Molecular Basis of 1,6-Anhydro Bond Cleavage and Phosphoryl Transfer by *Pseudomonas aeruginosa* 1,6-Anhydro-\(N\)-acetylmuramic Acid Kinase**†‡§

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Anhydro-\(N\)-acetylmuramic acid kinase (AnmK) catalyzes the ATP-dependent conversion of the Gram-negative peptidoglycan (PG) recycling intermediate 1,6-anhydro-\(N\)-acetylmuramic acid (anhMurNAc) to \(N\)-acetylmuramic acid-6-phosphate (MurNAc-6-P). Here we present crystal structures of *Pseudomonas aeruginosa* AnmK in complex with its natural substrate, anhMurNAc, and a product of the reaction, ADP. AnmK is homodimeric, with each subunit comprised of two subdomains that are separated by a deep active site cleft, which bears similarity to the ATPase core of proteins belonging to the hexokinase-hsp70-actin superfamily of proteins. The conversion of anhMurNAc to MurNAc-6-P involves both cleavage of the 1,6-anhydro ring of anhMurNAc along with addition of a phosphoryl group to O6 of the sugar, and thus represents an unusual enzymatic mechanism involving the formal addition of H\(_3\)PO\(_4\) to anhMurNAc. The structural complexes and NMR analysis of the reaction suggest that a water molecule, activated by Asp-182, attacks the anomeric carbon of anhMurNAc, aiding cleavage of the 1,6-anhydro bond and facilitating the capture of the \(\gamma\) phosphate of ATP by O6 via an in-line phosphoryl transfer. AnmK is active only against anhMurNAc and not the metabolically related 1,6-anhydro-anhydro-MurNAc and not the metabolically related 1,6-anhydro-MurNAc-oligopeptide. The 1,6-anhydroMurNAc-oligopeptidase (3, 4). The cytosolic amidaspeptide AmpD then processes muropeptides within the cytoplasm by cleaving the stem peptide from the 1,6-anhydro-MurNAc-oligopeptides (5). The resulting anhMurNAc is converted to MurNAc-6-P by AnmK (6) (Fig. 1A), however, whether AnmK can also act on 1,6-anhydro-MurNAc-oligopeptides has not been clarified. The PG degradation products are eventually recycled back to form UDP-MurNAc pentapeptide, a PG precursor that is exported to the periplasm and reincorporated into the cell wall (1, 7).

The bicyclic monosaccharide anhMurNAc is structurally similar to levoglucosan (1,6-anhydro-\(\beta\)-D-glucopyranosan) except that the 2-acetamido and 3-\(\alpha\)-lactyl groups of anhMurNAc take the place of hydroxyl groups on levoglucosan (8, 9). Levoglucosan is formed from the pyrolysis of carbohydrates such as starch and cellulose and is an important molecular marker for pollution from biomass burning (10). Both levoglucosan and anhMurNAc are known to be phosphorylated at O6 of the 1,6-anhydro sugar ring by levoglucosan kinase (LGK) and AnmK, respectively, in a bisubstrate reaction that is believed to result from the enzymatic cleavage of the 1,6-anhydro ring and phosphorylation of O6 (6, 11).

To understand the molecular basis for the recognition of anhMurNAc and related metabolites by AnmK, clarify the substrate specificity of AnmK, and gain insight into this unusual catalytic mechanism, we determined crystallographic structures of AnmK from *Pseudomonas aeruginosa* strain PAO1 in complex with anhMurNAc and ADP. Based on these crystallographic complexes and enzyme kinetic studies, we propose a mechanism wherein the base-catalyzed attack of a water molecule at the anomeric center of anhMurNAc facilitates opening of the 1,6-anhydro ring. ATP is positioned in space such that the adenine portion of the nucleotide is near O1 of anhMurNAc.

During bacterial growth, a considerable amount of \(N\)-acyetylglucosamine-1,6-anhydromuropeptide (tri-, tetra-, and penta-peptide) fragments are excised from the Gram-negative peptido- 

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The abbreviations used are: PG, peptidoglycan; anhMurNAc, 1,6-anhydro-\(N\)-acetylmuramic acid; AnmK, anhydro-\(N\)-acetylmuramic acid kinase; MurNAc-6-P, \(N\)-acetylmuramic acid-6-phosphate; LGK, levoglucosan kinase; PEG, polyethylene glycol; RMSD, root mean square deviation.
the terminal phosphoryl group can be transferred in-line to O6. We also demonstrate that AnmK is active only against free anhMurNAc and not 1,6-anhydro-N-acetylmuramyl peptides, suggesting that the cytosolic N-acetyl-anhydromuramyl-1-alanine amidase AmpD must first remove the stem peptide from these PG muropptide catabolites before the anhMurNAc moiety can be converted to MurNAc-6-P by AnmK.

