Catalytic process of anhydro-N-acetylmuramic acid kinase from *Pseudomonas aeruginosa*

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The bacterial cell envelope is the structure with which the bacterium engages with, and is protected from, its environment. Within this envelop is a conserved peptidoglycan polymer which confers shape and strength to the cell envelop. The enzymatic processes that build, remodel, and recycle the chemical components of this cross-linked polymer are preeminent targets of antibiotics and exploratory targets for emerging antibiotic structures. We report a comprehensive kinetic and structural analysis for one such enzyme, the *Pseudomonas aeruginosa* anhydro-N-acetylmuramic acid (anhNAM) kinase (AnmK). AnmK is an enzyme in the peptidoglycan-recycling pathway of this pathogen. It catalyzes the pairing of hydrolytic ring opening of anhNAM with concomitant ATP-dependent phosphoryl transfer. AnmK follows a random-sequential kinetic mechanism with respect to its anhNAM and ATP substrates. Crystallographic analyses of four distinct structures (apo AnmK, AnmK:AMPNP, AnmK:AMPNP:anhNAM, and AnmK:AT-PanhNAM) demonstrate that both substrates enter the active site independently in an un gated conformation of the substrate subsites, with protein loops acting as gates for anhNAM binding. Catalysis occurs within a closed conformational state for the enzyme. We observe this state crystallographically using ATP-mimetic molecules. A remarkable X-ray structure for dimeric AnmK sheds light on the precatalytic and postcatalytic ternary complexes. Computational simulations in conjunction with the high-resolution X-ray structures reveal the full catalytic cycle. We further report that a *P. aeruginosa* strain with disrupted anmK gene is more susceptible to the β-lactam imipenem compared to the WT strain. These observations position AnmK for understanding the nexus among peptidoglycan recycling, susceptibility to antibiotics, and bacterial virulence.

The Gram-negative bacterial cell wall is a cross-linked polymer that encases the entire bacterium. This wall is found in the periplasmic space between the outer and the inner membranes (Fig. 1A). Essentially, the dozens of enzymes involved in the biosynthesis, recycling, and regulation of cell wall influence the survival of the bacterium (1-3). The cell wall is remodeled and recycled not only in response to damage—as from exposure to antibiotics—but also during homeostasis (1). The onset of this recycling involves the catalytic action of lytic transglycosylases (LTs). LTs fragment the peptidoglycan polymer of the cell wall to give glycan strands of varying size (3-5). These strands are processed further by other enzymes and then internalized into the cytoplasm (3, 6). Additional structural transformations take place in the cytoplasm to give metabolites for the biosynthesis of lipid II (Fig. 1A). Lipid II is the key intermediate translocated from the cytoplasm to the periplasm for *de novo* peptidoglycan biosynthesis. The cell wall expands and matures by incorporating and cross-linking lipid II–derived nascent peptidoglycan into the existing cell wall structure.

The hallmark of the LT reaction is the formation of the glycan strands showing a bicyclic 1,6-anhydro-N-acetylmuramyl (anhNAM, obtained from the *N*-acetylmuramate [NAM] component of the peptidoglycan) terminus (3, 7). This bicyclic anhNAM (compound 1, Fig. 1A) motif targets the cell-wall-degradation products for import into the cytoplasm for recycling. A committed step in cytoplasmic recycling is performed by the enzyme anhydro-N-acetylmuramic acid kinase (AnmK). AnmK opens the glycosidic 1,6-anhNAM ring, concomitant with an ATP-dependent phosphorylation of the C6 hydroxyl (Fig. 1, A and B). This AnmK reaction enables direct entry of the newly formed NAM (compound 4, Fig. 1A) into the peptidoglycan biosynthetic processes. Here, we disclose the full catalytic cycle of the AnmK enzyme from *Pseudomonas aeruginosa* by insights from enzyme kinetics, high-resolution X-ray crystallography, and molecular dynamics (MD) simulations.

Results

**Cloning, expression of the gene, and purification of recombinant AnmK**

The gene *anmK* (gene locus PA0666) from *P. aeruginosa* PA01 was cloned into a pMAL-c6T plasmid. It was overexpressed in *Escherichia coli* LOBSTR-RIL cells to produce AnmK as a fusion protein with an N terminally hexahistidine-
tagged (6×His) maltose-binding protein (MBP) at the N terminus of AnmK, with an intervening tobacco etch virus (TEV) protease cleavage site. The 6×His and MBP tags were used for protein purification. TEV protease removed these tags. The purified AnmK was homogeneous by SDS-PAGE (Fig. S1C). Sedimentation velocity analytical ultracentrifugation analysis indicated that at a micromolar concentration in buffer solution AnmK was a homodimer (95%), showing also a higher mass component (mass is consistent with its assignment as a tetramer, 5%) (Fig. S2).

Catalytic activity assays of AnmK with natural substrates

The substrate anhNAM was synthesized by minor alteration of a literature method (Scheme S1) (8). Turnover of anhNAM by AnmK was monitored spectroscopically by consumption of NADH using a lactate dehydrogenase–coupled ATP-recycling assay. Steady-state kinetic parameters were determined for the turnover process: $K_{\text{M,anhNAM}}^{\text{app}} = 500 \pm 100 \, \mu M$, $K_{\text{M,ATP}}^{\text{app}} = 96 \pm 19 \, \mu M$, and $k_{\text{cat}}^{\text{app}} = 46 \pm 13 \, \text{s}^{-1}$. The values for $K_{\text{M,anhNAM}}^{\text{app}}$ and $k_{\text{cat}}^{\text{app}}$ are within 2-fold of those determined by Bacik et al. (a value for $K_{\text{M,ATP}}^{\text{app}}$ was not reported) (9). At the saturating concentration of anhNAM (4 mM), the accumulation of N-acetylmuramic acid-6-phosphate (NAM-6P) in the assay medium did not lead to AnmK inhibition, as seen by the unchanged rate of the catalytic reaction (10). We could not draw the same conclusion for ADP, as it was recycled to ATP under the assay conditions. Initial velocities obtained at different concentrations of ATP and of anhNAM intersected on the Lineweaver–Burk analysis, supporting a random-sequential mechanism for AnmK (Fig. S3) (11). Our efforts to confirm the random-sequential mechanism using a recently reported two-dimensional isothermal titration calorimetry experiment were unsuccessful (12, 13). Rapid decay of the thermogram peaks due to accumulation of ADP, with attendant AnmK inhibition, hindered this analysis (Fig. S4) (10). The random-order mechanism for AnmK was supported by X-ray crystallography. Crystal structures reported by Bacik et al. (9) and by us show that both anhNAM and adenosine 5’- (β,γ-imido) triphosphate (AMPPNP, an ATP analog) bind independently to AnmK. Their bound conformations are catalytically relevant (vide infra). The potential for ATPase activity of AnmK was evaluated, however, none was observed under the assay conditions.

