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Investigation of the *Methanosarcina thermophila* Acetate Kinase Mechanism by Fluorescence Quenching *

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Abstract

Acetate kinase, a member of the acetate and sugar kinase/Hsc 70/actin (ASKHA) structural superfamily, catalyzes the reversible transfer of the γ -phosphoryl group from ATP to acetate yielding ADP and acetyl phosphate. A catalytic mechanism for the enzyme from *Methanosarcina thermophila* has been proposed based on the crystal structure and kinetic analyses of amino acid replacement variants. The Gln⁴³Trp variant was generated to further investigate the catalytic mechanism *via* changes in fluorescence. The dissociation constants for ADP:Mg²⁺, ATP:Mg²⁺ were determined for the Gln⁴³Trp variant and double variants generated by replacing Arg²⁴¹ and Arg⁹¹ with Ala and Lys. The dissociation constants and kinetic analyses indicated roles for the arginines in transition state stabilization for catalysis but not in nucleotide binding. The results also provide the first experimental evidence for domain motion, and that catalysis is not as two independent active sites of the homodimer but the active site activities are coordinated in a half-the-sites manner.

Acetate kinase [E.C. 2.7.2.1] is a homodimer which catalyzes the reversible magnesium-dependent transfer of the γ -phosphoryl group from ATP to acetate, yielding ADP and acetyl phosphate ($\text{CH}_3\text{COO}^- + \text{ATP} \rightleftharpoons \text{CH}_3\text{CO}_2\text{PO}_3^{-2} + \text{ADP}$). The enzyme is widely distributed among fermentative prokaryotes in the Bacteria domain where it functions together with phosphotransacetylase ($\text{CH}_3\text{CO}_2\text{PO}_3^{-2} + \text{HS-CoA} \rightleftharpoons \text{CH}_3\text{COSCoA} + \text{HPO}_4^{-2}$) to convert acetyl-CoA to acetate and synthesize ATP. These enzymes also function to activate acetate to acetyl-CoA in the first step of the pathway for conversion of the methyl group to methane by *Methanosarcina* species from the Archaea domain (1).

Though acetate kinase was one of the earliest phosphoryl transfer enzymes to be identified (2) and investigated, a debate persisted in the literature until recently whether the catalytic mechanism is a triple displacement of the phosphate involving two covalent enzyme intermediates (3) or a direct in-line phosphoryl transfer (4,5). Support for the direct in-line mechanism accrues from studies with the acetate kinase from *Methanosarcina thermophila* (6-12). The enzyme co-crystallized with ADP, Al³⁺, F⁻, and acetate, shows a linear array of ADP-AlF₃-acetate in the active site cleft wherein the AlF₃ is proposed to mimic the *meta*-phosphate transition state (11). Kinetic analyses of Arg²⁴¹ and Arg⁹¹ replacement variants indicate these active site residues are essential for catalysis and important for binding acetate (11). In a direct in-line phosphoryl transfer, the hypothesized transition state is a trigonal bipyramidal structure of the γ -phosphoryl group, with the axial positions occupied by β -phosphoryl and acetyl oxygens. The guanidino groups of Arg²⁴¹ and Arg⁹¹ are proposed to

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stabilize this structure by interacting with oxygen atoms of the γ -phosphoryl and carboxyl groups of acetate (11). The guanidino group of Arg²⁴¹ is adjacent to AlF₃ consistent with the proposed role. However, the guanidino group of Arg⁹¹ is displaced 7 Å from AlF₃ placing doubt on the proposed role, although the structure may not accurately represent the position of Arg⁹¹ during catalysis (11).

Based upon structural similarities of the *M. thermophila* enzyme, acetate kinase was identified as a member of the acetate and sugar kinase/Hsc70/actin (ASKHA) structural superfamily (12). Each monomer of the homodimeric acetate kinase is characterized by a duplicated central β -sheet surrounded by α -helices ($\beta\beta\alpha\beta\alpha\beta\alpha$ core) and consisting of two domains (Fig. 1). The sugar kinase and actin family members are all known to undergo a catalytically essential domain closure upon ligand binding (13-18). Although there is no experimental evidence to support domain closure during catalysis of acetate kinase from *M. thermophila*, domain closure has been suggested to participate in stabilization of the transition state based upon the architecture of the crystal structure (12).

