Structural and Kinetic Analyses of Arginine Residues in the Active Site of the Acetate Kinase from Methanosarcina thermophila*

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Acetate kinase catalyzes transfer of the γ-phosphate of ATP to acetate. The only crystal structure reported for acetate kinase is the homodimeric enzyme from Methanosarcina thermophila containing ADP and sulfate in the active site (Buss, K. A., Cooper, D. C., Ingram-Smith, C., Ferry, J. G., Sanders, D. A., and Hasson, M. S. (2001) J. Bacteriol. 193, 680–686). Here we report two new crystal structures of the M. thermophila enzyme in the presence of substrate and transition state analogs. The enzyme co-crystallized with the ATP analog adenozine 5′-β,γ-thio]triphosphate contained AMP adjacent to thiopyrophosphate in the active site cleft of monomer B. The enzyme co-crystallized with ADP, acetate, AlF3 and F− contained a linear array of ADP-AlF3-acetate in the active site cleft of monomer B. Together, the structures clarify the substrate binding sites and support a direct in-line transfer mechanism in which AlF3 mimics the meta-phosphate transition state. Monomers A of both structures contained ADP and sulfate, and the active site clefts were closed less than in monomers B, suggesting that domain movement contributes to catalysis. The finding that His980 was in close proximity to AlF3 is consistent with a role for stabilization of the meta-phosphate that is in agreement with a previous report indicating that this residue is essential for catalysis. Residue Arg241 was also found adjacent to AlF3, consistent with a role for stabilization of the transition state. Kinetic analyses of Arg241 and Arg421 replacement variants indicated that these residues are essential for catalysis and also indicated a role in binding acetate.

Phosphoryl transfer is a key reaction in numerous biological processes, playing roles in signaling mechanisms, energy transfer, and energy storage in both eukaryotic and prokaryotic cells (1). One of the earliest phosphoryl transfers identified was the phosphorylation of acetate by ATP to form acetyl phosphate (AcP) and ADP, described in 1944 by Lippman (2). This reversible reaction is catalyzed by acetate kinase, which is widely distributed among anaerobic prokaryotes playing a central role in energy-yielding metabolism by synthesizing ATP from acetyl phosphate generated in fermentation pathways. The enzyme also plays an essential role in the fermentation of acetate to methane, which accounts for most of the one billion metric tons of methane produced annually from the decomposition of organic matter by anaerobic microbial consortia (3). In Methanosarcina thermophila, acetate kinase catalyzes the first step in the pathway by activating acetate to acetyl phosphate prior to transfer of the acetyl moiety to CoA catalyzed by phosphotrans-acytalyse (4, 5). In later steps of the pathway, the acetyl moiety is further metabolized to methane and carbon dioxide (6).

Although acetate kinase was one of the first enzymes to be investigated mechanistically, details remain elusive; indeed, the first crystal structure was obtained only recently for the M. thermophila enzyme, identifying acetate kinase as a member of the acetate and sugar kinase-Hsp70-actin (ASKHA) structural superfamily and the best candidate for the common ancestor of this family (7). The earliest kinetic studies of the enzyme from Escherichia coli suggested a ping-pong mechanism (8), and evidence for a covalent phosphoryl intermediate supported this mechanism (9, 10); however, it was later shown that the phosphoryl-enzyme complex is not kinetically competent (11). Additionally, the discovery that the E. coli acetate kinase is able to phosphorylate enzyme I of the phosphotransferase system (12) and CheY (13) in vitro indicates the phosphoenzyme functions in sugar transport. Later investigations reported inversion of the stereochemistry about the phosphorous (14) and isotope exchange kinetics inconsistent with the covalent mechanism (15) and supporting a direct in-line phosphoryl transfer. More recently, the acetate kinase from M. thermophila was shown to be inhibited by components of a putative transition state analogue ADP-AlF3-acetate (16) in which the AlF3 is proposed to mimic the meta-phosphate in a direct phosphoryl transfer mechanism. No structural evidence for either the covalent or in-line mechanism has been reported previously.

Access to the crystal structure (7) and production of the M. thermophila acetate kinase in E. coli (17) have allowed experimental approaches not previously employed to investigate the catalytic mechanism of this enzyme. The structure of the homodimeric acetate kinase co-crystallized with ATP (the ATP-AK structure) reveals ADP in a cleft with contacts that are conserved in the nucleotide binding sites of other ASKHA family members, which identifies the active site of the M. thermophila acetate kinase. The active site contains Arg901 and Arg421, a result consistent with roles for these residues in

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1 The abbreviations used are: AcP, acetyl phosphate; ATP; S, adenosine 5′-γ-thio]triphosphate; GdnHCl, guanidine hydrochloride; TPP, thiopyrophosphate; PIS, residue name for thiopyrophosphate in Protein Data Bank file; ATP-AK, structure of acetate kinase co-crystallized with ATP, ATP-S-AK, structure of acetate kinase co-crystallized with ATP-S; AlF3-AK, structure of acetate kinase co-crystallized with ADP, acetate, and AlF3; ASKHA, acetate and sugar kinase-Hsp70-actin.
substrate binding, catalysis, or both. It was hypothesized that Arg91 binds acetyl phosphate and Arg241 binds acetate based on a postulated binding site identified in the crystal structure (7). The low specific activity reported for Arg31 and Arg241 replacement variants relative to the wild type is consistent with a role for both arginines in stabilizing the pentacornerate transition state for the postulated direct in-line mechanism (16, 18); however, the low activity of the variants precluded a determination of the steady state kinetic parameters. The ATP-AK structure contains only ADP, with the β-phosphate repelled by a sulfate ion and pointing away from the arginines; thus, the catalytically competent orientation of the γ-phosphate of ATP relative to Arg31 and Arg241 is unknown. Here we present two novel M. thermophila acetate kinase structures obtained by co-crystallization with either the ATP analog ATPγS (the ATPγS-AK structure) or components of the putative transition state analog ADP-AlF3-acetate (the AlF3-AK structure) that, along with kinetic analyses utilizing an improved assay, allow us to further examine the roles for Arg31 and Arg241. The results also provide the first structural evidence for the proposed acetate binding site and a direct in-line phosphoryl transfer mechanism.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma, VWR Scientific Products, or Fisher. The pH values of ATP, ADP, and ATPγS stock solutions were adjusted to 7.0 with sodium hydroxide, and concentrations were determined using the extinction coefficient (ε248 = 15.4 × 103 M^-1 cm^-1). ATP and ADP stock solutions were prepared to be equimolar with magnesium chloride. Acetyl phosphate concentrations were determined by assay with hexokinase/glucose-6-phosphate dehydrogenase/acetate kinase. The pH of the acetate stock solution was adjusted to 7.0 with potassium hydroxide. Crystallization materials were obtained from VWR Scientific Products or Hampton Research.