Crystallographic and MD characterization of AnmK catalysis

Four different AnmK crystal structures were obtained from different crystallization conditions. One was apo AnmK. Three were complexes: with AMPPNP; with AMPPNP and anhNAM; and with ATP and anhNAM/NAM-6P (Table S1). The structures for the complexes were obtained by co-crystallizing AnmK either with MgAMPPNP or MgATP, followed by soaking of the resulting crystals with anhNAM (see Experimental procedures section). The resolutions of our structures (1.7 Å–2.2 Å) gave excellent electron-density maps for both the protein and the ligands (Fig. S5). In all of our structures, AnmK presented the characteristic homodimeric arrangement described previously (Fig. 2A) (9, 14). Within this dimeric arrangement an open conformational state was previously observed for AnmK when bound to AMPPCP (a non-hydrolyzable ATP analog) and when bound to AMPPCP and anhNAM (14). Also, low-resolution studies by small-angle X-ray scattering pointed to an open conformation for the apo AnmK (14). The closed conformation required for catalysis was, however, observed when crystallizing AnmK in complex with ADP or in complex with anhNAM (9, 14).

Herein, we report the first crystal structure of AnmK in its apo form (Fig. 2A). Surprisingly, our 2.2 Å resolution structure
Figure 2. Catalytic process of AnmK. A, crystal structure of apo AnmK homodimer of *P. aeruginosa*. One monomer is colored dark gray, and the other monomer is colored light gray. The top-right panel shows a close-up view of the substrate-binding cavities in the apo structure. The right-bottom panel shows the main changes in the loops L229–241 (anhNAM-binding region) and L8–14 (ATP-binding region) between the apo AnmK (colored in orange and blue, respectively) and the complex with AMPPNP and anhNAM (colored in purple and gray, respectively). B, our four crystallographic structures capture different states of AnmK (Structures I-IV, PDB codes 8BRE, 8CP9, 8C0U, and 8C0U for structures I, II, III, and IV, respectively), and the panels below detail the interactions between AnmK and substrates or products for each structure. Substrates and products are displayed with spheres or capped sticks, polar interactions are represented as dotted lines, and M2 corresponds to a second site for a magnesium ion. The four states are displayed in a different orientation related to panel A, in order to effectively highlight the important changes in the previously mentioned loops. Structure I represents the monomer of apo Catalysis of AnmK.
Catalysis of AnmK

shows the apo AnmK in a closed conformation (Figs. 2A and S6) in which both the ATP- and anhNAM-binding sites are preformed (Fig. 2A, right panels) and show mobility in loops L8–14 (amino acids 8–14) and L229–241 (amino acids 229–241) (Fig. S6). MD simulations (Movie S1) of apo AnmK sampled the open and closed conformations multiple times. Importantly, the individual subsites for anhNAM and for MgATP within the active site experienced opening and closure independently over the course of the simulation. The binding site for MgATP is un gated, whereas the binding site for anhNAM is gated by the loop L229–241 and the β5, β6-hairpin (βH: amino acids 96–110) (Fig. S6). In essence, access to the MgATP subsite is unrestricted, while that of anhNAM is controlled. As we discuss below, the X-ray structures of the ternary complexes along with the MD simulations of these complexes, underscore the importance of the gated access to the anhNAM subsite for catalysis.

The residues of the loop L229–241 and the βH located at the entrance of the anhNAM subsite were dynamic over the entire course of the apo AnmK dynamics simulation. These two regions experienced motions (root-mean-square fluctuation 6–8 Å) that were independent of that observed at the MgATP-binding site (Fig. S7). The opening and closing of anhNAM entry gate were also independent of the open and closed conformations of AnmK. Despite these observations, our simulations did not sample a conformational state in which the ATP-binding site would be closed and the anhNAM-binding site open. This observation is consistent with the random-sequential kinetic mechanism for AnmK, as determined in this study.

Considering that (i) our apo structure presented a closed conformation (Fig. S8A) and that (ii) AnmK was reported previously to adopt an open conformation when bound to AMPPCP or bound to both AMPPCP and anhNAM (14), we tried to obtain AnmK in the open conformation by using AMPPNP, an alternative nonhydrolyzable analog of ATP. However, both the crystal structures of AnmK with AMPPNP (structure II, Fig. 2B) and AnmK with AMPPNP and anhNAM (structure III, Fig. 2B) displayed the protein in a closed conformation (Fig. S8). This leads us to conclude that the open conformations of the earlier structures might be due to the pH in which the crystals were grown. In structure II (Fig. 2B), the anhNAM-binding site presents the L229–241 un gated, while the L8–14 shows a small change in the backbone with side chains of Ser12 and Asp14 oriented differently than in the apo state (box in Fig. 2A). The AMPPNP ligand presents an extended conformation that is stabilized by van der Waals and hydrogen-bonding interactions with the protein (box in Fig. 2B). Interestingly, residues Ser12 and Asp14 play roles in recognizing the α- and β-phosphate moieties of AMPPNP (box in Fig. 2B), something that was not observed in the previous open conformation of the AnmK:AMPPCP complex (PDB code 4MO4, Fig. S8B).

