Conformational Itinerary of *Pseudomonas aeruginosa* 1,6-Anhydro-\(N\)-acetylMuramic Acid Kinase during Its Catalytic Cycle*

**Background:** AnmK is an unusual sugar kinase that both cleaves and phosphorylates its 1,6-anhMurNAc substrate. Ligand binding at the active site of AnmK coordinates its conformational itinerary. **Conclusion:** Ligand binding at the active site of AnmK coordinates its conformational itinerary. **Significance:** The unique dual activities of AnmK coupled with its complex structural dynamics advance our understanding of the diverse reactions carried out by sugar kinases.

Anhydro-sugar kinases are unique from other sugar kinases in that they must cleave the 1,6-anhydro ring of their sugar substrate to phosphorylate it using ATP. Here we show that the peptidoglycan recycling enzyme 1,6-anhydro-\(N\)-acetylMuramic acid kinase (AnmK) from *Pseudomonas aeruginosa* undergoes large conformational changes during its catalytic cycle, with its two domains rotating apart by up to 32° around two hinge regions to expose an active site cleft into which the substrates 1,6-anhydroMurNAc and ATP can bind. X-ray structures of the open state bound to a nonhydrolyzable ATP analog (AMPPCP) and 1,6-anhydroMurNAc provide detailed insight into a ternary complex that forms preceding an operative Michaelis complex. Structural analysis of the hinge regions demonstrates a role for nucleotide binding and possible cross-talk between the bound ligands to modulate the opening and closing of AnmK. Although AnmK was found to exhibit similar binding affinities for ATP, ADP, and AMPPCP according to fluorescence spectroscopy, small angle x-ray scattering analyses revealed that AnmK adopts an open conformation in solution in the absence of ligand and that it remains in this open state after binding AMPPCP, as we had observed for our crystal structure of this complex. In contrast, the enzyme favored a closed conformation when bound to ADP in solution, consistent with a previous crystal structure of this complex. Together, our findings show that the open conformation of AnmK facilitates binding of both the sugar and nucleotide substrates and that large structural rearrangements must occur upon closure of the enzyme to correctly align the substrates and residues of the enzyme for catalysis.

The bacterial peptidoglycan cell wall is a mesh-like heteropolymer of alternating \(\beta1,4\)-linked GlcNAc and \(N\)-acetylMuramic acid (MurNAc) glycan strands that are cross-linked by short peptides (1, 2). Although the cell wall defines cellular shape and is necessary to resist intracellular turgor pressure, it is a remarkably dynamic structure that is continuously rebuilt as bacteria grow and divide. In Gram-negative bacteria, nearly 50% of the cell wall is turned over per generation by the combined action of amidases and lytic transglycosylases, which excise and release GlcNAc-\(\beta1\to4\)-1,6-anhydroMurNAc peptide fragments into the periplasmic space (3–5). The fragments are transported into the cytosol where they are catabolized and recycled. The terminal GlcNAc sugar is removed by the cytosolic glycosidase NagZ to produce free GlcNAc and 1,6-anhydroMurNAc peptides (5). The amidase AmpD then removes the peptide stem from the 1,6-anhydroMurNAc (anhMurNAc) sugar (6). Removal of the peptide allows the sugar kinase AnmK to convert anhMurNAc into MurNAc-6-phosphate (Fig. 1) (7, 8). The peptide and sugar catalytics are subsequently recycled into the synthesis of UDP-MurNAc-pentapeptide, a precursor of the lipid-anchored disaccharide-pentapeptide (lipid II) molecule that is reinserted into the cell wall (see Ref. 5 for a comprehensive review).

ATP-dependent phosphorylation of carbohydrate substrates by sugar kinases of the hexokinase-hsp70-actin superfamily is an essential step for the utilization of sugar in a multitude of metabolic functions in both eukaryotes and prokaryotes (9). AnmK and the functionally related enzyme levoglucosan kinase occurr upon closure of the enzyme to correctly align the substrates and residues of the enzyme for catalysis.
form a subfamily of sugar kinases that are capable of phosphorylating the O6 oxygen of pyranose sugars containing a 1,6-anhydro ring. Although AnmK adopts a two-domain fold that is structurally similar to proteins of the hexokinase-hsp70-actin superfamily, 1,6-anhydrosugar kinases are mechanistically unique in that they catalyze both the hydrolysis of the 1,6-anhydro ring and the transfer of the γ-phosphate group from ATP to O6 of sugar substrates (7). Previously determined crystal structures of AnmK from Pseudomonas aeruginosa bound to anhMurNAc and ADP revealed that the sugar and nucleotide bind to the protein at a deep active site cleft located between the two domains comprising the enzyme (8). An aspartate residue (Asp-182) at the bottom of the cleft was identified as the enzymatic base that catalyzes the attack of a water molecule on the anomeric carbon (C1) of anhMurNAc, thereby promoting cleavage of the 1,6-anhydro bond and transfer of the γ-phosphate of ATP to the O6 oxygen of the sugar (Fig. 1). The location of Asp-182 and associated water nucleophile suggested that hydrolysis of the 1,6-anhydro bond by the water would invert the anomeric configuration of MurNAc. Transient formation of an intermediate (Asp-182) at the bottom of the cleft was identified as the enzymatic base that catalyzes the attack of a water molecule on the anomeric carbon (C1) of anhMurNAc, thereby promoting cleavage of the 1,6-anhydro bond and transfer of the γ-phosphate of ATP to the O6 oxygen of the sugar (Fig. 1). The location of Asp-182 and associated water nucleophile suggested that hydrolysis of the 1,6-anhydro bond by the water would invert the anomeric configuration of the sugar, which was confirmed by NMR analysis of the MurNAc-6-phosphate product (8). Mutation of Asp-182 to asparagine reduced AnmK activity to an undetectable level, which further confirmed the importance of this residue in the hydrolysis of the 1,6-anhydro bond during phosphoryl transfer (8).