Heterologous Production and Purification of Acetate Kinase—Plasmids for the R91A, R91L, R91K, R241A, R241L, and R241K variant acetate kinases previously generated were utilized for this study (18). The wild-type and variant acetate kinases were overproduced in E. coli BL21(DE3) (F−ompT hsdS (r-bm-B-gal) l (DE3)) and purified as described previously (16, 18–20). Protein purity was examined by SDS-PAGE gel electrophoresis and protein concentrations were determined by the method of Bio-Rad dye and bovine serum albumin as the standard. The yields and dimeric state of the variants were similar to those of the wild type enzyme (data not shown).

Enzymatic Assays—The hydroxamate assay adaptation of the Lipmann and Rose methods (2, 23, 24) detects acetyl phosphate formation and was previously used to determine the kinetic parameters in the forward and reverse direction. In this study, the kinetic parameters were determined utilizing an enzyme-linked assay system with pyruvate kinase and lactate dehydrogenase as previously described by Allen et al. (25) and utilized by Aceti with slight modifications (24). Assay solutions contained 60 mM HEPES (pH 7.0), 5 mM MgCl2, 16.7 units of pyruvate kinase, 36 units of lactate dehydrogenase, 3 mM phospho(en)ylpyruvate, 0.2 mM NADH with the nonvariable substrate kept at 10× Km (unless otherwise noted). Fixed concentrations of ATP were equimolar with MgCl2 at 1 mM for wild-type or 10 mM for the variant enzymes, and the fixed concentration of acetate was 200 mM for wild type, 1 mM for Arg31 variants, and 2 mM for Arg241 variants. Assays contained 1–50 μg/ml wild-type or variant enzyme, depending upon its activity. Absorbance changes were monitored at 340 nm with a Beckman DU640 spectrophotometer, with assay times from 1 to 5 min, reading at 1-s intervals. Km and Vmax values were determined through nonlinear regression data analysis fit to the Michaelis-Menten equation utilizing the program Kaleidagraph (Synergy Software, Reading, PA).

The turnover (kcat, s^-1) was determined from the Vmax utilizing Equation 1:

\[
\Delta A_{340/min} = \frac{V_{\text{max}}}{K_{\text{cat}}} \times \frac{(\text{mol of ATP produced})}{(\text{mol of enzyme})} \times \frac{(60 \text{ s/min})(6.33 \text{ mM} \cdot \text{cm}^{-1})}{(1 \text{ cm})} \times (\mu\text{g/mol})(92,000 \text{ g})
\]

where E is concentration of enzyme in μg/ml, and Vmax is the maximum ΔA_{340/min} determined from the Michaelis-Menten equation.

When determining kinetic parameters in the reverse direction (ATP/acetate synthesis), the previously described enzyme-linked assay was used (24). Assay components were 100 mM Tris (pH 7.4), 0.2 mM dithiothreitol, 10 mM MgCl2, 4.4 mM glucose, 1 mM NADP, 10 units of hexokinase (yeast), and 10 units of glucose-6-phosphatase (yeast). The ADP concentration was held in excess at 5 mM when Km(ADP) was determined, and the AcP concentration was held in excess at 10 mM when Km(ADP) was determined. Enzyme concentrations varied from 1 to 50 μg/ml, depending upon enzyme activity to yield a linear rate over the duration of the assay. Kinetic constants were determined using nonlinear regression to fit data using the program Kaleidagraph (Synergy Software, Reading, PA).

Determination of Inhibition Constants for ATPγS and Hydroxylamine—Inhibition constants for hydroxylamine and ATPγS were determined by including 5 × 5 matrix of conditions that systematically varied inhibitor and substrate concentrations (hydroxylamine versus ATP or ATPγS versus ATP). Assays contained 60 mM HEPES, pH 7.0, 5 mM MgCl2, 16.7 units of pyruvate kinase, 36 units of lactate dehydrogenase, 3 mM phospho(en)ylpyruvate, 0.2 mM NADH, and 1 μg/ml wild-type acetate kinase, with 200 mM acetate when ATP varied and 5 mM acetate when ATP was varied. ATP concentrations were varied between 20 μM and 1 mM, whereas acetate concentrations were varied from 0.2 to 10 mM. ATPγS concentrations ranged from 0 to 300 μM. Hydroxylamine concentrations ranged from 0 to 1 mM. Kinetic parameters for inhibition were determined by linear regression using the MINITAB program and a value for α of 2.0 (26).

Guanine Rescue of Activity—The ability of the adenine-donating guanine to rescue the activity of the arginine variants was determined by including 5 × 5 matrix of conditions that systematically varied concentration and temperature. Wild-type acetate kinase was assayed in the presence of increasing concentrations of GdnHCl to determine maximum concentration permissible in the assay conditions before enzyme activity is affected, and 200 mM GdnHCl was determined to be the maximum concentration tolerated (data not shown). Kinetic constants for wild-type, R91A, and R241A acetate kinases were determined utilizing the forward reaction assay described under “Enzymatic Assays” in the presence of 200 mM guanidine hydrochloride. Substrate concentrations, enzyme concentrations, and assay times are as previously described.

Crystallization and Data Collection—The hanging drop method was used to co-crystallize acetate kinase with ATPγS as previously described for co-crystallization of the enzyme with ATP (7, 27). Wild-type acetate kinase (0.5 mg/ml) was incubated with 1 mM ATPγS, 1.5 mM MgCl2, 1.5 mM (NH4)2SO4, and 100 mM Tris (pH 7.4) in a drop that was equilibrated against a reservoir of 1.7 mM (NH4)2SO4 for 2 h at room temperature. The drop was then equilibrated against a reservoir of 0.8 mM (NH4)2SO4 at 37 °C, with small crystals first appearing overnight and reaching maximum size at 14 days. Crystals are stable for at least 3 months. A single ATPγS-AK crystal was transferred to a saturated glycerol solution as a cryoprotectant and flash frozen in a liquid-N2 stream. Data were collected at 100 K on the F2 beam line at the Cornell High Energy Synchrotron Source (Cornell University, Ithaca, NY), and image files were processed with DENZO/SCALEPACK (28).