EXPERIMENTAL PROCEDURES

Expression Plasmid Construction—The anmk gene (NCBI Gene ID: 882072) was PCR amplified from a laboratory stock of *P. aeruginosa* PA01 genomic DNA using Phusion DNA polymerase (New England Biolabs) and oligonucleotide primers 5’-GATATACATATGCGCGGTACCTGGGTT-3’ and 5’-GATATAGGATCCGGCGGATACGGCG-3’ (Alpha DNA, Montreal). The PCR amplicon and a modified version of a pET28 plasmid (Novagen) encoding a reduced multiclone site (Nde1/Spe1/BamH1) and a C-terminal His6 tag, were restricted with Nde1 and BamH1, and ligated using T4 DNA ligase (New England Biolabs). The ligation product was used to transform *E. coli* strain BL21(DE3) GOLD cells (Strategene). The recombinant plasmid, pPAAanmk, was isolated (Qiagen) from a single kanamycin resistant transformant and DNA sequencing confirmed the expected sequence of AnmK and incorporation of an in-frame His6 tag at the 3’-end of the gene.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange Lightning Site-directed Mutagenesis kit (Strategene). The primers used for the D182N mutation were the following: 5’-GAGGCGCCGGTCAACTGCGGCCGCGGC-3’ and 5’-GCCCCGGGCGCAGTTGAAGC-3’. Transformation, sequencing, and expression of the mutated plasmid were performed using the same protocols as described for pPAAanmk.

AnmK Expression and Purification—Recombinant *E. coli* BL21(DE3) GOLD cells harboring pPAAanmk were grown to an optical density of 0.5 at 37 °C, with shaking, in 500-ml volumes of LB media supplemented with 35 µg/ml kanamycin. Expression of AnmK was induced with 1 mM IPTG for 3 h at 28 °C, with shaking. Cells were pelleted by centrifugation and stored at -80 °C. Pellets were thawed in 20 ml of ice-cold lysis buffer (1 M NaCl, 20 mM Tris-HCl, pH 8, and 1 mM PMSF) and lysed using a French pressure cell press (Ameco). The lysate was clarified by centrifugation and loaded onto a Ni-NTA agarose (Qiagen) column that had been pre-equilibrated with binding buffer (1 M NaCl and 20 mM Tris-HCl (pH 8)). The column was washed with 10 column volumes of binding buffer, followed by 10 consecutive column volumes of binding buffer supplemented with 10 mM imidazole and then 25 mM imidazole. The AnmK protein was eluted from the column using binding buffer supplemented with 250 mM imidazole. The eluate was dialyzed overnight against 100 mM NaCl, 20 mM Tris-HCl (pH 8.0) and 0.5 mM DTT. The sample was further purified by gel filtration in 600 mM NaCl, 20 mM Tris HCl (pH 8.0), 0.5 mM TCEP, prior to concentrating and exchanging into crystallization buffer (600 mM NaCl, 20 mM Tris HCl (pH 8.0), 0.5 mM TCEP, 1–2 mM ADP, 2–4 mM MgSO4), using Amicon Ultra-15 concentrators with a 10,000 Da cut-off (Millipore). For the functional studies, ADP and MgSO4 were omitted from the final buffer solution and a NaCl concentration of 100 mM was used. Chromatographic steps were performed using an AKTA FPLC (GE Healthcare). All centrifugation, chromatography, and dialysis steps were conducted at 4 °C.