To determine the conformational itinerary of AnmK during its catalytic cycle, we investigated whether the enzyme could also adopt an open conformation before it closes into the catalytically competent conformation. Using a nonhydrolyzable ATP analog (AMPPCP), we were able to stabilize an open conformation of the enzyme that was amenable to crystallization and demonstrate that the two domains of AnmK rotate apart by as much as 32° to expose a large active site cleft within which the ATP analog binds. This conformational change was also confirmed in solution by applying small angle x-ray scattering analyses. Interestingly, it was also possible to soak crystals of the AnmK-AMPPCP complex with the 1,6-anhydroMurNAc sugar substrate and determine the structure of AnmK bound to both substrates in the open state. Taken together, we demonstrate that AnmK presents an open and accessible active site cleft that can bind both the sugar and nucleotide prior to closure to align the substrates and active site residues for catalysis.

**EXPERIMENTAL PROCEDURES**

AnmK Expression and Purification—AnmK from P. aeruginosa was produced in Escherichia coli strain BL21(DE3) GOLD (Stratagene) using the previously described expression plasmid pPAanmk (8). Cells were grown at 37 °C, with shaking, in 500-ml volumes of liquid LB medium supplemented with 35 μg/ml kanamycin. Once the cell density reached an A600 of ~0.5, the culture was supplemented with isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM and incubated for an additional 3 h at 28 °C, with shaking, to induce expression of AnmK. Cells were then pelleted by centrifugation and stored at −80 °C. Pellets were thawed in 20 ml of ice-cold lysis buffer (0.5 M NaCl, 20 mM Tris, pH 8, 0.5 mM PMSF, 2 mM imidazole) and lysed using a French pressure cell press (American Instrument Company). The lysate was clarified by centrifugation and mixed with 2 ml of TALON metal affinity resin (Clontech) with gentle shaking for 30 min on ice. The TALON resin was centrifuged and resuspended in binding buffer (0.5 M NaCl, 20 mM Tris, pH 8.0) supplemented with 2 mM imidazole before being poured into a 20-ml gravity column. The column was washed...
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with 20 ml of binding buffer supplemented with 5 mM imidazole (Sigma), followed by 20 ml of binding buffer supplemented with 10 mM imidazole. AnmK was eluted from the column with 10 ml of binding buffer supplemented with 250 mM imidazole. The eluate was dialyzed overnight against 20 mM Tris, pH 8.0, 100 mM NaCl, 0.5 mM DTT. The protein was further purified by gel filtration (Superdex 75) in 20 mM Tris, pH 8.0, 100 mM NaCl, 0.5 mM Tris(2-carboxyethyl)phosphine prior to concentration using an Amicon Ultra-15 concentrator with a 10,000-Da cutoff (Millipore). Gel filtration was performed using an AKTA FPLC (GE Healthcare) at 4 °C.

AnmK Crystallization, Data Collection, and Structure Determination—AnmK crystals were grown at room temperature using the hanging drop vapor diffusion method. For the structure of AnmK bound to the nonhydrolyzable ATP analog AMPPCP, equal volumes of mother liquor (26% PEG 4000, 0.2 M MgCl2, 0.1 M Tris, pH 9.0) were mixed with AnmK (at 6.5 mg/ml in 600 mM NaCl, 2 mM AMPPCP, 4 mM MgCl2, 0.5 mM TCEP, 20 mM Tris, pH 8.0). For the structure of AnmK bound to AMPPCP and the anhMurNAc sugar, AnmK was first co-crystallized with AMPPCP by mixing equal volumes of mother liquor (24% PEG 4000, 0.2 M MgCl2, 0.1 M Tris, pH 8.2) with the enzyme (at 6.5 mg/ml in 600 mM NaCl, 2 mM AMPPCP, 4 mM MgCl2, 0.5 mM TCEP, 20 mM Tris, pH 8.0). A single crystal of the AnmK-AMPPCP complex was then transferred to a drop of reservoir buffer supplemented with 8 mM anhMurNAc, 2 mM AMPPCP, and 4 mM MgCl2 and soaked for 1 h. 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), and 8% ethylene glycol (v/v)), prior to being flash-cooled in liquid nitrogen. X-ray diffraction data were collected at Beamline 08ID-1 at the Canadian Light Source (Saskatoon, Canada), integrated using XDS (14), and scaled and merged using SCALA (15).

