cutoff). The NMR samples typically consisted of ~150 μl (in 4-mm Shigemi tubes) of ~100 μM U-²H,¹⁵N-labeled WzcCDAC. Ligands (Mg²⁺, 200 molar equivalents; ADP, up to 20 molar equivalents) were added directly to the NMR tubes from stock solutions prepared in the same buffer. ¹H,¹⁵N H-TROSY experiments (64 scans, 1.5-s recycle delay) were collected using 2048 and 256 complex points, with sweep widths of 15 and 32 ppm in the direct and indirect dimensions, respectively. All experiments were acquired at 25°C on a 700-MHz Avance III Bruker spectrometer equipped with cryogenic probes capable of applying pulse field gradients along the z axis. The spectra were processed using nmrPipe (42) and analyzed using nmrViewJ (43). Amide chemical shift perturbations were calculated using the formula

\[ \Delta \delta = \sqrt{\Delta \delta_{\text{HN}}^2 + (0.154 \Delta \delta_{\text{N}})^2} \]

\( \Delta \delta_{\text{HN}} \) and \( \Delta \delta_{\text{N}} \) represent the perturbations for amide ¹H and ¹⁵N chemical shifts, respectively. The Δδ values represent the differences between well-resolved, free, and ADP-bound resonances of WzcCDAC under partial ADP saturation or the perturbations induced by Mg²⁺ on ADP-saturated WzcCDAC.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://advances.sciencemag.org/cgi/content/full/6/51/eabd3718/DC1

**REFERENCES AND NOTES**

1. C. Grangeasse, S. Nessler, I. Mijakovic, Bacterial tyrosine kinases: Evolution, biological function and structural insights. *Philos. Trans. R. Soc. B* **367**, 2640–2655 (2012).

2. C. Grangeasse, R. Terreux, S. Nessler, Bacterial tyrosine-kinases: Structure-function analysis and therapeutic potential. *Biochim. Biophys. Acta* **1804**, 628–634 (2010).

3. F. Jadeau, E. Bechet, A. J. Cozzone, G. Deléage, C. Grangeasse, C. Combet, Identification of the idiosyncratic bacterial protein tyrosine kinase (BY-kinase) family signature. *Bioinformatics* **24**, 2427–2430 (2008).
5. R. F. Collins, K. Beis, C. Dong, C. H. Botting, C. McDonnell, R. C. Ford, B. R. Clarke, D. C. Lee, J. Zheng, Y.-M. She, Z. Jia, Structure of...Sci. Adv. 2020; 6 : eabd3718
6. J. E. Walker, The ATP synthase: The understood, the uncertain and the unknown. Biochem. Soc. Trans. 43, 1–16 (2015).