Crystallization and X-ray Data Collection—AnmK crystals were grown at room temperature using the hanging drop vapor-diffusion method by mixing equal volumes of reservoir buffer (25% polyethylene glycol (PEG) 4000, 0.2 M (NH4)2SO4, and 0.1 M tri-sodium citrate (pH 5.6)) and protein solution (9 mg/ml) in crystallization buffer. Crystals were cryoprotected by dragging them through a drop containing cryoprotectant solution, reservoir buffer supplemented with 9% sucrose (w/v), 2% glucose (w/v), 8% glycerol (v/v), 8% ethylene glycol (v/v)), prior to being flash-cooled in liquid nitrogen. For the AnmK structure in complex with anhMurNAc, crystals grown in the presence of 2 mM ADP were soaked overnight in the equilibrated crystallization/reservoir buffer supplemented with 20 mM anhMurNAc. X-ray diffraction data were collected at beamline 08ID-1 at the Canadian Light Source (Saskatoon, Canada), integrated using XDS (ADP complex) or MOSFLM (anhMurNAc complex) (12, 13) and scaled and merged using SCALA (14) in space group P63.

Structure Determination and Refinement—Phase estimates were obtained by molecular replacement using Phaser and the apo structure of AnmK from *Shewanella oneidensis* as a search model (PDB identifier: 3CQY). The *P. aeruginosa* AnmK model bound to ADP was rebuilt using the PHENIX autobuild routine (15) with the twinning operator: h, -h, k, -l, and this twinning operator was used in all stages of refinement. The AnmK crystal structure in complex with anhMurNAc was determined using the ADP-bound structure as a starting model for rigid body refinement, followed by simulated annealing, prior to being rebuilt using the PHENIX autobuild routine. Further iterative model building and refinement of the protein structures was performed using Coot and REFMAC (16, 17). To minimize model bias in the electron density maps, models for ADP and anhMurNAc were not included in the structure refinement until the protein structure refinement was nearly complete. The ligands were placed using PHENIX Ligandfit (15). Following placement of the ligands, REFMAC-generated omit maps were routinely used to verify the electron density ascribed to both ligands and solvent molecules within the enzyme active site. Final rounds of refinement were performed using Coot and REFMAC and the stereochemical quality of the final models was assessed using MolProbity (18). Refinement statistics are presented in Table 1. All structural figures were prepared using PyMOL (19).

Synthesis of anhMurNAc—The synthesis of anhMurNAc was achieved using a combination of literature procedures (see supplemental Fig. S1) (20–24). The synthesis of 1,6-anhMurNAc-1-Ala-d-Glu-meso-DAP-(d-Ala)-d-Ala and 1,6-anhMurNAc-1-Ala-d-Glu-meso-DAP will be reported elsewhere.

Enzyme Kinetics—Steady-state kinetic studies were performed using a continuous coupled assay similar to that described by Gosselin et al. (25) in which UDP release is coupled to the oxidation of NADH, which can be conveniently monitored by spectrophotometry (340 nm, 6.22 mm⁻¹ cm⁻¹). All assays were carried out in triplicate at 37 °C in a buffered
solution of 50 mM HEPES, pH 7.5, 10 mM MgCl₂, and 50 mM KCl containing 2 mM phosphoenolpyruvate (PEP), 0.4 mM NADH, 10 mM ATP, 9–14 units of lactic dehydrogenase, 6–10 units of pyruvate kinase, and 0.75 μg of AnmK. The reaction mixture was pre-incubated at 37 °C for 3 min and then the reaction was initiated by the addition of 1,6-anhMurNAC, 1,6-anhMurNAC- L-Ala-D-Glu-meso-DAP( D-Ala)- D-Ala or 1,6-anhMurNAC- L-Ala-D-Glu-meso-DAP (ranging from 0.05 $K_m^{app}$ to 30 $K_m^{app}$), bringing the final volume to 200 μl in the quartz reaction vessel. The change in absorbance at 37 °C over a 5 min time period was measured using a Cary 3E spectrophotometer equipped with a Peltier temperature controller.

**NMR Determination of Anomeric Configuration — ¹H-NMR spectroscopy (Bruker Avance III 500-MHz) was used to follow the progress and identify the products of the AnmK-catalyzed reaction.** The reaction was carried out at 17 °C, in 0.7 ml of HEPES buffer (50 mM HEPES, 50 mM KCl, 10 mM MgCl₂), pH 7.4, made up in D₂O containing 6.0 mM anhMurNAC, 5 mM phosphoenolpyruvate (PEP), 0.3 mM ATP, 3–5 units of lactic dehydrogenase, 2–6 units of pyruvate kinase. The reaction was initiated by the addition of 50 μl of a 20.0 mg/ml stock of AnmK. The phosphorylation of anhMurNAC was monitored until the PEP was consumed and the equilibrium between α- and β-anomers of MurNAC-6-P was reached. All enzymes used in this experiment were buffered exchanged by dialysis (5 times) into the deuterated HEPES buffer so as to minimize the intensity of the HOD peak observed at 4.68 ppm in the spectra.