The hanging drop method was also used to co-crystallize acetate kinase in the presence of acetate, ADP, and AlF3. Prior to use in crystallization trials, 100 mM AlCl3, and 50 mM NaF were pre-equilibrated overnight. Wild-type acetate kinase (0.5 mg/ml) was incubated with 1 mM ADP, 1.5 mM LiCl, 0.1 mM AlCl3, plus 0.5 mM NaF, 10 mM acetate, 315 mM (NH4)2SO4, and 25 mM Tris (pH 7.4) in a drop that was equilibrated against a reservoir of 1.7 mM (NH4)2SO4 for 2 h at room temperature. The drop was then transferred to a reservoir of 0.8 mM (NH4)2SO4. Crystallization was allowed to proceed as described above, although the crystals reached a smaller macroscopic size. The crystals were transferred to a saturated glucose solution as a cryoprotectant and frozen in liquid N2. Data were collected at 100 K at Argonne National Synchrotron (Argonne, IL), and image files were processed with DENZO/SCALEPACK (28).

Structure Solution and Refinement—Since the unit cell dimensions of both the ATPγS-AK and AlF3-AK structures were nearly identical to the previously solved ATP-AK structure, molecular replacement was used to determine the structures of the complexes. The program AMORE (CCP4 suite, version 1.1) (29) was used to perform molecular replacement using as a search model the ATP-AK structure deposited in the Protein Data Bank data base (identifier 1GG9) (7). Refinement was performed with CNS solve (version 4.1) (30), and molecular models were built and visualized with O (31). Ideal parameter and topology

\[
V_{\text{max}} = 248.08 \frac{\text{μg/mol}}{E[\mu\text{g}]} \quad (\text{Eq. 1})
\]
files for AMP, ADP, SO\textsubscript{4}\textsuperscript{2-}, NH\textsubscript{3}, AlF\textsubscript{3}, and acetate for CNS\_solve were obtained from HIC-Up (32). Parameter and topology files for pyrophosphate were modified to include sulfur in place of one oxygen of the γ-phosphate for thio pyrophosphate (TPP; Protein Data Bank residue name PIS) for use in CNS\_solve. Designations for chains A and B in both structures were assigned so that chain A contains the same heteroatoms as in the ATP-AK structure. Model suitability was determined with PROCHECK (33), and overall molecular refinement statistics are presented in Table IV. The rotation matrix and root mean square values between either the ATP\_S-AK or AlF\textsubscript{3}-AK structure and the ATP-AK structure were calculated with LSQ\_MAN (27). Coordinate and structure files have been deposited at the RCSB Data bank with the PDB identifiers 1TUU for the acetate kinase-ATP complex and ITUY for the acetate kinase-ADP-AlF\textsubscript{3}-acetate complex.

**RESULTS**

**Kinetic Parameters of the Wild-type and Variant Acetate Kinases**—Prior to assessment of the kinetic parameters of the variant acetate kinases, the accuracy of the hydroxamate assay was tested utilizing the enzyme-linked assay described under "Experimental Procedures." Hydroxylamine was found to inhibit wild-type acetate kinase in a nonlinear and noncompetitive fashion versus either acetate or ATP (Fig. 1), as described by Equation 2,

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_i}{V_{\text{max}}}[I] + \frac{1}{K_m[A]} + \frac{1}{V_{\text{max}}[A]K_i} \quad (\text{Eq. 2})
\]

where \(v\) represents the reaction velocity, \(V_{\text{max}}\) is the maximal reaction velocity, \(K_i\) is the Michaelis constant (\(K_m\)) for substrate A, \(K_m\) is the concentration of substrate A, \([I]\) is the concentration of inhibitor, \(K_i\) is the equilibrium constant for inhibition of the enzyme, and \(K_m\) is the kinetic constant for inhibition of the enzyme-substrate complex. Since the data indicated that hydroxylamine binds to multiple sites other than the substrate binding sites and that hydroxylamine inhibition could have introduced errors in the kinetic parameters previously reported utilizing the hydroxamate assay (16, 18–20, 24, 34), the enzyme-linked assays were utilized to measure the kinetic parameters reported in this study.

A search of the data bases revealed that Arg\textsuperscript{241} and Arg\textsuperscript{91} of the *M. thermophila* enzyme are each conserved in 218 of 219 acetate kinase sequences retrieved (data not shown), strongly suggesting a role for these active site residues in substrate binding, catalysis, or both. The kinetic parameters in either direction have not been determined for Arg\textsuperscript{241} variants of the *M. thermophila*, and parameters are reported for only the R91K variant in the direction of ADP synthesis utilizing the hydroxamate assay that could have introduced errors (18). The kinetic parameters for the wild-type acetate kinase from *M. thermophila* in the direction of ADP synthesis are only reported (18, 19, 24) utilizing the hydroxamate assay. Furthermore, kinetic parameters for the wild-type enzyme in the direction of ATP synthesis have not been determined. Thus, kinetic constants for the wild-type and arginine replacement variants (Tables I and II) were determined in both directions utilizing the enzyme-linked assays.

Although the \(k_{\text{cat}}\) determined for the wild-type acetate kinase approximated the values (1050–1596 s\(^{-1}\)) reported using the hydroxamate assay, the \(K_{m(\text{ATP})}\) and \(K_{m(\text{acetate})}\) values determined with the enzyme-linked assay in the direction of ADP synthesis (Table I) were at least 12- and 7-fold lower than those previously reported (18, 19, 24). When assayed in the direction of ATP synthesis (Table II), the wild-type \(k_{\text{cat}}\) approximated the value determined in the direction of ADP synthesis (Table I). The \(K_{m(\text{ADP})}\) approximated the \(K_{m(\text{ATP})}\); however, the \(K_{m(\text{AcP})}\) was nearly 6-fold less than the \(K_{m(\text{AcP})}\).