Our structure III of the ternary complex AnmK:AMPPNP:AnhNAM also presented a closed conformation in which the L8–14 interacts with AMPNP (as observed in structure II) and the loop L229–241 undergoes a large conformational change that occludes the anhNAM-binding site in the gated conformation (Fig. 2B). The binding of anhNAM nudges AMPNP to a tighter conformation, observed in chain A of this complex, for the three phosphates with the γ-phosphate of AMPNP within 3.2 Å of the O6 atom of the 1,6-anhydro ring (box in Fig. 2B). It is noteworthy that this conformation for the AMPNP in the presence of anhNAM is tight, and in the chain B of this complex, in which two conformations for the triphosphate moiety (one as in chain A and the other with the γ-phosphate moving away from anhNAM) were observed (Fig. S5B). Again, the full recognition of both the ATP analog and anhNAM observed in our ternary closed complex is not observed in the earlier open AnmK:AMPPCP:AnhNAM structure (PDB code 4MO5)(Fig. S8C).

One crystal structure presented intriguing mechanistic insight. The cocystal of AnmK with MgATP was soaked with the substrate anhNAM (structure IV). While loops L8–14 and L229–241 were disposed similarly to those of structure III (Fig. 2B), the structure of the resulting homodimer revealed that one of the monomers was bound to NAM-6P and MgADP (structure IV, Fig. 2B; Fig. 3, right panel), the products of in crystalllo turnover. Surprisingly, the other protein monomer was captured in complex with the substrate anhNAM and the product MgADP (Fig. 3, left panel). The latter is likely the product of in crystalllo turnover and rebinding of the product ADP. This is consistent with the random-sequential mechanism and the observation of inhibition by ADP, as discussed above. Considering that one could build the structure of MgATP from that of MgADP, in essence, this crystal structure of AnmK reveals in a single snapshot the ternary complexes of the two substrates and the two products bound in the separate active sites of the monomers. The ternary complex with the two products is especially interesting, considering that a repulsive force may be imposed by the proximity of phosphate of NAM-6P to the β-phosphate of ADP. As previously stated (14), a Mg2+ ion is expected between the ATP molecule and Glu326 to coordinate and stabilize the transfer of the γ-phosphate group from ATP to anhNAM, as shown in Figure 1B.

The multiple X-ray structures in Figure 2 define the full catalytic cycle of AnmK. We addressed the interplay between the open and closed conformations in the context of these structures by two target-dynamics simulations. Movie S2 shows the conformational transition from the open to closed states for the ternary complex of anhNAM and ATP.
In the MgATP-binding site, MgATP coordinates residues from both domains. This interaction can be seen as enabling the conformational closing of AnmK. Within the closed conformation, ATP adopts a compact conformation that is accommodated by changes in the loop L8–14. This observation is in agreement with the compact crystallographic conformation of the AnmK:AMPPNP:anhNAM complex (structure III, Fig. 2B). At the end of the simulation, ATP and anhNAM approach each other at a distance of ~3.4 Å. This distance is suitable for catalysis and is similar (~3.2 Å) to that observed in our crystallographic AnmK:AMPPNP:anhNAM complex. The structural alignment of the two substrates, the crystallographic water molecule that attacks the anomeric carbon of anhNAM and the side chain carboxylate of Asp182 that promotes the water molecule, are well documented. The reaction likely goes through an oxocarbenium species. However, as the incoming water molecule is a structurally conserved water, and already poised for capture by the electrophilic species, scrambling of the anomeric carbon is not seen while the product is bound to the active site. Movie S3 illustrates the targeted dynamics for the ternary complex of the two products within the active site spanning the closed-to-open conformations. The key feature in this simulation is the repulsion of the two products leading to the open conformation (along with the loops L229–241 and the βH), which ultimately releases the products. The interactions of the substrates and products with AnmK are summarized in Table S2.

We emphasize the catalytic water molecule coordinated with the catalytic general base Asp182. This water molecule is observed in multiple crystal structures when the anhNAM site is occupied. This water molecule is present and poised for catalysis in the AnmK:ADP:anhNAM, AnmK:AMPPNP:anhNAM, and the simulated structure AnmK:ATP:anhNAM structures (Fig. S9). Its presence—and its orientation—reveal its nucleophilic role in the 1,6-anhydro ring cleavage.

**The effect of annK inactivation on growth and on β-lactam susceptibility**

*P. aeruginosa* MPAO1 can dispense with the recycling pathway. This is evidenced by the ability to grow the strain *annK::Tn* MPAO1, a strain from the Manoil library in which...
Catalysis of AnmK

AnmK was inactivated by transposon insertion (15). The loss of anmK is not lethal. Nonetheless, the transposon-mutant strain does not behave as does the WT background strain. As a means to explore the difference between the two strains, we tested the susceptibility of WT P. aeruginosa (background strain MPAO1) and its transposon mutant (anmK::Tn MPAO1) for the β-lactam antibiotic imipenem by determination of minimal-inhibitory concentration (MIC). MICs were determined by the broth-microdilution method (see Experimental procedures section) (16). The MICs were 4 μg·mL⁻¹ and 0.5 μg·mL⁻¹ for MPAO1 and anmK::Tn MPAO1 strains, respectively. The transposon-inserted strain was 2-fold to 8-fold more susceptible to imipenem compared to the WT.