**RESULTS AND DISCUSSION**

**Catalytic Activity and Crystallographic Structures of P. aeruginosa AnmK—** Full-length P. aeruginosa AnmK was expressed as a C-terminal His₆-tagged recombinant protein in *Escherichia coli.* The purified protein was shown to be catalytically active (Fig. 1B) using synthetic anhMurNAC (20–24) (supplemental Fig. S1) as a substrate in an NADH-coupled assay that is similar to that described in reference (25). We established a $K_m^{app}$ of 0.2 mM for anhMurNAC (determined under a constant ATP concentration of 10 mM), which is lower than the 1 mM $K_m$ for anhMurNAC reported for *Escherichia coli* AnmK (1 mM) (6). The basis for this difference is unclear; however, there is notable sequence variation between these enzymes, which are also from different bacteria. We also used synthetic anhMurNAC rather than material isolated from bacteria, which could also contribute to the difference.

We were unable to crystallize *P. aeruginosa* AnmK in the absence of a substrate. However, crystals of the kinase were obtained using the hanging drop vapor diffusion method by mixing equal volumes of AnmK in crystallization buffer that included 1 mM ADP, 2 mM MgSO₄ with reservoir buffer (25% polyethylene glycol 4000, 0.2M ammonium sulfate, and 100 mM tri-sodium citrate (pH 5.6)). In an attempt to observe a ternary complex with anhMurNAC bound, crystals were first grown in the above mother liquor and subsequently soaked for 24 h with 20 mM anhMurNAC. Phases for the diffraction data collected from these crystals were determined by molecular replacement using the *S. oneidensis* apo AnmK structure as the search model (PDB entry: 3CQY). Although the crystals appeared to belong to space group P6₁,22, twinning analyses with phenix.xtriage (15) revealed the possibility of twinning, and the space group was subsequently determined to be P6₁ with the twinning operator (h, h, k, l) and twinning fractions of 42 and 45% for the anhMurNAC and ADP bound structures, respectively. To determine if twinning was affecting our ability to reliably build and refine the AnmK structures, portions of the AnmK model and bound ligands were omitted from refinement to verify that the electron density maps were not being biased by the protein model or ligands. Generation of these omit maps demonstrated that the presence of twinning was not adversely affecting our ability to interpret the experimental data, because clear and unambiguous electron density could be routinely generated in regions where the protein model or ligands (see below) had been omitted. Diffraction and refinement statistics for the ADP and anhMurNAC bound complexes are presented in Table 1.

AnmK is homodimeric, with an extensive buried surface area at the dimer interface: 3100 Å² and 3270 Å² (calculated using PISA analysis of protein interfacing structures (26)) for the AnmK-anhMurNAC and AnmK-ADP structures, respectively (Fig. 2A). The subunits exhibit a topology similar to the ATPase core of proteins belonging to the hexokinase-hsp70-actin superfamily. Each subunit is comprised of two domains, I (residues 1–141, 319–363) and II (residues 152–318), which are separated by a deep cleft that houses the enzyme active site (Fig. 2, B and C). Each domain contains a central β-sheet surrounded by a number of α-helices. The β-sheets of each domain form either side of the central active site cleft and two helices, α6 (residues 142–150) and α15 (residues 322–339), connect the domains together to form the base of the cleft. A β-hairpin within domain I closes off one side of the cleft, creating a deep
1,6-Anhydro-N-acetylMuramic Acid Kinase