It was reported previously that all of the variants shown in Tables I and II purified according to the wild-type are dimeric, and the CD spectra of the R91A and R241A variants are nearly identical to wild type, indicating no gross conformational changes in the variants relative to wild type (18). All of the Arg\textsuperscript{91} and Arg\textsuperscript{241} variants showed large decreases in \(k_{\text{cat}}\) relative to wild type when assayed in the direction of ADP synthesis, ranging from 250-fold for R91K to 8200-fold for R91A (Table I). The \(K_{m(\text{ATP})}\) values determined for all of the Arg\textsuperscript{91} variants changed little relative to the wild type, with the largest effect being a 5-fold decrease for the R91A variant; however, the \(K_{m(\text{AcP})}\) values increased 93–156-, and 26-fold for the R91A, R91L, and R91K variants, respectively (Table I). Only a modest increase in \(K_{m(\text{ATP})}\) compared with wild-type was determined for the R241A variant, arguing against an important role in binding ATP. In contrast, large increases were observed in \(K_{m(\text{ATP})}\) for the R241L (213-fold) and R241K (143-fold) variants. The Arg\textsuperscript{241} variants also displayed substantial increases for \(K_{m(\text{AcP})}\); 263-fold for R241A, 100-fold for R241L, and 29-fold for R241K. Notably, the increases in \(K_{m(\text{AcP})}\) were severalfold less when Arg\textsuperscript{91} or Arg\textsuperscript{241} was replaced with a Lys as opposed to the other residues tested.

When assayed in the direction of ATP synthesis (Table II), large decreases in \(k_{\text{cat}}\) relative to wild-type were observed for all of the variants that were similar in magnitude to the decreases in \(k_{\text{cat}}\) in the direction of ADP synthesis (Table I). The minor deviations in \(K_{m(\text{ATP})}\) for all of the variants relative to wild type argue against a role for these residues in binding acetyl phosphate. Although the 2-fold increase in \(K_{m(\text{ADP})}\) observed for the R241A variant relative to wild-type was also minor, moderately larger increases were observed for the R241L (13-fold) and R241K (6-fold) variants.

**Guandine Hydrochloride Rescue of Variants**—GdnHCl is reported to rescue the \(k_{\text{cat}}\) of arginine replacement variants of several enzymes for which arginine is essential (35–40); thus, rescue of the R91A and R241A variants of the *M. thermophila* acetate kinase was investigated using the enzyme-linked assay to further address the role of these residues. The \(k_{\text{cat}}\) of the wild-type acetate kinase was reduced to approximately one-
that ATP hydrolysis occurred during crystallization, the non-

![Image](340x338 to 540x496)

half in the presence of 200 mM GdnHCl with no significant change in \( K_{\text{m(ADP)}} \) (Table III), indicating that GdnHCl does not significantly compromise the enzyme active site (Tables I and II). However, a 10-fold increase in \( K_{\text{m(acetate)}} \) was observed in the presence of GdnHCl, for which the most straightforward explanation is that GdnHCl occupies space near the acetate binding pocket. Analysis of the R91A variant in the presence of GdnHCl showed a 250-fold decrease in \( k_{\text{cat}} \) and only modest changes in \( K_{\text{m(ATP)}} \) and \( K_{\text{m(acetate)}} \) as compared with the wild-type parameters in the presence of GdnHCl (Table III). However, a comparison of R241A in the presence of GdnHCl (Table III) revealed a 15-fold increase in \( k_{\text{cat}} \), a 4-fold decrease in \( K_{\text{m(ATP)}} \), and a 3-fold decrease in \( K_{\text{m(acetate)}} \) relative to the parameters obtained for this variant in the absence of GdnHCl (Table I).

Analysis of the R241A variant in the presence of GdnHCl revealed an 807-fold decrease in \( k_{\text{cat}} \), a 4-fold increase in \( K_{\text{m(ATP)}} \), and no significant change in \( K_{\text{m(acetate)}} \) compared with parameters for the wild-type enzyme in the presence of GdnHCl (Table III). Comparison of the R241A variant kinetic parameters in the presence and absence of GdnHCl showed no appreciable differences in the \( k_{\text{cat}} \) and \( K_{\text{m(ATP)}} \) whereas the \( K_{\text{m(acetate)}} \) decreased 24-fold in the presence of GdnHCl.

**Induction by ATP-S**—The first acetate kinase structure (ATP-AK) was obtained by co-crystallization of the \( M. \ thermophila \) enzyme with ATP; however, only ADP was identified in the active site with the \( \beta \)-phosphate repelled by a sulfate ion precluding the catalytically competent location of the \( \gamma \)-phosphate of ATP relative to Arg\(^{81} \) and Arg\(^{241} \) (7). Since it is likely that ATP hydrolysis occurred during crystallization, the non-hydrolyzable ATP analogue ATP\(_{\gamma}S\) was co-crystallized with the enzyme in anticipation of generating a more catalytically relevant complex. In order to better interpret the ATP\(_{\gamma}S\)-AK structure, the influence of ATP\(_{\gamma}S\) on acetate kinase activity was evaluated. No acetate kinase activity was detected with the coupled assay system when ATP\(_{\gamma}S\) replaced ATP, a result consistent with no hydrolysis of the \( \gamma \)-thiophosphate. Inhibition of the wild-type enzyme by ATP\(_{\gamma}S\) was investigated to indicate whether the analog bound to the catalytic ATP binding site. Inhibition was determined in assays with five concentrations of ATP, each versus five concentrations of ATP\(_{\gamma}S\) (a 5 \( \times \) 5 matrix) and holding acetate (Fig. 2A) at a 100-fold excess relative to \( K_{\text{m}} \) (Fig. 2B). Data were fit utilizing the equation for competitive inhibition (Equation 3),

\[
\frac{1}{v} = \frac{1}{v_{\text{max}}} \left( 1 + \frac{[I][K_{\text{I}}]}{[A][K_{\text{m}}]} \right)
\]

where \( v \), \( v_{\text{max}} \), \( K_{\text{I}} \), [A], [I], and \( K_{\text{m}} \) are as previously defined for Equation 2. The value determined for \( K_{\text{m(ADP)}} \) (65.0 ± 3.4 \( \mu M \)) approximates that determined for the \( K_{\text{m(ATP)}} \) (240 ± 17 \( \mu M \)). In conjunction with the competitive inhibition data, this result indicates that the ATP\(_{\gamma}S\) binds to the catalytic ATP binding site. Furthermore, the absence of parallel lines in the double reciprocal plots adds credence to the argument against a ping-pong kinetic mechanism.