The growth curves for the two strains were determined in both the absence and the presence of one-half MIC concentration of imipenem in cation-adjusted Müller Hinton broth (CAMHB) (Fig. 4). Growth curves in the absence of antibiotic reveal parity at the outset through the end of the logarithmic phase of the growth (Fig. 4). However, the transposon-mutant strain cannot reach the same levels of growth in the stationary phase. The more interesting aspect of the growth profile is in the presence of imipenem as a representative cell-wall-active β-lactam antibiotic. The mutant strain experience impaired growth from the outset in the presence of the antibiotic, compared to the WT strain. In the presence of sub-MIC imipenem, the mutant strain should, in principle, grow unimpeded, but it would appear to be less able to tolerate the condition. This observation, together with the 2-fold to 8-fold attenuation in MIC for imipenem, argues that an inhibitor of AnmK could serve as a potentiator of β-lactam antibiotics in the treatment of P. aeruginosa infections.

Discussion

Decades of study with the model bacterium E. coli—a Gram-negative rod-shaped bacterium—show that growth of the cell envelope uses simple accretion of its nonpolymeric components (proteins, lipids, lipopolysaccharides). In contrast, complementary studies demonstrate emphatically that the mechanism for the growth of its polymeric component, the peptidoglycan, is more than the simple addition of new monomer to the existing polymer. Rather, peptidoglycan growth requires extensive disassembly of old polymer to allow the insertion of new polymer. The hypothesis presented by Höltje in 1998 remains prescient: it is as if preexisting peptidoglycan polymer structure is used as a template for new peptidoglycan synthesis (17). While the molecular events of such a growth process are hardly better understood today as when Höltje hypothesized, all subsequent experimental studies confirm the presence of robust pathways for the recycling of excised peptidoglycan not just in E. coli, but in other pathogenic Gram-negative bacteria (such as Pseudomonas, Acinetobacter, Stenotrophomonas, and Francisella).

While the pathways within each of these bacteria are distinctive, the pathways have key commonalities (Fig. 1A). One such key is the AnmK-catalyzed conversion of anhNAM to NAM-6P (10, 18).

AnmK is one of two anhydrosaccharide kinases with mechanistic study. The second is the levoglucosan kinase (LGK) of Lipomyces starkeyi, a fungus (19). The sequence identity between E. coli AnmK and LGK is 33.6% (20). Indeed, numerous fungi encode both levoglucosan and anhydroMur kinases, coinciding with speculation that the ability of fungi to recycle bacterial peptidoglycan is as advantageous to them, as it is to bacteria (19, 20). Prior mechanistic study with the L. starkeyi LGK (21) and P. aeruginosa AnmK (9, 14) established a fundamental mechanistic framework. Water addition to the anhydrosaccharide is general-base catalyzed by a protein aspartate, with water addition to the anomeric carbon occurring with inversion; and the 1C₄ conformation of the anhydroglucose progresses through a likely 3H₄ conformation at the transition state to give the saccharide-6-phosphate in an 3S₁ conformation (21). The 3S₃ conformation is characterized by axial configuration for all of the pyranose ring substituents, with the pyranose itself in a skewed conformation. The final and extremely energetically favorable 3S₃ to 4C₁ (all equatorial) conformational relaxation is surmised to occur no later than saccharide-6-phosphate release (9, 14, 21) and (given the estimated magnitude of the free-energy change of ≥32 kJ·mol⁻¹ for this relaxation) (21) could occur even earlier as a driving force for the transition from the closed to open state of the active site.

Our AnmK:ATP:anhNAM crystal structure captures this sequence of conformational state at the intermediate 3S₃ conformation for the product NAM-6P. Similar to the proposed mechanism for the fungal enzyme (21), the AnmK-catalyzed anhNAM reaction also appears to follow a 1C₁ → 3H₄ → 3S₃ conformational itinerary, proceeding through an oxocarbenium transition state (Fig. 5). The 3S₃ conformation is stabilized by important active-site interactions (Table S2), which provide added thermodynamic incentive for this conformational itinerary for the transformation. Our product-bound cocrystal structure provides experimental support for this sequence of conformational states. Furthermore, as seen in our targeted MD, NAM-6P swiftly proceeds to adopt the

![Figure 4. Growth curves of wild-type MPAO1 (blue) and anmK::Tn MPAO1 (red) were determined in both the absence (●) and the presence (▲) of one-half the MIC of imipenem. Cells with transposon-disrupted anmK gene demonstrated impaired growth compared to the wild-type cells under imipenem pressure. Data are presented as mean ± SD of three replicates.](image-url)
Catalysis of AnmK

Figure 5. Mechanistic insights into the AnmK reaction. A, conformational itinerary of the anhNAM → NAM-6P reaction captured by crystallography. The AnmK:AMPPNP:anhNAM complex captures the alignment of the reacting entities. Following the $^1C_4 \rightarrow ^3H_4$ step, we observe the $^5S_1$ conformation for NAM-6P in the AnmKATP:anhNAM complex, indicating that the $^1C_4 \rightarrow ^3H_4 \rightarrow ^5S_1$ itinerary is most likely. The $^5S_1 \rightarrow ^3C_1$ transformation can proceed via the envelope $E_1$ or $^3E$ conformations (21, 48). B, Cremer-Pople spherical coordinates displaying the conformational itinerary of the anhNAM → NAM-6P transformation.

In the apo conformation, our kinetic studies show that either anhNAM or ATP can bind independently of each other. This ability is simulated well by our MD. The binding of anhNAM requires the additional motion of gatekeeper residues and is stabilized by a crystallographic water molecule sequestered between its anomeric carbon and an aspartate within the active site (Fig. S9, Movie S3). This same aspartate is the catalytic general-base of turnover (9, 14, 21). Following closure and catalysis, the combination of an energetically favorable conformational change in the NAM-6P product, per comments above, and other possible unfavorable steric interactions between the two products, facilitate the domain opening required for product release and catalytic turnover. Our crystallographic studies reveal a catalytic importance for magnesium ions. In the AnmK:AMPPNP complex, a second site for another magnesium ion appears coordinating the residue Asp14, the α- and β-phosphates of the AMPPNP molecule, and two water molecules (structure II, Fig. 2B). Previous crystallographic and kinetic studies of the analogous enzyme LGK agree with this observation, an identical magnesium ion coordinates Asp26 (22). Structural alignment of all crystal structures reported in this study with LGK supports the presence of two magnesium-ion-binding sites in AnmK (Fig. S10). Indeed, the utility of two magnesium ions in phosphoryl transfer is of precedent (23).