Phase estimates were obtained by molecular replacement using PHASER (16) and the structure of AnmK from P. aeruginosa as a search model (PDB code 3QBW). To locate the two domains of each AnmK monomer, each domain was used as an independent search model and delineated as domain 1 (residues 2–149 and 320–362) and domain 2 (residues 151–319). AnmK crystallized as a homotetramer, and thus a total of eight domains needed to be located to complete the tetrameric structure and fully define the crystallographic asymmetric unit. The molecular replacement model was subsequently rebuilt using the PHENIX autobuild routine (17) and further optimized using iterative rounds of rebuilding and refinement using Coot (18) and PHENIX (17), respectively. TLS partitions describing domain motions were determined using the TLSMD server (19), and these partitions were used throughout refinement. The structure of AnmK in complex with AMPPCP was used as the starting model for the structure bound to both AMPPCP and anhMurNAc and, following rigid body refinement, refined in a similar fashion. To minimize model bias, the AMPPCP and anhMurNAc ligands were not included in the structural models until their refinements were nearly complete. Ligand restraints were generated using the programs GRADE and MOGUL (20).

Following placement of the ligands, PHENIX-generated omit maps were routinely used to verify the electron density ascribed to both ligands and solvent molecules within the enzyme active site. The stereochemical quality of the final models were assessed using MolProbity (21). Domain motions and analyses of hinge regions was performed using DYNDOM (22). Protein superpositions were performed using the CCP4 program, SUPERPOSE (23). All structural figures were prepared using PyMOL (24).

Fluorescence Spectroscopy—steady-state fluorescence spectra were measured on a Fluorolog-3 Horiba Jobin Yvon spectrophotometer (Edison, NJ). The sample was held in a 10 × 3-mm² quartz cuvette. The cuvettes were thermostatted using a water bath (JEIO TECH, Seoul, Korea). The fluorescence data were analyzed with Sigma Plot (Point Richmond, CA) software. All samples contained 1 μM protein dissolved in 50 mM MOPS buffer, pH 7, containing 100 mM NaCl and 10 mM MgCl2; all samples were prepared in triplicate. Fluorescence spectra were collected at 290-nm excitation, and the excitation and emission slits were set at 1.5- or 2-nm band pass resolution.

The binding of the fluorescent N'-methylthraniloyl substituted ATP analog (MANT-ATP) to AnmK was characterized by monitoring the quenching of the intrinsic fluorescence of the protein by MANT-ATP at 340 nm. Protein fluorescence quenching was corrected for inner filter effects at the excitation and emission wavelengths. If the ligand binds to the protein through simple hyperbolic behavior, the fraction of MANT-ATP-bound protein can be defined as follows,

\[
\text{Fraction Bound} = \frac{F - F_0}{F_n - F_0} = \frac{[\text{ligand}]}{K_d^\text{MANT-ATP} + [\text{ligand}]} + n[\text{ligand}]
\]

where \( F \) is the intrinsic protein fluorescence measured at a certain formal concentration of MANT-ATP ligand, \( F_0 \) is the protein fluorescence when there is no ligand present, \( F_n \) is the protein fluorescence measured at infinite ligand concentration, the constant \( K_d \) characterizes the binding of MANT-ATP to protein, and the constant \( n \) corrects for any nonspecific interactions existing between probe and protein.

The displacement fluorescence assay was based on a single-site displacement model in which nucleotides displace protein-bound MANT-ATP molecules. In this case, the following equilibria compete.

\[
K_d^\text{MANT-ATP} = \frac{[\text{MANT-ATP}][\text{AnmK}]}{[\text{AnmK} - (\text{MANT-ATP})]}
\]

\[
K_d^\text{nucleotide} = \frac{[\text{nucleotide}][\text{AnmK}]}{[\text{AnmK} - \text{nucleotide}]}
\]