**Crystal Structures**—The \( M. \ thermophila \) acetate kinase that was co-crystallized with either ATP\(_{\gamma}S\) or components of the

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**Table I**

| Enzyme   | \( k_{\text{cat}} \) | \( K_{\text{m(ATP)}} \) | \( K_{\text{m(acetate)}} \) |
|----------|----------------------|--------------------------|-----------------------------|
| Wild type | 913 ± 52             | 80 ± 9                    | 2.7 ± 0.3                    |
| R91A     | 0.11 ± 0.004         | 16 ± 0.6                  | 250 ± 38                     |
| R91L     | 0.22 ± 0.04          | 145 ± 3                   | 420 ± 40                     |
| R91K     | 3.7 ± 0.2            | 76 ± 3                    | 70 ± 3                      |
| R241A    | 0.68 ± 0.10          | 297 ± 27                  | 710 ± 130                    |
| R241L    | 1.38 ± 0.10          | 17,000 ± 2800             | 270 ± 67                     |
| R241K    | 1.3 ± 0.06           | 11,400 ± 1900             | 77 ± 7                       |

**Table II**

| Enzyme   | \( k_{\text{cat}} \) | \( K_{\text{m(ADP)}} \) | \( K_{\text{m(acetate)}} \) |
|----------|----------------------|--------------------------|-----------------------------|
| Wild type | 1260 ± 60            | 98 ± 7                    | 0.47 ± 0.05                  |
| R91A     | 3.5 ± 0.8            | 63 ± 6                    | 1.36 ± 0.20                  |
| R91L     | 7.7 ± 0.4            | 63 ± 5                    | 2.3 ± 0.1                    |
| R91K     | 23 ± 0.2             | 168 ± 6                   | 0.61 ± 0.06                  |
| R241A    | 4.5 ± 0.4            | 210 ± 28                  | 0.84 ± 0.02                  |
| R241L    | 0.42 ± 0.06          | 1260 ± 89                 | 1.64 ± 0.40                  |
| R241K    | 4.5 ± 0.4            | 578 ± 21                  | 0.92 ± 0.03                  |

**Table III**

| Enzyme   | \( k_{\text{cat}} \) | \( K_{\text{m(ATP)}} \) | \( K_{\text{m(acetate)}} \) |
|----------|----------------------|--------------------------|-----------------------------|
| Wild type | 428 ± 13             | 68 ± 5                    | 24 ± 7                      |
| R91A     | 1.7 ± 0.3            | 68 ± 7                    | 83 ± 9                      |
| R241A    | 0.53 ± 0.09          | 264 ± 26                  | 30 ± 4                      |

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**Fig. 2** Inhibition of \( M. \ thermophila \) acetate kinase by ATP\(_{\gamma}S\). For each reaction, acetate was held in excess at 200 mM while the concentration of ATP was varied. The inhibitor, ATP\(_{\gamma}S\), was present at fixed concentrations of 0 \( \mu M \) (●), 25 \( \mu M \) (□), 75 \( \mu M \) (▲), 150 \( \mu M \) (×), and 300 \( \mu M \) (○).
putative transition state analog, ADP-AlF₃-acetate, had the same C2 space group and similar unit cell dimensions as previously reported for the enzyme co-crystallized with ATP (16) (Table IV). The ATPγS-AK structure was solved by molecular replacement starting with the backbone coordinates reported for the published ATP-AK structure (7), and rigid body refinement was performed utilizing each monomer as the rigid body. The AlF₃-AK structure was solved by molecular replacement as described above; however, rigid body refinement utilized each of the two domains within each monomer as the rigid body. Refinement of both the ATPγS-AK and AlF₃-AK structures resulted in models with a similar Cα backbone trace to each other and to the previously reported ATP-AK structure (Fig. 3). The overall structure of each acetate kinase homodimer resembles a bird with its wings spread. The “body” of the bird contains the dimer interface and is formed by the C-terminal domains of each monomer. The “wings” are formed by the N-terminal domains, and the active site of each monomer is located in the cleft between the two domains (Fig. 3). As in the ATP-AK structure, the wings of each monomer in both the ATPγS-AK and AlF₃-AK structures were closed onto the body to different extents. As reported for the ATP-AK structure, electron density in the active site of one of the monomers (designated monomer A) for both the ATPγS-AK and AlF₃-AK structures was fit to ADP and SO₄²⁻, and the domains were closed less than in monomer B (Fig. 4). Alignments of the Cα backbone of monomers A from the ATPγS-AK and AlF₃-AK structures (ATPγS-AK[A] and AlF₃-AK[A]) with monomer A of the ATP-AK structure (ATP-AK[A]) yielded root mean square differences of 0.48 and 0.76 Å for the ATPγS-AK[A] and AlF₃-AK[A] structures, respectively. Alignments of the Cα backbone of monomer B of the ATP-AK structure (ATP-AK[B]) with the Cα backbone of the ATPγS-AK[B] and AlF₃-AK[B] structures yielded root mean square differences of 1.06 and 1.39 Å for the ATPγS-AK[B] and AlF₃-AK[B] structures, respectively.

Electron densities in the monomer A active sites of both the ATPγS-AK[A] and AlF₃-AK[A] structures were fit to ADP and sulfate (Fig. 4). The nucleotide base and ribose of ADP in both structures were found to have the same contacts as the ADP in

![Fig. 3. The Cα trace overlay of the ATPγS-AK and AlF₃-AK structures onto the ATP-AK structure. In both A and B, the ATP-AK structure is shown in green (ATP-AK[A]) and violet (ATP-AK[B]), and the arrows indicate the active site clefts of each monomer, A, the ATPγS-AK structure overlaid on the ATP-AK structure with ATPγS-AK[A] in blue and ATPγS-AK[B] in orange. B, the AlF₃-AK structure overlaid on the ATP-AK structure with AlF₃-AK[A] in blue and AlF₃-AK[B] in orange. In both A and B, the active site cleft of each monomer is indicated by an arrow.](image-url)
the published ATP-AK[A] structure (7). The phosphates of ADP and the sulfate in both new structures were positioned in the active site similarly to the published ATP-AK[A] structure. Also as reported for the ATP-AK[A] structure, Arg241, Arg91, and His180 were directly adjacent to sulfate in both the ATPγS-AK[A] and AlF3[A] structures (Fig. 4). Monomer B of both the ATPγS-AK and AlF3-AK structures contained electron densities not reported for the ATP-AK[B] structure that will be discussed separately for each new structure.