The presence of a catalytic activity is necessary, but alone is insufficient, to demonstrate that it is advantageous for survival. Strong circumstantial evidence in a Pseudomonas species (Pseudomonas putida) (24) and in Acinetobacter baumannii directly correlate AnmK activity to bacterial virulence (25, 26). Complementary phenotypic studies have not been done yet in P. aeruginosa. Moreover, we alluded earlier to the presence of multiple synthesizing pathways for peptidoglycan. Two pathways are present in P. aeruginosa; a de novo synthesis pathway and a recycling pathway to which AnmK is central (18, 27).

Our evaluation of the susceptibility of P. aeruginosa anmK::Tn MPAO1 cells to the β-lactam antibiotic imipenem demonstrates an enhancement of susceptibility upon anmK disruption (Fig. 4). This observation underscores the centrality of AnmK to peptidoglycan recycling and contribution to inherent antibiotic resistance. Acinetobacter has these same two pathways (28) and possibly a third AnmK-independent pathway (29). How these different pathways may be compensatory; how they contribute individually and collectively to bacterial virulence, antibiotic resistance, and antibiotic tolerance; and how the selection of suitable antibiotic combination strategies can be discerned to impede peptidoglycan recycling are all open research efforts. The foundations to this progress are the understanding of the catalyst(s) of the pathway(s) and the pathway interplay among these catalysts, at a molecular level. Our work builds upon seminal studies by others (9, 14, 21) who reported the first structures for AnmK. We report the full catalytic cycle of this enzyme by identifying the distinct states in catalysis and documenting the transitions among them. These results dovetail with kinetics of AnmK and computation, which disclose the full spectrum of events enabling catalysis.

Experimental procedures
Cloning of the anmK gene

The anmK gene was cloned following a procedure previously described (13). The anmK gene encoding full-length AnmK was amplified from the genomic DNA of P. aeruginosa PAO1 strain using the primers oREF14 and oREF15. An expression vector pMALc6T (New England Biolabs) was amplified using the primers oREF12 and oREF13, followed by assembly of the vector and the insert using NEBuilder HiFi DNA Assembly Kit (New England Biolabs). The resulting plasmid contained an N terminally 6×His MBP fused at the N terminus of AnmK with an intermitting TEV protease cleavage site; 6×His-MBP–TEV–AnmK. Unfortunately, TEV cleavage of the protein expressed from this plasmid was incomplete, so a Gly-Ala-Gly-Ala-Gly sequence...
Catalysis of AnmK

was inserted between the TEV cleavage site and AnmK. This insertion was made by self-ligation of the PCR products amplified with primers oREF38 and oREF39 using KLD Enzyme Mix (New England Biolabs). The final construct would produce 6×His–MBP–TEV–GAGAG–AnmK. The pMALc6T plasmid containing the final construct was used to transform competent *E. coli* LOBSTR-RIL cells for protein production. The primers are collected in Table S3.

Overexpression and protein purification of AnmK

The purification of AnmK followed a reported procedure (13). Transformed *E. coli* LOBSTR-RIL were grown in 1 l of LB medium supplemented with 50 μg·ml⁻¹ ampicillin and 34 μg·ml⁻¹ chloramphenicol with shaking at 37 °C and the absorbance was monitored at 600 nm (*A*₆₀₀). When an *A*₆₀₀ value of 0.6 was reached, the medium was supplemented with 0.3 mM IPTG to induce the production of 6×His–MBP–TEV–GAGAG–AnmK, followed by incubation for 18 h at 16 °C with continued shaking. At the end of the induction process, the cells were harvested by centrifugation at 5500g for 10 min at 16 °C, and the pellet was frozen at −80 °C. The pellet was then thawed and resuspended in a washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol) and 1× protease inhibitor cocktail (APEXBio) and lysed by sonication on ice (10 cycles, 1 min sonication, and 1 min rest) (Branson Sonifier 450, VWR). The cell lysate was centrifuged (18,000 g, 1 h, 4 °C) to remove any unbroken cells, and the supernatant was loaded onto 5 ml of Protino nickel–nitrilotriacetic acid (Ni-NTA) Agarose resin (Macherey-Nagel). The resin was washed extensively with the washing buffer. The protein of interest was cationically bound to the Ni-NTA resin. The fusion protein was diluted with an equal volume of 500 mM. The fractions containing the fusion protein were pooled, and concentrated using Amicon Ultra-15 centrifugation filters (Millipore Sigma; 10-kDa molecular weight cut-off), followed by removal of imidazole. The fusion protein was then eluted using a gradient of imidazole (0–500 mM). The fractions containing the fusion protein were determined by SDS-PAGE, pooled, and concentrated using Amicon Ultra-15 centrifugation filters (Millipore Sigma; 10-kDa molecular weight cut-off), followed by removal of imidazole. The fusion protein was diluted with an equal volume of the cleavage buffer (20 mM Tris–Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol), and TEV protease was added in a 20:1 (protein:TEV) ratio. The reaction mixture was incubated overnight at 4 °C with gentle shaking, followed by loading on 5 ml of fresh Protino Ni-NTA Agarose resin. The flow-through was collected and the resin was washed with the washing buffer three times. The protein of interest (GAGAG–AnmK) was collected from the flow-through and the first washing. The protein was exchanged to the cleavage buffer. Finally, to remove any remaining MBP, the protein was loaded onto Amylose Resin High Flow (New England Biolabs). GAGAG–AnmK was present in the flow-through. The protein was concentrated and buffer-exchanged to the cleavage buffer prior to flash-freezing and storage at −80 °C.