The *M. thermophila* acetate kinase has no tryptophan residues which has precluded intrinsic fluorescence measurements as an approach to investigate the catalytic mechanism. Here we report K_d values for ADP:Mg and ATP:Mg for the *M. thermophila* acetate kinase enabled by fluorescence quenching of the Gln⁴³Trp variant. Double variants, with either Arg²⁴¹ or Arg⁹¹ replaced in the Gln⁴³Trp variant, had K_d values consistent with previously proposed roles for these arginines in catalysis and substrate binding. The Gln⁴³Trp variant has also provided evidence for domain motion with implications for the catalytic mechanism. The results indicate that catalysis is not as two independent active sites, but that the active site activities are coordinated in a half-the-sites manner.

Materials and Methods

Materials

Chemicals were purchased from Sigma Chemical, VWR Scientific Products, or Fisher Scientific. Oligonucleotides for DNA sequencing and site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). ATP and ADP solutions were adjusted to pH 7.0 with sodium hydroxide, and concentrations determined utilizing the extinction coefficient ($\epsilon_{259} = 15.4 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$). All ATP and ADP solutions were equimolar with magnesium chloride. Acetate (Ac) stock solutions were adjusted to pH 7.0 with sodium hydroxide. Acetyl phosphate (AcP) solutions were made fresh daily, and concentrations were verified *via* kinetic assay ((19)) from maximal change in absorbance.

Site-directed Mutagenesis

Mutagenesis was performed by the oligonucleotide-directed *in vitro* mutagenesis method (20) using the QuikChange mutagenesis kit (Stratagene). The plasmid pET-AK, a derivative of pET-15b with the *M. thermophila ack* gene from pML703 (21) in the multicloning site (7), was the target for mutagenesis. Mutations were verified by dye termination cycle sequencing using an ABI PRISM 377 DNA sequencer (Applied Biosystems) at the Nucleic Acid Facility at Pennsylvania State University.

Heterologous Production and Purification of Acetate Kinases

The wild-type and variant acetate kinases were overproduced in *E. coli* BL21(DE3) (F⁻ *dcm ompT hsdS* ($r_B^- m_B^-$) *gal* λ (DE3)) and purified as described previously (21). All variants were further purified *via* gel filtration in a 100 mL Sephadex G-200 column (1.6 cm diameter) 1.0 mL flow rate. Protein purity was examined by SDS-polyacrylamide gel electrophoresis (22)

and protein concentrations determined by the Bradford method (23) using Bio-Rad dye reagent with bovine serum albumin as the standard.

Kinetic Parameters of Wild-type and Variant Acetate Kinases

Kinetic parameters for wild-type and variant acetate kinases were determined as previously described with assays performed utilizing the coupled assay systems for both the ADP/AcP synthesizing (11) and ATP/Ac synthesizing (19,24) reaction directions holding the concentration of the non-variable substrate at 10X its K_m . Data was fit using non-linear regression analysis (Kaleidagraph, Synergy Software), with the exception of Arg⁹¹Ala/Gln⁴³Trp variant. Due to inability to saturate substrate at 5 M acetate concentrations for the Arg⁹¹Ala/Gln⁴³Trp variant, when $K_m^{\text{ATP:Mg}}$ was determined data was fit using Lineweaver-Burk regression analysis, holding acetate at 2 M.

Steady-State Fluorescence Measurements

The intrinsic fluorescence of wild-type and variant acetate kinases was determined using a Hitachi F-200 fluorometer, 1 cm pathlength cuvettes, with 0.07 to 1 μM enzyme, 50 mM Tris, 130 mM NaCl, 10 mM MgCl_2 , total volume of 2 mL. Initial scans of the wild-type and tryptophan variants were taken at a constant 20°C using a circulating water bath, with 2 nm steps for both excitation and emission wavelengths with each data point an average of 10 measurements. Fluorescence was measured in arbitrary intensity units.