TABLE 1
Crystalllographic data and model refinement

|                         | AnmK-ADP (PDB: 3QBW) | AnmK-anhMurNAc (PDB:3QBX) |
|-------------------------|-----------------------|---------------------------|
| **Data Collection**     |                       |                           |
| Space group             | P6_3                  | P6_3                      |
| a = b = c = 90.22,     |                       |                           |
| a = b = 91.48, c = 174.82 |                       |                           |
| α = β = γ = 90°,       |                       |                           |
| α = β = γ = 90°, γ = 120° |                       |                           |
| Wavelength (Å)          | 0.9795                | 0.9795                    |
| Resolution range (Å)    | 46.71–2.23 (2.35–2.23) | 46.68–2.10 (2.21–2.10)    |
| Total observations      | 335432                | 242954                    |
| Unique reflections      | 39204                 | 47575                     |
| I/σ (all)               | 16.7 (4.6)            | 11.3 (3.9)                |
| Completeness (%)        | 100.0 (99.8)          | 99.5 (100.0)              |
| Rmerge (%)              | 0.082 (0.397)         | 0.080 (0.332)             |
| Redundancy              | 8.6 (6.8)             | 5.1 (5.0)                 |
| **Refinement Statistics** |                       |                           |
| Resolution (Å)          | 46.71–2.23            | 40.45–2.10                |
| Reflections (work)      | 37465                 | 45429                     |
| Reflections (test)      | 1691                  | 2055                      |
| Twin fraction           | 0.45                  | 0.42                      |
| Total atoms refined     | 5431                  | 5553                      |
| Solvent                 | 139                   | 166                       |
| Rwork (Rfree)           | 0.19 (0.21)           | 0.19 (0.22)               |
| Mean B values (Å²)      | 35.2                  | 33.9                      |
| Overall                 | 35.2                  | 33.9                      |
| Bound ligand (A/B subunit) | 41.3/56.9           | 28.7/27.1                |
| **RMSDs**               |                       |                           |
| Bond lengths (Å)/angles (°) | 0.009/1.173        | 0.008/1.126               |
| Ramachandran plot       | 96.5/3.2              | 98.6/1.1                  |

* Values in parentheses refer to the high-resolution shell.

Pocket at its base. The homodimer interface is formed exclusively from interactions between domain I of each subunit, where the central β-sheets of these domains align to form a continuous 12-stranded β-sheet that spans across the homodimer and is stabilized by additional helical packing interactions between the subunits.

By performing structure-based alignment using the Dali server (27), we found that AnmK from P. aeruginosa and S. oneidensis belong to the functionally diverse hexokinase-hsp70-actin superfamily of proteins. Proteins comprising this superfamily all have an ATP binding cleft positioned between two structurally conserved domains, which have the capacity to rotate relative to each other in response to substrate binding (28–30). Conformational changes that occur along the reaction coordinate of these enzymes appear to position the ATP phosphate donor and substrate phosphoryl acceptors in optimal orientations to facilitate catalysis, and also likely contribute to defining the substrate specificity of each kinase (29). Although these enzymes show similar overall structural topologies, differences in catalytic mechanism of phosphoryl transfer and substrate specificity have been found within the superfamily. Of the known sugar kinases, AnmK and LGK appear unique in their ability to both cleave the 1,6-anhydro ring and catalyze phosphoryl transfer from ATP to O6 of the sugar substrate.

Following modeling and refinement of the anhMurNAc and ADP ligands into their respective densities of each structure, the ligands and solvent were removed from the asymmetric unit and Fo–Fc omit maps were generated using REFMAC. These maps showed clear density for bound ADP and anhMurNAc in each of the complexes (Fig. 2, B and C). ADP was observed within the entryway of the active site cleft, blocking access to a pocket located at its base, which was assumed to be the anhMurNAc binding site (Fig. 2B). Interestingly, soaking crystals of the AnmK-ADP complex with anhMurNAc resulted in only anhMurNAc bound to the enzyme, binding of which presumably disfavors binding of ADP even though it was present at a concentration of 2–4 mM during the soaking experiments. This seems reasonable since under physiological conditions, binding of ADP product would need to be weak in the presence of substrate to avoid significant product inhibition.

**ADP Complex**—Electron density for the ADP was observed in both subunits of AnmK when crystallized in the presence of ADP alone, although it was noticeably weaker in the B subunit. Magnesium was also included in the crystallization solution since it is a known co-factor for the AnmK reaction (6). However, similar to several other sugar kinase crystal structures that have been determined in complex with ADP (29–31), electron density could not be identified for magnesium within the active site of either subunit of AnmK.