Synthesis of anhNAM

The synthesis of anhNAM (6) was carried out according to a known procedure with minor modifications (8). The modified procedures are provided below. ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE III HD 400 Nanobay (400 MHz) spectrometer in the indicated solvent. Electrospray ionization mass spectra were recorded on a BRUKER micrOTOF II mass spectrometer. Chemical reagents and solvents were purchased from Sigma-Aldrich Inc or TCI America Inc and used without purification. Routine TLC was performed on silica gel 60 F254 plates (Merck). Flash chromatography used silica-gel 60 (spherical, particle size 40–63 μm; Sigma-Aldrich). Chemical shifts are given in ppm and were calibrated on residual solvent peaks as internal standard. Multiplicities were specified as s (singlet), d (doublet), t (triplet), or m (multiplet). ¹H NMR (400 MHz, MeOD) δ 5.17 (s, 1H, H-1), 4.39 (d, *J* = 5.7 Hz, 1H, H-5), 4.18 (q, *J* = 6.8 Hz, 1H, α-Lac), 4.03 (d, *J* = 7.2 Hz, 1H, H-6a), 3.86 (s, 1H, H-2), 3.63 – 3.51 (m, 2H, H-4, H-6b), 3.26 (d, *J* = 17.7 Hz, 1H, H-3), 1.88 (s, 3H), 1.29 (d, *J* = 6.8 Hz, 3H). High-resolution mass spectrometry (ESI): *m/z* 276.107 [M + H]⁺ (exact mass 275.100).

2-acetamido-1,6-anhydro-2-deoxy-a-D-glucopyranose

A solution of 2 (4.26 mmol) in methanol (32 ml) was treated with potassium carbonate (17.05 mmol) at room temperature (3) The solution was stirred at 20 °C for 20 h. The reaction mixture was concentrated to one-third of its original volume, at which point diethyl ether (100 ml) was added and the mixture was cooled to 4 °C overnight. The precipitate was filtered out and discarded and the filtrate was subjected to flash-chromatography purification using a methanol in ethyl acetate gradient (0–10% methanol). The fractions containing 3 were concentrated to remove solvent under reduced pressure at 30 °C. This method provided 3 as a colorless oil in a 25% isolated yield. Purity was adequate for the next step of the synthesis.¹H NMR (400 MHz, D₂O) δ 5.46 (d, *J* = 2.3 Hz, 1H, H-2), 4.06 (dd, *J* = 7.6, 1.1 Hz, 1H, H-6a), 3.84 – 3.78 (m, 2H, H-2 + H-6b), 3.75 (d, *J* = 2.3 Hz, 1H, H-3), 3.67 (m, *J* = 1.9 Hz, 1H, H-4), 2.07 (s, 3H, Me). HRMS (ESI): *m/z* 226.068 [M + Na]⁺ (exact mass 203.079).

Sedimentation-velocity analytical ultracentrifugation

GAGAG–AnmK was buffer-exchanged into 20 mM Tris–HCl pH 7.5, 200 mM NaCl and 1 mM EDTA and the concentration was adjusted to give an *A*₂₈₀ value of 0.12, 0.5, and 1.2 (2.6, 10.7, and 26 μM, respectively). Samples were then spun at 15,000 rpm for 10 min at 4 °C. Sedimentation-velocity experiments were performed in a PROTEOMELAB XL-1 AUC (Beckman) at 42,000 rpm and 4 °C using an AN-50 Ti rotor. Double sector cells equipped with 1.2-cm charcoal-epon centerpieces (Beckman) and sapphire windows were used. Samples were allowed to equilibrate for 2 h prior to starting the run. Absorbance at 280 nm was measured with 0.003-cm radial step size, 1 replicate. AnmK partial specific volume and buffer density and viscosity at 4 °C were calculated using SEDNTERP (30). Data were analyzed using the c(s) distribution and c(M) distribution models in SedFit (31, 32). Data were plotted using GUSSI (33).
Enzymological studies using continuous-coupled assay

Steady-state kinetic parameters of AnmK were evaluated by a continuous-coupled assay, as previously reported (13, 34, 35). Briefly, a mixture containing 100 mM Tris–HCl pH 7.5, 60 μg·ml⁻¹ pyruvate kinase, 32 μg·ml⁻¹ lactate dehydrogenase, 9 mM phosphoenol pyruvate, 0.3 mM NADH, 10 mM MgCl₂, and variable concentrations of ATP and anhNAM were dispensed in half-area 96-well plates (Greiner Bio-One). In the case of evaluation of ATP kinetics, anhNAM was adjusted to 4 mM and anhNAM concentrations ranging 0.03 to 2 mM were used. In case of evaluating the ATPase activity of AnmK, a serial dilution of ATP was used (0.6–90 μM) without any other substrates. The enzymatic reaction was initiated by adding AnmK at a final concentration of 1 μM·ml⁻¹ (10 μM in case of ATPase assay) and bringing the final volume in each well to 90 μl. AnmK was used fresh after production without prior freezing. The reaction was followed for 10 min at room temperature by measuring the absorbance of NADH at 340 nm using an Epoch Microplate Reader (BioTek Instruments). Wells containing all the reaction components except one of the two substrates were used as blank. To calculate the rate of NADH consumption was adjusted to 4 mM and ATP concentrations ranging 0.03 to 2 mM were used. In case of evaluating anhNAM kinetics, ATP concentration was adjusted to 4 mM and anhNAM concentrations ranging 0.03 to 2 mM were used. In case of evaluating ATP kinetics, anhNAM was adjusted to 4 mM and ATP concentrations ranging 0.03 to 2 mM were used. In case of evaluating the kinetic mechanism, different ATP concentrations (0.05, 0.1, 0.2, and 4 mM) and variable anhNAM concentrations (0.03–2 mM) were used. In case of evaluating the ATPase activity of AnmK, a serial dilution of ATP was used (0.6–40 mM) without any other substrates. The enzymatic reaction was initiated by adding AnmK at a final concentration of 1 μM·ml⁻¹ (10 μM in case of ATPase assay) and bringing the final volume in each well to 90 μl. AnmK was used fresh after production without prior freezing. The reaction was followed for 10 min at room temperature by measuring the absorbance of NADH at 340 nm using an Epoch Microplate Reader (BioTek Instruments). Wells containing all the reaction components except one of the two substrates were used as blank. To calculate the rate of NADH consumption, a standard plot of NADH absorbance at different concentrations (39–625 μM) was generated using linear regression ($R^2 > 0.99$). The rate of NADH consumption is equivalent to the rate of anhNAM turnover by AnmK (1:1), therefore, the rate of the AnmK reaction was determined using the slope of the NADH standard absorbance plot. The calculated reaction rates were blank-subtracted. The blank-subtracted rate data was fit to the Michaelis-Menten equation to determine the values of $k_{cat}$ and $K_M$. Each experiment was done in triplicate with two technical replicates. Data analysis was performed using Origin (V. 2022b, OriginLab) (https://www.originlab.com/), while figures and plots were generated using GraphPad Prism 5 (Insight Partners) (https://www.graphpad.com/).