In the ATPγS-AK[B] structure, electron density in the active site was fit to AMP (Fig. 5) for which contacts (not shown) were the same as reported for the nucleotide base, ribose ring, and γ-phosphate of the ADP that was reported in the ATP-AK[B] structure. These results, combined with the inhibition results, indicate that ATPγS bound analogously to the catalytically competent binding of ATP. Additional electron density in the active site of the ATPγS-AK[B] structure was fit to NH3 that probably originated from the crystallization solution. The NH3 was located at the mouth of the acetate binding pocket predicted from the ATP-AK structure (7) and supported by kinetic analyses of replacement variants.\(^2\) The binding pocket is composed of Val93, Leu122, and Pro232 and is proposed to accept the methyl group of acetate, thereby positioning the carboxyl group at the mouth of the pocket adjacent to Arg241 in the approximate position of NH3 (Fig. 5). Although kinetic analyses of replacement variants suggested a role for Arg91 in binding acetate, this residue is positioned ~7 Å away from NH3 (Fig. 5). Additional electron density was observed in the active site of ATPγS-AK[B] that could not be fit to any component of the crystallization conditions. The presence of AMP in this active site suggested the possibility of hydrolysis of the ester bond between the α- and β-phosphates of ATPγS, resulting in AMP and thiopyrophosphate (TPP, H₂PO₃⁻O-HPO₂S⁻). For description, the phosphates of TPP are named β’ and γ’, reflecting their original positions in the ATPγS. Since TPP had not been described previously, the structural topologies and parameters for pyrophosphate were manually modified to simulate TPP.

\(^2\) Ingram-Smith, C., Gorrell, A., Lawrence, S. H., Iyer, P. P., Smith, K., and Ferry, J. G. (2005) J. Bacteriol., in press.
and used to fit the postulated TPP to the unidentified electron density in monomer B (Fig. 5), with a subsequent decrease in $R_{\text{free}}$. The model showed a distance of 3.68 Å from the β'-phosphate of TPP to the proximal oxygen of the AMP phospho-ryl group. These results suggest in situ hydrolysis of the ester bond between the α- and β'-phosphates of ATPγS. The β'- and γ'- phosphates of TPP were positioned in line with the α-phosphate of AMP and NH₃ (Fig. 5). Notably, the electron density for the γ-phosphate of TPP showed an interaction with the ε amine group of Arg²⁴¹ and proximity to His¹⁸⁰ (Fig. 5). The position of the γ-phosphate of TPP was similar to the position of sulfate in the ATPγS-AK[A] structure (Fig. 4A) and published ATP-AK structure (not shown). Furthermore, the β'-phosphate of TPP was positioned distinct from the β-phosphate of ADP determined for the ATPγS-AK[A] structure (Fig. 4A) and published ATP-AK structure (not shown).

Electron density in the active site of the AlF₃-AK[B] structure was fit to ADP, AlF₃, and acetate (Fig. 6) in an arrangement consistent with a transition state analog of the direct in-line phosphoryl transfer mechanism in which the AlF₃ has been proposed to mimic the meta-phosphate (16). The ribose ring and α-phosphate of ADP were positioned in the active site similarly to AMP in the ATPγS-AK[B] structure (Fig. 5), and the β-phosphate of ADP and AlF₃ were positioned in the active site similarly to the β'- and γ-phosphates of TPP in the ATPγS-AK[B] structure. Acetate was found adjacent to AlF₃ and contained in the active site hydrophobic pocket formed by Val⁹³, Leu¹²², and Pro²³² placing the carboxyl group of acetate within hydrogen bonding distance of Arg²⁴¹ (Fig. 6). This binding site for acetate was previously postulated (7) based on analogy to the substrate binding sites of other ASKHA family members and recently supported by kinetic analyses of site-specific variants.² The trigonal planar electron density of aluminum fluoride in the structure indicates that it formed with the stoichiometry AlF₃ as opposed to AlF₄, consistent with the pH dependence of aluminum fluoride formation (42) and with the stoichiometry suggested by inhibition of the M. thermophila acetate kinase by the transition state analog (16). The plane of AlF₃ is oriented parallel to the β-phosphoryl group of ADP and acetate (Fig. 6) and therefore does not represent a true transition state, since a direct in-line transfer mechanism (S₉₋₂ reaction) requires the plane of the AlF₃ to be perpendicular to both ADP and acetate. Although no contacts were evident, the AlF₃ was adjacent to Arg²⁴¹ and His¹⁸⁰, two candidates for stabilizing the transition state (18, 20). The results

**Fig. 5.** Stereo view of the monomer B active site of *M. thermophila* acetate kinase co-crystallized with ATPγS (ATPγS-AK[B]). A, the electron density contoured at 1.5 σ calculated from 2$F_\text{o}$−$F_\text{c}$ maps for TPP, NH₃, the α-phosphate of AMP, and residues Arg⁹¹, Asp¹⁴⁸, Thr¹⁸², His¹⁸⁰, Arg²⁴¹, and Glu³⁸⁴. B, key distances discussed under “Results.” The α-carbon of each residue is shown as a ball.
presented here provide the first structural evidence supporting the previously proposed direct in-line phosphoryl transfer mechanism (14–16).

Several additional nuances of the AlF₃-AK structure merit further description. Although the ribose of ADP in the AlF₃-AK[B] active site had a similar position and interacted with the same side chains as in ATP-AK[B], the plane of the purine ring was rotated −14°, resulting in a 1.6-Å shift from the hydrophobic nucleotide-binding pocket (not shown). Whereas the phosphates of the ADP in the active site of AlF₃-AK[B] were in approximately the same locations in as in the ATP-AK[B] structure (Fig. 6), the shift of the purine ring produced two noteworthy changes. A hydrogen bond between the backbone amide of Gly331 and the phosphate reported in the ATP-AK[B] structure was not observed in the AlF₃-AK[B] structure; however, a hydrogen bond between the backbone amide of Gly331 and the phosphate of ADP, not present in the published ATP-AK[B] structure, is observed in the AlF₃-AK[B] structure. The backbone φ and ψ angles for Gly331, a conserved feature of the ASKHA superfamily (43), are maintained in the crystal structure even when contacts to the substrates have changed. Additional electron density, not observed in any other acetate kinase crystal structure, was found within hydrogen bonding distance of acetate in the active site of AlF₃-AK[B] and was fit to a water molecule (Wat2 in Fig. 6). The function of this water is unknown at present.