If the fluorescence (at 440 nm) of a given sample containing AnmK and MANT-ATP is measured to be \( F_{(0)} \) and the fluorescence of the same solution measured at infinitely high nucleotide concentration is \( F_{(-)} \), we can define a value of \( FC_{\text{ap}} \) which corresponds to the total concentration of nucleotide added that results in a fluorescence intensity value midway between \( F_{(0)} \) and \( F_{(-)} \). The value of \( K_d^\text{nucleotide} \) can then be calculated from...
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TABLE 1
Crystallographic data processing and refinement statistics

| Protein (ligand) | AnmK AMPPCP (PDB code 4MO4) | AMPPCP/anhMurNAc (PDB code 4MO5) |
|-----------------|--------------------------|--------------------------|
| **Data collection** | | |
| Space group | P1 | P1 |
| Unit cell (Å) | 44.86–1.67 | 44.81–1.75 |
| Wavelength (Å) | 0.979 | 0.9795 |
| Resolution range (Å) | (1.76–1.67) | (1.84–1.75) |
| Total observations | 504,436 | 391,372 |
| Total unique observations | 151,753 | 130,828 |
| Completeness (%) | 97.2 (95.2) | 97.5 (96.2) |
| Rmerge | 0.050 (0.593) | 0.059 (0.493) |
| Redundancy | 3.3 (3.3) | 3.0 (3.0) |
| **Refinement statistics** | | |
| Resolution (Å) | 44.86–1.67 | 44.81–1.75 |
| Reflections (total) | 151,721 | 130,811 |
| Solvent | 7765 | 6689 |
| Total atoms refined | 11,707 | 11,637 |
| Rwork (Rfree) | 0.177 (0.213) | 0.174 (0.210) |
| Mean B values (Å²) overall | 10.0 (2.2) | 10.0 (2.2) |
| Root mean square deviations (bond lengths (Å)/angles (°)) | 0.007/1.160 | 0.007/1.153 |
| Ramachandran plot (favored/allowed (%)) | 98.6/1.1 | 98.3/1.4 |

* The values in parentheses refer to the high resolution shell.

$F_{C_{50}}$ by simultaneously solving for the two competing equilibria:

$$K_{d}^{\text{nucleotide}} = \frac{F_{C_{50}}}{1 - \frac{[\text{MANT-ATP}]_{\text{total}}}{K_{d}^{\text{MANT-ATP}}}}$$  \hspace{1cm} \text{(Eq. 4)}$$

In this equation, $[\text{MANT-ATP}]_{\text{total}}$ is the total concentration of fluorescent probe. Equation 2 is valid when: 1) the concentration of total protein is much less than that of the ligands, and 2) $K_{d}^{\text{MANT-ATP}}$ is more than a factor of 4 larger than that of the fluorescent probe concentration.

Small Angle X-ray Scattering—Prior to SAXS data collection, the quality of samples was checked using a dynamic light scattering instrument (Malvern Instruments) as previously described (25). The solution x-ray scattering data for AnmK alone and AnmK bound with ADP or AMPPCP were collected at 2 mg/ml as described previously (26) using an in-house Rigaku instrument for 3 h. Primary data analysis was performed using the program PRIMUS (27) followed by application of program DAMMIN (28) to calculate low resolution *ab initio* models. For all three systems, multiple models were calculated which were rotated, aligned, and filtered using the program DAMAVER (29). The quality of individual models was assessed by χ values for each model.

RESULTS

Crystal Structure of AnmK in the Open Conformation Bound to an ATP Analog—AnmK exists as a homodimer in solution, and similar to other hexokinases, each monomer is comprised of two distinct domains, with each domain composed of a central β-sheet surrounded by helices. The domains are separated by a deep active site cleft region that binds ATP and the anhMurNAc sugar substrates. We previously determined the crystal structure of AnmK in a closed state, whereby the two domains were near together and the active site cleft was occupied by either ADP (bound near the entrance of the cleft) or anhMurNAc (bound at the base of the cleft) (8). We were, however, interested in the possibility that AnmK could also exist in an open conformation similar to other hexokinases, and thus we continued to pursue new crystallization conditions to capture the enzyme in such a conformation. We considered the possibility that a nonhydrolyzable ATP analog could stabilize AnmK in an open state. After extensive screening, conditions for crystallizing AnmK in an open conformation were discovered by co-crystallizing the enzyme in the presence of the nonhydrolyzable ATP analog AMPPCP (Table 1 and Fig. 2A). The structure was determined using molecular replacement by separating the two domains of the previously determined closed AnmK structure and using these as separate search models. A dramatic difference in the relative orientation of domains 1 and 2 was observed in the AMPPCP-bound structure of AnmK compared with AnmK bound to either ADP or anhMurNAc. In the AMPPCP-bound structure, the two domains rotate apart by as much as 32° (relative to closed structures) to expose the active site cleft, clearly revealing that the enzyme binds AMPPCP in an open conformation (Fig. 2A).