Enzymological studies using two-dimensional isothermal titration calorimetry

To evaluate the kinetic mechanism of AnmK, we attempted a previously reported two-dimensional isothermal titration calorimetry method (12, 13). The sample cell of a MicroCal PEAQ-isothermal titration calorimetry instrument (Malvern Panalytical) was filled with a mixture of 13.5 mM ATP, 100 mM Tris–HCl pH 7.5, 10 mM MgCl₂, and 650 nM AnmK. AnmK was used fresh after production without prior freezing. The reference power was adjusted to 10 μcal/s and stirring was adjusted to 750 rpm and high feedback mode was selected. The sample cell was then equilibrated at 25 °C for 10 min with an additional 5 min of preinjection delay time to allow the complete stability of the baseline. The titrant solution contained 230 mM anhNAM in 100 mM Tris–HCl pH 7.5 and 10 mM MgCl₂. Following equilibration, a preinjection of 0.1 μl of the titrant was made into the sample cell, followed by 18 consecutive injections of 1 μl. Unfortunately, the thermogram signal completely decayed by the sixth injection due to accumulation of ADP and inhibition of AnmK by it (Fig. S4). Although the data produced cannot be utilized for fitting to elucidate the random mechanism by this methodology, it is still clear that ADP accumulation inhibits AnmK.

Antimicrobial susceptibility testing

The MIC of imipenem was determined using the broth microdilution assay on 96-well round-bottom plates following the Clinical and Laboratory Standards Institute guidelines (16). Briefly, the WT MPAO1 and the anmK::Tn mutant MPAO1 P. aeruginosa (15) strains were streaked on LB-agar plates, followed by incubation overnight at 37 °C. Imipenem was prepared in CAMHB and serially diluted with the same volume (100 μl) of CAMHB on the 96-well plates. Several colonies of the strains on the LB-agar plates were picked and resuspended in 3 ml of saline, followed by the measurement of absorbance at 600 nm ($A_{600}$). The $A_{600}$ was adjusted with saline to be 0.01 (1 × 10⁷ colony-forming unit·ml⁻¹) (36). Five microliters of the adjusted bacterial solution were transferred to each well of the 96-well plates to be 5 × 10⁶ colony-forming unit·ml⁻¹. The plates were incubated for 18 h at 37 °C to determine the MIC values.

Growth curves were determined to evaluate the effect of imipenem on the growth rate of the WT MPAO1 and the anmK::Tn MPAO1 cells. The cells were incubated with imipenem at one-half the value of the MIC (4 μg·ml⁻¹ and 2 μg·ml⁻¹ for WT MPAO1 and anmK::Tn MPAO1 strains, respectively) in CAMHB broth with shaking at 180 rpm at 37 °C. The $A_{600}$ was monitored for 22 h (one measurement every 15 min) using a Stratus plate reader (Cerillo). Cells incubated without antibiotic were also monitored in the same manner as a control. All experiments were performed in triplicate. Growth curves were then generated (GraphPad Prism 5, Insight Partners) by plotting the measured $A_{600}$ values against time.

Crystallization

The crystallization of the apo AnmK was performed using sitting drop vapor-diffusion technique at 18 °C with the Oryx crystallization robot (Douglas Instruments Inc). Various commercial screens from Hampton Research, Jena Bioscience, Qiagen and Molecular Dimensions were tested. All crystallization droplets were set up by mixing 200 nl of the protein solution with 200 nl of precipitant solution and equilibrated against 65 μl of precipitant solution in the reservoir. Crystals appeared, typically, after 3 days in several conditions. Crystal size and quality were further optimized by mixing 1 μl of AnmK (7 mg·ml⁻¹) with 1 μl of the precipitant solution and equilibrated against 150 μl of precipitant solution in the reservoir. The best crystals of AnmK apo form grew in the presence of a precipitant solution comprised of 10% PEG 8000, 0.2 M LiCl, and 50 mM MgSO₄. Finally, crystals were dipped into a cryoprotectant buffer composed of the reservoir buffer.
supplemented with 25% glycerol and then flash-frozen in liquid nitrogen.

With the aim of crystallizing different complexes of AnmK with substrates and ligands, we used the co-crystallization technique in which the protein solution was mixed with 10 mM MgSO₄ and 10 mM of ATP or AMPPNP (natural substrate, and nonhydrolysable homolog, respectively) and incubated for 1 h on ice. Then, the mixing solution was equilibrated in a droplet with a precipitant solution (1:1 ratio), that contained 20% PEG 3350, 0.2 M Na/K tartrate, and 0.2 M Li₂SO₄/MgSO₄. Cocrystals appeared after 3 days. To obtain the ternary complexes, cocrystals containing either ATP or AMPPNP were further soaked for 15 to 30 min with a solution of 15 mM anhNAM.