A number of hydrogen-bonding interactions stabilize ADP at the entrance of the AnmK active site cleft (Fig. 2B). The α and β phosphates of ADP protrude deep into the cleft and form hydrogen bonds with the main chain amides of Ser-12, Gly-165, Gly-292, and the side chain of Asp-14 (Fig. 2B). The adenosine and ribose moieties of ADP bind near the cleft opening and form fewer interactions with AnmK than the pyrophosphate moiety. However hydrophobic interactions with Phe-202 and Phe-295 likely stabilize the adenyl moiety, while Asp-191 forms hydrogen bonds with the 2′′-hydroxy group of the ribose unit, which appears to assist in orienting this moiety within the substrate binding cleft (Fig. 2B).

Unlike other members of this enzyme superfamily, AnmK does not appear to undergo large conformational rearrangements when binding either ADP or anhMurNAc. A superposition of the two complexes using the DaliLite server (32) reveals an RMSD of only 0.5 Å (342 α-carbons) and 0.5 Å (355 α-carbons) for the A and B subunits of each structure, respectively. Furthermore, superposing the apo structure of AnmK from S. oneidensis with the ADP bound structure of our P. aeruginosa AnmK structure, reveals an RMSD of 1.1 Å for 346 α-carbons for both subunits, suggesting that the apo form of P. aeruginosa AnmK adopts a conformation similar to that seen for the AnmK-ADP complex.

**anhMurNAc Complex**—Soaking AnmK crystals with anhMurNAc revealed the 1,6-anhydro sugar occupies a well-defined pocket at the base of the active site cleft (Fig. 2C). The amino acids Thr-97 and Arg-129 from domain I, Asp-182 from domain II, and the main chain amides of Leu-142 and Val-143 of helix α6, contribute to an extensive hydrogen-bonding network that positions anhMurNAc within its binding pocket (Fig. 2C). Hydrogen-bonding interactions between the main chain amides of Leu-142 and Val-143 stabilize the carbonyl group of the 3-O-lactyl moiety of anhMurNAc. The side chain of Arg-129 also appears to engage this carbonyl in an electrostatic interaction and also hydrogen bond with the carbonyl oxygen of the 2-acetamido group of anhMurNAc. This carbonyl group forms an additional hydrogen bond with the hydroxyl group of Thr-97. The pyranose ring itself interacts with Asp-182 through a hydrogen bond with O4. Strikingly, at the base of the AnmK active site cleft, a water molecule (Watα) is positioned in line with the C1-O6 bond of the 1,6-anhydro ring of anhMurNAc.
This water molecule is present in both monomers of the dimer (water 376 in the A subunit and 387 in the B subunit of PDB entry 3QBX). It is held in position by a 2.8–2.9 Å hydrogen bond with the carboxyl group of Asp-182 and lies 4.0–4.6 Å from the anomeric center (C1) of the bound anhMurNAc. The mechanistic significance of this observation is discussed below.

The deep pocket into which anhMurNAc binds strongly suggests that AnmK would be unable to accommodate and process the anhMurNAc moieties of either anhMurNAc-pentapeptide or anhMurNAc-tripeptide, two PG recycling intermediates that accumulate in the Gram-negative cytosol during exposure to β-lactam antibiotics (33). Indeed, consistent with the
structure, AnmK exhibited no detectible activity against synthetic anhMurNAC-tripeptide or anhMurNAC-pentapeptide PG catabolites, demonstrating that AnmK can only efficiently process free anhMurNAC. This observation clarifies a distinct and stepwise catabolic pathway that is widely conserved among Gram-negative bacteria. First, cytosolic NagZ must remove the non-reducing GlcNAc unit from imported N-acetylglucosamine-1,6-anhydromuropeptides, after which the amidase AmpD must remove the stem peptide from anhMurNAC. Only after this step can AnmK convert anhMurNAc to MurNAc-6-P.