The two monomers comprising the AnmK homodimer interact through an extensive protein-protein interface that is a continuous β-sheet that spans both monomers. Interestingly, the crystallographic asymmetric unit of the AnmK-AMPPCP complex contains two copies of the AnmK dimer, resembling a tetrameric structure; however, analysis using PISA (30) revealed that the buried surface area between the AnmK dimers is relatively small and is likely to be comprised of only crystallographic contacts. Comparing the open and closed structures of AnmK revealed that the contacts formed within the dimer interface are unaltered between the two conformational states. The interface is comprised exclusively of residues from domain 1 of each monomer. Because domain 1 is firmly locked in a dimeric interface with the opposite monomer, the domain...
motion is characterized by domain 2 of each monomer swinging outwards away from the dimer interface (Fig. 2A). This rotation is asymmetric relative to each AnmK dimer, with rotations of 25 and 20° in monomers A and B and of 30 and 27° in monomers C and D of the asymmetric unit.

Two hinge regions responsible for modulating the conformation of AnmK were identified at the base of the active site cleft: residues 144–149 (hinge region 1) and residues 324–327 (hinge region 2). The involvement in protein domain motion was corroborated by computational analysis using DYNDOM, a program that identifies hinge regions within conformationally flexible proteins (22) (Fig. 2B). Superposing the closed and open conformations of AnmK monomers clearly shows how the two domains of the enzyme move about these hinge regions (Fig. 2B). Interestingly, hinge region 2 forms a region at the base of the active site cleft that participates in binding to the ATP analog (Fig. 2C), which also allows for the possibility of nucleotide interactions with residues comprising the hinge region to affect the conformational dynamics of AnmK.

The closed state of the AnmK-ADP complex is defined by the close packing of the two domains of each AnmK monomer (8). This creates a narrow active site cleft, which allows for multiple binding interactions between the ADP and the protein. In contrast, the open conformation of the AnmK-AMPPCP complex contains fewer interactions between the nucleotide and enzyme. For example, the hydrogen bonding interactions observed between residues Ser-12 and Asp-14 and the β-phosphate of ADP are not observed between AnmK and AMPPCP in the open state. Although there are fewer interactions between AnmK and AMPPCP versus ADP, the electron density for the adenylic moiety of AMPPCP is well defined, suggesting that it is bound at high occupancy to the enzyme through the same interactions as the analogous moiety of ADP (Fig. 2C) (8). The ribose ring interacts through hydrogen bonding with residues Asp-191 and Asp-205 of domain 2, and additional hydrophobic interactions also occur between the adenyl and ribose sugar moieties of the AMPPCP molecules. However, main chain hydrogen bonding interactions with Gly-165 and Gly-192 do stabilize the phosphates in monomer B of the asymmetric unit (Fig. 2C), suggesting that closure of the enzyme promotes these bonding interactions, because this is the most closed of the four AnmK monomers bound to AMPPCP. In addition, residue Glu-326 of monomer B is also within hydrogen bonding distance (3.0 Å) of the β-phosphate of AMPPCP (Fig. 2C). Because Glu-326 comprises part of hinge region 2, this observation suggests that this residue may also be involved in promoting ligand-induced domain closure.

The AnmK-AMPPCP Complex Also Binds anhMurNAc—

The crystal structure of the AnmK-AMPPCP complex revealed that the enzyme adopts an open conformation with a defined binding site for ATP on domain 2. However, this structure did not resolve whether the open conformation of AnmK is also
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FIGURE 3. Crystal structure of AnmK in the open conformation bound to anhMurNAc and AMPPCP. A, structure of AnmK monomeric subunit shown in cartoon format with domain 1 colored dark gray and domain 2 in light gray. AMPPCP and anhMurNAc are shown in stick format with carbon, nitrogen, oxygen, and phosphorous atoms shown in yellow, blue, red, and orange, respectively; carbon atoms for anhMurNAc are shown in green. B, anhMurNAc binding site in chain D. Electron density is shown as a maximum-likelihood weighted 2Fo − Fc map contoured to 1σ. Carbon atoms are shown colored gray for the protein. Hydrogen bonds are shown as yellow dashed lines with distances in angstroms. The distance between the anomeric carbon and Asp-182 is shown as a black dashed line. C, AMPPCP binding site in chain D. Electron density is shown as a maximum-likelihood weighted 2Fo − Fc map contoured to 1σ. Glu-326 carbon atoms are shown in blue to highlight their position in hinge region 2. Magnesium and water atoms are shown as green and red spheres, respectively. All water to magnesium bond distances are 2.1 Å.

capable of binding anhMurNAc. This was investigated by soaking a crystal of the AnmK-AMPPCP complex in mother liquor containing anhMurNAc. The X-ray structure of the soaked crystal was determined to 1.75 Å resolution using the structure bound to AMPPCP as the starting model. The resulting structure was found to retain an open conformation similar to the AnmK-AMPPCP complex (Fig. 3A) with only the C chain showing a slight change in rotation to 32° from 30° when compared with the closed structure. Well defined electron density was observed for anhMurNAc bound to each monomer of AnmK in this structure (Fig. 3B). Accordingly, atoms of the anhMurNAc sugars modeled into these densities exhibited considerably lower B-factor values than those of bound AMPPCP. The majority of electrostatic interactions that hold anhMurNAc in place in the open conformation are conserved with those observed in the closed conformation of the enzyme (Fig. 3B), except for a hydrogen bond with Asp-182. Similar to the AnmK-AMPPCP complex, several of the phosphate groups for AMPPCP were not well defined in the electron density maps, and the terminal γ-phosphate could be reliably modeled in only one monomer (D), where a magnesium ion was identified that appears to stabilize the phosphate chain of AMPPCP (see below).