**Data collection and structural determination**

The diffraction datasets were collected at BL13-XALOC beamline at ALBA synchrotron using a Pilatus 6 M detector at a wavelength of 0.97926 Å. All collected images were indexed and integrated using XDS (https://xds.mr.mpg.de/) (37). As previously described for AnmK crystals (9, 14), even though the crystals seemed to belong to the P6₃22 space group, a deep data analysis with phenix.xtriage (38) revealed fractions of twinning. Because of that, the space group was consequently determined to be a P6₃.

For the AnmK apo and AMPPNP/AMPPNP:anhNAM datasets, scaling was performed using AIMLESS from the CCP4 package (https://www.ccp4.ac.uk/) (39). However, the AnmK:ATP:anhnAM dataset, displayed some anisotropy in its diffraction limits, so it was further processed with the STAR-ANISO server from Global Phasing (40). After cutting off the ellipsoidal resolution limits were 2.09, 2.09, and 2.24 Å along the a*, b*, and, c* axes, respectively, and the final resolution was cut to 2.11 Å. All the structures were solved by molecular replacement with Phaser (41) using the structure of the AnmK monomer previously reported (PDB: 3QBW). Two monomers were found in the asymmetric unit with 52 to 55% solvent content. In the complexes, the Fo-Fc map, revealed the presence of extra electron density in the catalytic site corresponding to the ligands. The final structure models were refined with REFMAC5 (42) and PHENIX (43) programs, using the twin refinement intensity based in all steps and translation/libration/screw calculated groups. Manual modelling was done with Coot program (44). Electron density for all substrates and products was of great quality (Fig. S6), allowing even modeling of a double conformation for the triphosphate moiety in one of the monomers of the AnmK:AMPPNP:anhnAM complex (Fig. S6B). As observed previously (9), the electron density for the catalytic Mg⁴⁺ was, in most of the cases, not visible or not very strong. In that case, a water molecule was included at this position. In one of the monomers of the AnmK:AMPPNP complex an electron density compatible with a Mg²⁺ cation was included coordinating Asp14, phosphates α and β, and two water molecules. Geometry and bond distances are characteristic of magnesium coordination with bond lengths between 1.9 and 2.4 Å. Details for all data collection and refinement statistics are presented in Table S1.

**Catalysis of AnmK**

The apo AnmK structure was simulated to observe the conformational sampling of the protein. This crystal structure is a dimer, where monomer B is missing the loop region 229 to 240, while in monomer A this loop is intact. For this reason, monomer A was isolated and used in our simulations. First, monomer A was prepared using the Protein Preparation Wizard (Schrödinger), where bond orders were assigned, hydrogen atoms were added and the protein was energy minimized at pH 7.0 using the OPLS-2005 force field. The protein was then solvated in an octahedral box of TIP3P water molecules, energy minimized and subjected to MD simulations using PMEMD module of AMBER18 and ff99sb force field (45). The PMEMD module energy-minimized the system in successive stages, each of 500 steps of steepest descent, followed by 2000 steps using the conjugate-gradient method and simulation was performed for 100 ns. In Movie S1, loop L229–241 and β5, β6-hairpin (βH: amino acids 96–110) are colored in yellow and red, respectively.

**Targeted MD simulations**

We have conducted two targeted MD simulations to study conformational dynamics associated with the closing and opening of the enzyme upon substrate binding and product release. To obtain enzyme complexes, ligands in existing complexes were manually edited to arrive at substrates or products for the simulation (Table S4). The ligand–protein complexes were then edited using the Prime module (Schrödinger, LLC) to construct missing amino acid side chains and loops. The protein complexes were then prepared using the Protein Preparation Wizard (Schrödinger LLC). The complexes were maintained at pH 7, and energy minimized by applying the following steps: (i) hydrogen atoms were energy-minimized, (ii) restraining the rest of the atoms, and (iii) the entire system was energy minimized without restraints. The system was then equilibrated in five stages, starting with a heating stage, in which the temperature was raised gradually from 0 K to 300 K over 50 ps in NVT ensemble. During the next 50 ps, water molecules were allowed to equilibrate, restraining the protein in order to sufficiently solvate the protein surface cavities. Over a subsequent 300 ps interval, the restraints on the protein residues were gradually released. The final equilibrated system was used for the targeted MD simulation (46). This stage of the simulation was performed using a force constant of 1 kcal/mol Å with linearly decrementing the target RMSD value to zero. Simulation of the trajectory was visualized using the Visual Molecular Dynamics program (45, 47). In Movies S2 and S3, loop L229–241 and β5, β6-hairpin (βH: amino acids 96–110) are colored in yellow and red, respectively, anhnAM, NAM-6P, ATP, ADP, Asp182, and Glu326 are displayed as capped sticks. The reactive water molecule and magnesium ion are displayed as red and magenta spheres, respectively.

**Data availability**

All the data are published in the article. Additional requests for data could be made to the corresponding authors.
Supporting information—This article contains supporting information (8, 14). The crystallographic coordinates are deposited in the Protein Data Bank (PDB codes 8BRE, 8CP9, 8CPB, and 8C0U for structures I, II, III, and IV respectively).

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Abbreviations—The abbreviations used are: 6×His, hexahistidine tag; βH, β-hairpin; AMPPPN, adenosine 5′-(β,y-imido)triphosphate; anhNAM, 1,6-anhydro-N-acetylmuramic acid; Ammk, anhydro-N-acetylmuramic acid kinase; CAMBH, cation-adjusted Müeller Hinton broth; LDK, levoglucosan kinase; LT, lytic transglycosylase; MBP, maltose-binding protein; MD, molecular dynamics; MIC, minimum-inhibitory concentration; NAM, N-acetylmuramylate; NAM-6P, N-acetylmuramic acid-6-phosphate; Ni-TNT, nickel nitritotriacetic acid; TEV, tobacco etch virus.

References