In P. aeruginosa and most Enterobacteriaceae spp., either anhMurNAc-tripeptide or anhMurNAc-pentapeptide is believed to competitively displace UDP-MurNAc-pentapeptide from a chromosomally encoded LysR-type transcriptional regulator known as AmpR (34) thereby converting the protein into an activator of AmpC β-lactamase production, an enzyme that confers high-level resistance to penicillins and cephalosporins (35). In the absence of β-lactams, the amidase activity of AmpD appears sufficient to reduce the cytosolic concentration of these PG recycling muropeptides to avoid AmpR activation. It is possible that the anhMurNAc moiety of these muropeptides may play a key role in converting AmpR into an activator of ampC gene expression (36) and the presence of the stem peptide simply restricts AnmK from converting the anhMurNAc moiety into MurNAc-6-P. Genetic inactivation of anmK offers an obvious means of testing this hypothesis, however, Uehara et al. (6) found that inactivation of the anmK gene in E. coli resulted in very little anhMurNAc accumulation in the cytosol, observing instead that the compound is rapidly exported from the cell into the growth medium. Thus, the presence of the stem peptide not only appears to retain anhMurNAc within the cytosol, it also appears to restrict AnmK from catalyzing the conversion of anhMurNAc to MurNAc-6-P. Accordingly, AnmK may have evolved selectivity for stem peptide-free anhMurNAc in order to avoid compromising fitness through inappropriate inactivation of AmpR inducer molecules.

The AnmK catalyzed ring opening and phosphorylation of anhMurNAc to MurNAc-6-P also may ensure that this product remains within the cytosol (6). In E. coli, the 3-O-lactyl group of MurNAc-6-P is removed by MurQ following phosphorylation to form GlcNAc-6-P (37). However, MurQ is not strongly conserved and is not encoded in P. aeruginosa (1), suggesting that the product of the AnmK reaction, MurNAc-6-P, is salvaged via an alternate route in this organism.

Catalytic Mechanism—To gain insight into the unusual catalytic mechanism of AnmK, the ADP and anhMurNAc bound structures were superimposed to reveal the relative positions of ADP and the anhMurNAc sugar within the active site cleft. In the AnmK-anhMurNAc complex, two water molecules in the B subunit, WatB and WatC, (waters 409 and 419 in the B subunit of PDB entry 3QBX), form hydrogen bonds with O6 of anhMurNAc and the carboxylate side chain of Glu-326, while a sulfate ion is present in the structure at the same position as the β phosphate of the corresponding ADP-bound structure (Fig. 3A). This ion is bound through a similar set of interactions as observed for the β phosphate observed within the ADP-AnmK complex. We postulate that the WatB and WatC waters occupy a similar position as two of the oxygen atoms of the γ phosphate of ATP. The γ phosphate of ATP was thus modeled, using the ADP-bound structure as a template, with two of the phosphate oxygens near in space to the positions of WatB and WatC (Fig. 3B). This model predicts that the γ phosphate of ATP lies approximately 3.0 Å from O6 of anhMurNAc, placing the two atoms in an ideal position for an in-line phosphoryl transfer, as has been predicted for other kinases (38). Despite efforts to observe magnesium in the structures, this ion was not visible in our crystallographic complexes. It is known, however, to be essential for catalytic activity of AnmK (6) and we expect that it would be positioned within the active site to stabilize the developing negative charge of the pentacoordinate transition state structure of the terminal ATP γ-phosphate moiety during transfer. In this regard, the conserved cationic residue, Arg-238, is positioned on a flexible loop close to the active site and is close enough to enable its rearrangement and interaction with the γ phosphate of ATP. This residue could also therefore aid phosphoryl transfer by stabilization of charge development in the transition state.

Notably, Asp-182 is conserved in AnmK and LGK enzymes (supplemental Fig. S2) and is ideally oriented to act as a general base to activate a strategically positioned water molecule (WatA) observed to lie within striking distance (4.0 Å in the B subunit) of the anomeric center of anhMurNAc (Figs. 2C, A, B, and B). Attack of water at the anomeric center would promote expulsion of O6 and consequent opening of the 1,6-anhydro ring (Fig. 4). Interestingly, the conformation adopted by the 1,6-anhydro ring results in the C1-O6 scissile bond adopting a pseudo-axial orientation in a manner reminiscent of that observed for glycoside substrates bound within the active site of certain glycoside hydrolases (Fig. 2C) (39–41). This conformation enables in-line attack of the anti-bonding orbital at the anomeric center and also places the nonbonding lone pair of electrons on O5 in a favorable antiperiplanar arrangement with the anhydro bond thereby enabling O6 to capture the γ phosphate of ATP via an associative in-line phosphoryl transfer reaction. Consistent with the proposed mechanism, mutation of Asp-182 to asparagine produced a mutant enzyme with undetectable catalytic activity. Indeed, anhMurNAc concentrations as high as 5 mM did not result in any product formation when incubating large amounts of the mutant enzyme with substrates over a 30-min assay period.