Although sugar binding has been proposed to induce closure of sugar kinases (11), we believe that AnmK remains held in an open, pre-Michaelis state in the above crystal structure because of the presence of the nonhydrolyzable ATP analog and crystal packing contacts. A remarkable observation made from this structural complex is the position of the catalytic nucleophile, Asp-182, relative to the anomeric carbon of anhMurNAc. Although the anhMurNAc sugar is bound to domain 1 of AnmK in the open state, Asp-182 is located on domain 2 and is displaced 9–11 Å from the anomeric center (C1) of the sugar (Fig. 3B). Comparing the open and closed states of AnmK clearly shows that closing of the active site cleft allows for correct placement of Asp-182 near the anomeric center (C1) of the sugar (8). Moreover, in monomer D of AnmK, where electron density for the complete phosphate chain of AMPPCP was visible, the distance of the γ-phosphate phosphorus atom to the O6 oxygen of anhMurNAc is 9 Å, again revealing that closure of the active site is necessary to align these reactive sites as well (Fig. 3, A and C). Together, these observations indicate after AnmK binds ATP and anhMurNAc, the enzyme must undergo considerable conformational changes to attain an operative Michaelis complex.

Structural Evidence for a Magnesium-binding Site—Previous crystal structure determinations of AnmK did not reveal electron density that could be reliably ascribed to an active site magnesium ion, even though the ion is essential for catalysis (7). Indeed, magnesium-binding sites have been difficult to identify in most crystal structures of sugar kinases, with the notable exception of the well defined hexa-coordinated magnesium ion in the active site of Sulfolobus tokodaii hexokinase (PDB code 2E2Q) (13). Interestingly, a magnesium-binding site is evident in monomer D of the ternary complex of AnmK bound to AMPPCP and anhMurNAc (Fig. 3C). Five of six expected hexa-coordinate hydrogen bonds can be observed between residues of AnmK and a putative magnesium ion, including four waters that bind in a plane around the metal. The metal also coordinates directly with the β-phosphate AMPPCP, which stabilizes the phosphate chain of the ATP analog and is likely a key interaction that helps promote phosphoryl transfer (Fig. 3C). The position of the magnesium is similar to that observed in the
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*S. tokodaii* hexokinase structure bound to ADP, with an analogous hydrogen bond between the metal and the β-phosphate of ADP and a comparable water bonding arrangement (13). The above structural observations confirm a role for both water bonding networks and metal coordination in ordering the phosphate chain of ATP within the AnmK active site, an interaction that is critical for catalyzing phosphate transfer to anMurNAc.

**Fluorescence Analysis of the Binding of ATP, ADP, and AMPPCP to AnmK**—The binding of ATP and various analogs to AnmK was studied using the fluorescently labeled ATP analog MANT-ATP. The effects of MANT-ATP binding on AnmK fluorescence are shown in Fig. 4A and demonstrate that binding of MANT-ATP quenches the intrinsic fluorescence of AnmK and commensurately causes an increase in the fluorescence of the MANT moiety. This change in fluorescence is shown in Fig. 4B and was used to estimate the fraction of ligand-bound protein according to Equation 1. Fig. 4C depicts these data and the subsequent regression analysis to Equation 1, which yielded: $n = 0.4 \pm 0.2 \text{ mm}^{-1}$ and $K_{d}^{\text{MANT-ATP}}$. Given that the $r^2$ (coefficient of determination) value was larger than 0.997, MANT-ATP appears to bind AnmK through a simple one-site binding model.

Because the fluorescence of MANT-ATP bound to the AnmK is significantly higher than that of the free ligand (Fig. 4A), we were able to use the change in fluorescence to characterize the binding of AMPPCP, ATP, and ADP to AnmK via a competitive assay. Adding these nucleotides to solutions containing the AnmK-(MANT-ATP) complex were found to displace the MANT-ATP ligand from the protein and lead to a measurable decrease in fluorescence (Fig. 4D–F). The decrease in fluorescence followed hyperbolic behavior from which $F_{50}$ values could be determined (Fig. 4D–F) and used in Equation 2 to calculate $K_{d}$ nucleotide values for each ligand.

\[
K_{d}^{\text{AMPPCP}} = 0.060 \pm 0.008 \text{ mm} \tag{Eq. 5}
\]

\[
K_{d}^{\text{ATP}} = 0.032 \pm 0.005 \text{ mm} \tag{Eq. 6}
\]

\[
K_{d}^{\text{ADP}} = 0.05 \pm 0.01 \text{ mm} \tag{Eq. 7}
\]

These values demonstrate that all three ligands have very similar affinities for the AnmK protein.