### DISCUSSION

**Kinetic Analysis of Site-specific Replacement Variants**—The only kinetic parameters previously reported for the acetate kinase from *M. thermophila* were determined in the direction of ADP synthesis with the hydroxamate assay where hydroxylamine is a component of the assay mixture (18–20, 34). Using the enzyme-linked assay in the direction of ADP synthesis, it was shown that hydroxylamine inhibits activity and could influence the kinetic parameters; therefore, the more sensitive and accurate enzyme-linked assay was used to measure the activity of the variants. The *k*₅₆ values obtained for all of the Arg⁹¹ and Arg²⁴¹ variants in both reaction directions establish that these residues are essential for catalysis and support the previously hypothesized role for these active site residues in stabilization of a meta-phosphate transition state in a direct in-line mechanism for phosphoryl transfer from ATP to acetate (16, 18). It was previously postulated that Arg²⁴¹ also interacts with the carboxyl group of acetate and that Arg⁹¹ interacts with the phosphophoryl group of acetyl phosphate based on features of the ATP-AK crystal structure identifying putative binding sites for these substrates (7). However, the consequential changes in *K*₅₆(AcP) relative to the wild-type enzyme in all of the Arg⁹¹ and Arg²⁴¹ variants argue against a role for either residue in binding the phosphoryl group of acetyl phosphate. In contrast, substantial increases in *K*₅₆(acetate) com-
pared with wild-type were observed for all of the Arg\(^{91}\) and Arg\(^{241}\) variants, supporting the previously proposed role for Arg\(^{241}\) in binding acetate and suggesting the same role for Arg\(^{91}\). The increases in \(K_{m\text{acetate}}\) for the R91K and R241K variants were severalfold less than for the variants in which the arginines were replaced with Ala or Leu, a result consistent with the requirement for a positive charge in positions 91 and 241 to interact with the carboxyl group of acetate.

Interpretation of the \(K_{m\text{ATP}}\) and \(K_{m\text{ADP}}\) values obtained for the Arg\(^{91}\) and Arg\(^{241}\) variants is not straightforward. The marginal increases relative to wild type for the R241A variant indicate a minor involvement for Arg\(^{241}\) in binding ATP or ADP; however, large increases in both parameters were observed for the R241L and R241K variants. One possible explanation for these results is that, compared with Ala, the larger side chains of Leu and Lys sterically hinder ATP binding consistent with results with the Arg241 variants is not straightforward. The marginal involvement for Arg241 in binding acetate and suggesting the same role for Arg91. The increases in \(K_{m\text{acetate}}\) for the R91K and R241K variants were severalfold less than for the variants in which the arginines were replaced with Ala or Leu, a result consistent with the requirement for a positive charge in positions 91 and 241 to interact with the carboxyl group of acetate.

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The \(K_{m\text{acetate}}\) values for the R91A and R241A variants were found to be significantly lower in the presence of GdnHCl, a result further supporting a role for the guanidino groups of the arginines interacting with the carboxyl group of acetate. Both the lower \(K_{m\text{acetate}}\) and substantially higher \(k_{\text{cat}}\) of the R91K variant compared with the other Arg\(^{91}\) variants indicate that the positive charge of the guanidino moiety of Arg\(^{91}\) enhances the catalytic efficiency. Indeed, the presence of GdnHCl decreased the \(K_{m\text{acetate}}\) of the R91A variant while increasing the \(k_{\text{cat}}\). Thus, in addition to the proposed role in stabilization of the transition state, another potential role for Arg\(^{91}\) may be to orient the carboxyl group of acetate for nucleophilic attack on the \(\gamma\)-phosphate of ATP.

**Analysis of Acetate Kinase Crystal Structures**—The published ATP-AK structure co-crystallized with ATP contained only ADP in the active site clef adjacent to sulfate that is proposed to displaced the \(\beta\)-phosphate and the inferred \(\gamma\)-phosphate of ATP from the catalytically competent position (7). The ATP\(^\gamma\)S-AK[A] and AlF\(^{3-}\)AK[A] structures reported here also contained only ADP and sulfate positioned in the active site similarly to the ATP-AK structure; however, monomer B of both new structures was void of sulfate in the active site and revealed new structural information advancing an understanding of substrate binding and catalysis (16, 18). The nucleotide base and ribose of AMP in the ATP\(^\gamma\)S-AK[B] structure were positioned in the active site similarly to ADP in the published ATP-AK structure, which, combined with results showing inhibition of acetate kinase activity by ATP\(^\gamma\)S, indicates that ATP\(^\gamma\)S bound to monomer B of the ATP\(^\gamma\)S-AK structure analogously to ATP. The close proximity of the AMP phosphate to the \(\beta\)-phosphate of TPP suggests that ATP\(^\gamma\)S had bound to the active site followed by in situ hydrolysis of the ester bond between the \(\alpha\)- and \(\beta\)-phosphates, yielding AMP and TPP. Although the TPP may have shifted slightly away from AMP after hydrolysis, the results suggest that the \(\gamma\)-phosphate of TPP approximates the active site position of the \(\gamma\)-phosphate of ATP poised for catalysis.

The \(\beta\)-phosphate of ADP in the AlF\(^{3-}\)AK[B] structure and the \(\beta\)-phosphate of TPP in the ATP\(^\gamma\)S-AK[B] structure were positioned similarly in the active site although differently from the \(\beta\)-phosphate of ADP in the published ATP-AK[A] structure and for monomer A of both the ATP\(^\gamma\)S-AK and AlF\(^{3-}\)AK structures that contain sulfate. In the ATP-AK[A] structure, the sulfate is proposed to displace the \(\beta\)-phosphate and the inferred \(\gamma\)-phosphate of ATP from the catalytically competent position. Furthermore, the \(\gamma\)-phosphate of TPP in the active site was in the approximate position of the sulfate in the ATP-AK structure, consistent with the previous proposal that sulfate occupies the position of the \(\gamma\)-phosphate of ATP (7). Although the ATP\(^\gamma\)S-AK[B] structure indicates contact of the \(\gamma\)-phosphate of TPP with Arg\(^{241}\), kinetic analysis of the R241A variant suggests that this residue is not involved in binding ATP; instead, the kinetic results suggest that Arg\(^{241}\) binds acetate.