The proposed attack of water and resulting cleavage of the 1,6-anhydro bond by AnmK would necessitate inversion of stereochemistry at the anomeric center of MurNAc; from a β- to an α-configuration. Thus, we expect that AnmK generates α-MurNAc-6-P and ADP as the first formed products (Fig. 4). To test this hypothesis, the 1H-NMR analysis of the enzyme-

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**1,6-Anhydro-N-acetylmuramic Acid Kinase**

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**Catalytic Mechanism**

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**AnmK**

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**Structure**

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**Mechanism**

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**Conformation**

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**Analysis**

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catalyzed hydrolysis of anhMurNAc by AnmK (Fig. 5) was carried out and revealed the initial appearance of the α-anomer of MurNAc-6-P (initial measurable ratio of 7 to 1; α- to β-anomer). This finding is consistent with a catalytic mechanism resulting in inversion of the anomeric center. The slightly slower appearance of the β-anomer of MurNAc-6-P (final ratio of 3.7 to 1; α- to β-anomer) likely stems from mutarotation of MurNAc-6-P.

Based on our data, two mechanistic scenarios can be envisioned as to how AnmK catalyzes opening of the 1,6-anhydro ring and phosphoryl transfer from ATP to O6 of anhMurNAc. The first scenario is a one step reaction, in which opening of the 1,6-anhydro ring and phosphoryl transfer occur in a concerted manner (Fig. 4). Here, the O6 atom becomes more nucleophilic as cleavage of the 1,6-anhydro bond occurs, enabling it to accept the γ phosphorus of ATP. Since opening the 1,6-anhy-
hydro ring is formally the cleavage of a glycosidic bond, it is useful to consider what is known for the superfamily of inverting glycoside hydrolases. For these enzymes, cleavage of glycosidic bonds is accelerated by general acid catalysis furnished by a strategically positioned enzymic residue that interacts with the glycosidic oxygen. For AnmK however, presence of the phosphate may obviate the need for such a residue, because the phosphate could instead act as an electrophilic group to directly stabilize the negative charge that develops on O6 in the transition state. Moreover, the thermodynamically unfavorable all axial conformation of the 1,6-anhydro ring may contribute significant strain within the substrate, resulting in its destabilization and thereby facilitating 1,6-anhydro bond cleavage and capture of the phosphate of ATP by O6 (Fig. 4).

Alternatively, the reaction may proceed in two discrete steps. The first step would involve opening of the 1,6-anhydro ring as described above but lead to the formation of MurNac as a discrete intermediate. The second step of the reaction would involve base-catalyzed in-line attack of the MurNac O6 atom onto the γ phosphate, leading to formation of MurNac-6-phosphate. These two steps could be aided by an amino acid residue positioned within hydrogen bonding distance of O6, which might serve as a general acid-base catalytic group. Glu-326 is a possible candidate; however, it is somewhat distant to O6 (4.6 Å). Nevertheless, it is possible that small structural rearrangements occurring during catalysis could allow the residue to interact productively with O6 of the substrate. For a two-step mechanism, the result of the first step would be the transient formation of an intermediate, MurNac, within the active site.

The atomic coordinates and structure factors have been deposited to the Protein Data Bank under entries 3QBW (AnmK-ADP complex) and 3QBX (AnmK-anhMurNac complex).

**CONCLUSIONS**

We have determined the crystal structures of *P. aeruginosa* AnmK in complex with ADP and 1,6-anhydro-N-acetylmuramic Acid Kinase.
ramic acid and performed functional studies to elucidate the molecular basis for the conversion of cell wall derived \textit{an}MuN\textit{Na}c to MuN\textit{a}c-6-P. These studies provide a foundation for a mechanistic understanding of the apparent dual activities of AnmK as a glycoside hydrolase and a kinase. Our work provides the basis for future studies that will further clarify details of the catalytic mechanism and the role of various active site residues. It is interesting to consider that the diverse range of naturally occurring carbohydrates has given rise to the evolution of diverse active site architectures among sugar kinases. Although these enzymes share a similar structural fold, they have diversified over time to catalyze the phosphorylation of a variety of substrates. The unusual catalytic mechanism of AnmK provides an extraordinary example of this evolution and highlights the diversity of phosphoryl transfer carried out by the family of sugar kinases.

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