**Small Angle X-ray Scattering Analyses of the Conformational States of AnmK**—To further examine the impact of ligands on solution conformations, solution x-ray scattering methods were employed to investigate free AnmK, as well as AnmK in
complex with either AMPPCP or ADP. Scattering data from each species were used to generate the pair distance distribution function (a histogram of all observed inter-electron distances), which enabled determination of the maximum particle dimension ($D_{\text{max}}$) and the radius of gyration ($r_G$) (Fig. 5A and Table 2). Although similar $D_{\text{max}}$ (97–99 Å) and $r_G$ (31.0 ± 0.6 to 32.0 ± 0.4 Å) values were obtained for each species, ab initio models generated using these parameters and the original scattering data as restraints enabled observation of significantly different average solution conformations of AnmK depending on the ligand present. Excellent fit of individual models to the original scattering data (reflected in $\chi^2$ values of ~1 for all models) and low discrepancy between individual ab initio models relative to each other (reflected by normalized spatial discrepancy values less than 0.6 for all species) strengthens our confidence in the models presented (Table 2).

AnmK bound to AMPPCP adopts an extended and symmetrical solution conformation with indents proximal to the center of the structure (Fig. 5B). Superposition of the high resolution crystallographic structure on the low resolution models obtained from SAXS confirms that the AnmK dimer presents an open conformation in solution, with the hinge regions of each monomer corresponding to the indentations observed. Conversely, AnmK dimer bound to ADP adopts a closed conformation in solution with a markedly different overall solution conformation from the AMPPCP-bound form (Fig. 5C). Near perfect superposition of the previously determined AnmK-ADP crystallographic structure on the ab initio model demonstrates that the closing of the hinge region is reflected in a more globular overall conformation. Interestingly, AnmK devoid of ligand adopts a solution conformation that is consistent with an open conformation observed in the presence of nonhydrolyzable ATP analog. As with the AMPPCP-bound form, significant indents proximal to the center of the symmetrical envelope are observed, likely corresponding to the open hinge region orientation (Fig. 5D). Taken together, the SAXS results support a mechanism wherein the AnmK dimer adopts an open conformation in solution capable of ATP binding, ultimately closing to enable catalysis to occur.

Comparison of Open and Closed Active Site Architectures—Superposition of the AnmK closed and open structures shows that the binding interactions that hold the anhMurNAc and nucleotide are retained in both states, with the exception of the AMPPCP phosphates that are for the most part flexible in the open structure. A single hydrogen bond between Asp-182 and anhMurNAc is also abrogated in the open structure. Although we note the dramatic movement that Asp-182 must translocate to act as a base for catalysis (Fig. 6), we also observe a more discreet structural rearrangement of the superposed structures whereby Glu-326 can rotate to come in to close contact with both substrates. For example, the residue forms a hydrogen bond with AMPPCP in the open structure B chain but also has the capability of coming closer than 2 Å to the terminal carbon of the anhMurNAc lactyl moiety in a closed superposed structure (Fig. 6). The observed binding interaction between this residue and the nucleotide in addition to the possible clash with anhMurNAc suggests a ligand mediated mechanism for cross-talk between the two hinge regions because Glu-326 is located in hinge region 2. Concomitantly, binding of anhMurNAc in solution could also affect the backbone dynamics of AnmK by making main chain interactions

![FIGURE 5. SAXS analysis of AnmK in the presence and absence of nucleotide. A, the pair distribution function ($p(r)$) for individual proteins obtained from GNOM analysis. B–D, averaged low resolution ab initio models in the presence of AMPPCP (B), ADP (C), and no ligand (D).](image)

| Table 2: Small angle x-ray scattering data statistics |
|-----------------|-----------------|-----------------|
|                | AnmK            | AnmK-ADP        | AnmK-AMPPCP     |
| $R_g$ (Å)      | 31.0 ± 0.6      | 31.87 ± 0.53    | 32.0 ± 0.46     |
| $D_{\text{max}}$ (Å) | 98.7           | 98.0           | 96.6           |
| $\chi^2$       | ~0.94           | ~0.97           | ~1.0           |
| NSD            | 0.60 ± 0.06     | 0.66 ± 0.02     | 0.37 ± 0.03     |

* From $p(r)$ distribution function analysis of SAXS data.
* Describes discrepancy between experimental data and data calculated from ab initio model.
* NSD, normalized spatial discrepancy between multiple ab initio models.
With two residues (Leu-142 and Val-143) immediately upstream from hinge region 1.