The AlF\(^{3-}\)AK[B] structure contained acetate in the active site, the first reported for any acetate kinase structure, positioned in a hydrophobic pocket as previously postulated (7) and supported by recent kinetic analyses of site-specific replacement variants of the enzyme. The active site also contained AlF\(^{3-}\), shown previously to mimic the planar phosphoryl group derived from the \(\gamma\)-phosphate of NTPs in the catalytic transition state for a variety of kinases (16, 49–51). The proximity to the carboxyl group of acetate and the \(\beta\)-phosphate of ATP in the AlF\(^{3-}\)AK[B] structure suggests that AlF\(^{3-}\) also mimics the meta-phosphate transition state in a direct in-line phosphoryl transfer mechanism for the acetate kinase from *M. thermophila*. These results provide the first structural evidence supporting
the previously proposed direct in-line mechanism for acetate kinase (16, 18). The AlF₃ was positioned in the active site of the AlF₃-AK[B] structure approximately the same as the γ-phosphate of TPP and the inferred γ-phosphate of ATP.

The direct in-line mechanism predicts a requirement for residues to stabilize the trigonal bipyramidal phosphate transition state by coordination with the three equatorial oxygen atoms. Residues Arg²⁴¹ and Arg⁹¹ are candidates, based on the kinetic analyses of variants presented here. A role for His¹₈⁰ in stabilizing the transition state has been proposed based on

**FIG. 8.** Postulated mechanism of acetate kinase from *M. thermophila* for the forward (AcP-producing) reaction direction. A, ATP and acetate substrate interactions with acetate kinase; B, interactions for the proposed direct in-line mechanism transition state; C, interactions of the products ADP and AcP with acetate kinase. The arrows indicate direction of electron movement described under “Discussion.”
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kinetic analysis of replacement variants of the M. thermophila acetate kinase (18, 20). Although in close proximity, neither Arg241 or His180 was within strict bonding distance to AlF3. However, in a true transition state for an S_{2}2 reaction, the plane of AlF3 is perpendicular to the plane connecting the substrate and product (42, 52–54). This preferred orientation of the plane of AlF3 relative to ADP and acetate was not observed in the AlF3-AK[B] structure, indicating that the structure does not accurately indicate all contacts that would be expected if it truly captured the transition state. Nonetheless, the close proximities of His180 and Arg241 to AlF3 are consistent with roles for these residues in stabilization of a meta-phosphate transition state. The proximity of Arg241 to the carboxyl group of acetate is also consistent with a role for this residue in binding acetate consistent with the kinetic results reported here. An interpretation of the position of Arg91 relative to AlF3 and acetate is less straightforward. The 7-Å distance of the side chain of Arg91 from acetate and AlF3 is inconsistent with the kinetic results, which suggest a role for acetate binding, and with the previously proposed role for stabilization of the meta-phosphate transition state during catalysis (18). As previously discussed, AlF3 was not found in the expected orientation for a true transition state analog, indicating that this structure may not accurately reflect the position of Arg91 during substrate binding or catalysis.

The new structures also indicate novel interactions involving the catalytically essential active site residues Glu384 and Asp148 (19, 34) located in the domain connection motifs (7, 16). Although the role of Asp148 is unknown, Glu384 is implicated in Mg2+ binding (34). In monomer B of both structures, Glu384 and Asp148 are linked through hydrogen bonds to Thr182. Residue Glu384 is located in the α4 helix of the C-terminal “body” domain, whereas Thr182 is located in the α3 helix in the N-terminal “wing” domain (7). Hydrogen bonding has been shown for the corresponding α3 helices in other ASKHA family members, where it facilitates the domain closure necessary to prevent ATP hydrolysis and promote phosphoryl transfer (43, 55, 56). Therefore, a postulated role for Glu384 is to facilitate closure of the “wing” onto the “body” via an interaction of Glu384 and Mg2+ with Thr182 upon nucleotide binding. Thr182 was found to be highly conserved in the 219 acetate kinase sequences available from the data bases, strongly indicating an essential role for this residue. An indication of further side chain movements required for acetate kinase activity is found in the AlF3-AK[B] structure in the rearrangement of side chains in the β4-β5 loop that connects the two domains (Fig. 7).

Proposing a Catalytic Mechanism—Fig. 8A shows substrates in the active site poised for catalysis in the direction of acetate synthesis. As previously proposed (7), and further supported by the new structures reported here, ATP binding is solely through hydrophobic interactions with the adenine base (not shown). His180 is presented in Fig. 8A interacting with the γ-phosphate of ATP, and Asn7 and Glu384 are shown interacting with the magnesium of ATP in a bidentate coordination as previously suggested for the enzyme from E. coli (11, 16, 41). Acetate is shown with the methyl group bound in the hydrophobic pocket formed by Val193, Pro232, and the carboxylate group interacting with Arg91 and Arg241. Once the substrates have bound in the active site, there is a postulated domain closure as demonstrated for other members of the ASKHA superfamily to position the reactants and exclude water from the active site (43). The mechanism is proposed to proceed via an S_{2}2 nucleophilic attack of the carboxyl group of acetate on the γ-phosphate of ATP through the trigonal bipyramidal transition state shown in Fig. 8B. The evidence reported here supports stabilization of this transition state by coordination of the equatorial oxygen atoms through interactions with His180 and Arg241, and previous studies with the enzyme from E. coli have suggested that the continued bidentate coordination of magnesium during phosphoryl transfer provides further stabilization (11, 41). Upon collapse of the transition state, the γ-phosphate of ATP is formally transferred to acetate, forming ADP and acetyl phosphate as shown in Fig. 8C, with ADP being released from the active site with magnesium in a monodentate coordination with the β-phosphate. Acetyl phosphate is proposed to bind in a similar fashion to acetate, and kinetic evidence suggests an interaction between His180 and the phosphoryl group of acetyl phosphate, which could be significant for acetyl phosphate binding, orientation of the phosphate group for attack by ADP, or both.

Conclusions—The data presented here add to the structural and mechanistic understanding of acetate kinase, the founding member of the ASKHA superfamily, and strongly support a direct in-line transfer of the γ-phosphoryl group of ATP to acetate. The AlF3-AK structure revealed acetate in the proposed binding pocket for the first time and furthermore contained the transition state analog AlF3 proximal to Arg241 and His180, two residues with potential to stabilize the meta-phosphate transition state during catalysis. Indeed, kinetic results suggested that Arg241 is a catalytically essential residue. Kinetic analyses also suggest roles for Arg241 and Arg244 in binding or positioning acetate. Different conformations between the monomers were found in both of the structures, implying a potential role for domain closure during catalysis. Hydrogen bond interactions revealed in the new structures indicate that Asp148 and Glu384 could potentially play a role in domain closure and identified Thr182 as an additional residue for further investigation. As the proposed founding member of the ASKHA structural superfamily (7), the results presented here broaden understanding of the superfamily and provide a foundation for continuing investigations.

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