**DISCUSSION**

AnmK and levogluosan kinase are unique from other sugar kinases in that they must hydrolyze the 1,6-anhydro ring of the sugar substrate to phosphorylate the sugar using ATP. We previously gained insight into the structural and functional basis for how AnmK carries out the hydrolysis and phosphoryl transfer (8). However, the conformational itinerary of AnmK and the mechanisms by which the enzyme captures and releases substrates and products remained unknown. Using a nonhydrolyzable ATP analog to “trap” the enzyme in a conformation that allows for binding of both substrates, we now have also been able to show the dynamic conformational changes that occur during the catalytic cycle of AnmK, with its two domains rotating apart up to 32° to expose a deep active site cleft into which the 1,6-anhydroMurNAc sugar and ATP bind can both bind prior to closure of the cleft and formation of a productive Michaelis complex (Fig. 7). Indeed, the crystal structures of AnmK bound to nucleotides and anhMurNAc sugar, combined with SAXS analyses, demonstrate that AnmK alternates between closed and open conformations during the catalytic cycle and that these conformations are stabilized by the ligands with which the enzyme interacts. In summary, the available data for AnmK indicate that: 1) the enzyme can adopt a closed conformation when bound to ADP or anhMurNAc; 2) the enzyme adopts an open conformation when bound to AMPPCP alone or when bound to both AMPPCP and anhMurNAc; and 3) catalysis requires the open ternary complex to collapse into a closed state in which the substrates and active site residues are properly aligned to form a Michaelis complex. Product release might be mediated by electrostatic repulsion and potential steric clashing upon the conformational change of anhMurNAc to MurNAc-6-phosphate, and a similar mechanism has been proposed for yeast hexokinase II (31). Because the competitive binding assay also showed that ATP and ADP bind with similar affinities to the enzyme, the much higher cellular concentration of ATP in bacterial cells suggests that ADP release is promoted through competition with ATP following catalysis (32, 33).

The hinge regions connecting domains 1 and 2 of AnmK can rotate when the enzyme is bound to AMPPCP because the copies of AnmK in the asymmetric unit of our crystal structures were found with domains rotated apart to various degrees when compared with the closed structure (20–32°) (Fig. 2B). The range of motion for the two domains of AnmK is comparable to the crystallographic structures of other sugar kinases. For these latter enzymes, substrate-mediated domain closure has been correlated to the binding of the sugar ligand (12, 13, 31, 34, 35). For example, N-acetylgalactosamine kinase was shown to bind the sugar substrate N-acetylgalactosamine only in the closed conformation with several bonding interactions between the sugar and amino acid residues from both domains of the enzyme (10). For other sugar kinases that form binding interactions with their sugar substrates primarily with only one of the two domains, it has been suggested that sugar binding promotes a closed conformation by compensating for an unfavorable loss of hydration in this conformation (11). For AnmK, other mechanisms that promote domain closure also appear likely, including a role for nucleotide binding (because binding of ADP stabilizes the closed form) and possible cross-talk between bound ligands and the hinge regions of the enzyme. The hydrogen bonding interactions that stabilize the nucleotide phosphates in the closed conformation bound to ADP (Ser-12, Asp-14, Gly-165, and Gly-292) (8) and that also form with AMPPCP in some of the open structures (including Glu-326; Fig. 2C) could play a role in promoting domain closure upon binding of ATP. The close proximity of the nucleotide and the anhMurNAc binding sites to both hinge regions also suggests how cross-talk between the two sites can be achieved because the binding of anhMurNAc in solution would likely promote Glu-326 to adopt a conformation that is more amenable for interactions with ATP (Fig. 6). In the open crystal structure of AnmK, we posit that the enzyme is held in the open state through nonspecific clashes.
between the phosphates and residues of domain 1, because of the nonhydrolyzable nature of the analog, or that crystal contacts preclude closing of the enzyme when bound to both AMPPCP and anhMurNAc.

Protein domain movement is an extraordinary attribute of enzymes that allows for highly complex and stereospecific chemical reactions to be carried out during the catalytic cycle of an enzyme. It is noteworthy that the active site nucleophile of AnmK, Asp-182, is located on domain 2 of the enzyme and thus positioned 9–11 Å away from the anomeric carbon of the sugar bound to domain 1 in the open ternary complex of the enzyme. With the nucleophile located on domain 2, catalysis cannot proceed until domain closure has occurred, which allows AnmK to correctly align the substrates and residues of the enzyme into an operative Michaelis complex prior to the reaction. The complex structural dynamics and unique mechanism for the cleavage and phosphorylation of anhMurNAc places AnmK and the functionally related levoglucosan kinase in a unique subclass of kinases, providing a thought-provoking example of protein evolution and highlighting the diversity of phosphoryl transfer reactions carried out by the family of sugar kinases.

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