Equilibrium Binding Studies

The equilibrium dissociation constants (K_d) of the Gln⁴³Trp and Gln⁴³Trp double variants were determined by measuring the observed decrease in fluorescence intensity upon addition of the ligand. Ligands utilized were ATP:Mg and ADP:Mg. Buffer conditions were as described in the above section for steady-state fluorescence measurements, where excitation and emission wavelengths were 295 and 340 nm. Each enzyme was held at the same concentration used for kinetic assay (see above) and was titrated with each nucleotide ligand, measuring the fluorescence intensities after an incubation time of 10 minutes. The time was chosen as equilibrium where no further change in fluorescence was observed. ATP:Mg hydrolysis by acetate kinase over the time frame of the binding studies was insignificant (data not shown). All fluorescence intensities were corrected for protein dilution.

Results

Generation of the Fluorescent Variant

As the *M. thermophila* acetate kinase has no tryptophan, several residues (Tyr¹⁵, Gln⁴³, Phe¹⁵¹, and Phe²⁵²) were individually replaced with tryptophan to generate variants to investigate the catalytic mechanism *via* fluorescence quenching. Selection of residues for replacement was based on three criteria: *i*) location near the substrate binding site, *ii*) space for a tryptophan residue, and *iii*) probability of environmental change. Further, the *Escherichia coli* K12 acetate kinase contains a single tryptophan residue (Trp⁴⁶) in the equivalent position of Gln⁴³ in the *M. thermophila* enzyme.

Wild-type and all variant enzymes were over-expressed in *E. coli* BL21(DE3) and purified to apparent homogeneity as judged by SDS-PAGE (data not shown). The yields of the purified variants were similar to that of the wild-type enzyme and native gel filtration chromatography indicated that all variants were dimeric in accordance with the wild-type (data not shown). These results indicate that the variants were not compromised by major structural changes.

The $K_m^{\text{ATP:Mg}}$ and K_m^{Ac} values for the variants were compared to the wild-type values to determine the affect of the replacements on the kinetic parameters. The parameters of the

Tyr¹⁵Trp, Phe¹⁵¹Trp, and Phe²⁵²Trp variants deviated too far from wild-type to be valid experimental tools (data not shown). However, the differences in kinetic parameters of the Gln⁴³Trp variant vs. wild-type enzyme (Tables 1 and 2) were not large enough to preclude the use of this variant to investigate the catalytic mechanism *via* fluorescence quenching.

An λ_{max} for excitation of 295 and λ_{max} emission of 340 nm was determined for both the Gln⁴³Trp variant and wild-type acetate kinase (Fig. 2). However, the intensity of the variant was increased several fold over the wild-type.

Kinetic Analysis of Double Variant Acetate Kinases

The fluorescent Gln⁴³Trp variant was used to generate double variants by replacement of Arg⁹¹ or Arg²⁴¹ with Ala and Lys to probe the roles of these residues. Each variant was purified to apparent homogeneity as judged by SDS-PAGE. The yields and properties of the variants were similar to wild-type (data not shown) indicating that the variants were not compromised by major structural changes.

For the ADP/acetylphosphate producing direction (Table 1), all the double variants showed a substantial decrease in k_{cat} vs. the Gln⁴³Trp variant commensurate with previously published values for the single Arg⁹¹ and Arg²⁴¹ variants vs. wild-type that further supports the previously proposed role for these arginines interacting with the *meta*-phosphate and stabilizing the transition state (11). In most cases, the trends in differences for K_m values between the double variants and the Gln⁴³Trp variant were consistent with trends between the single variants vs. wild-type which lead to the previous proposal that neither arginine binds ATP (11). However, exceptions were observed for the $K_m^{\text{ATP:Mg}}$ of the Arg⁹¹Ala/Gln⁴³Trp variant and the K_m^{Ac} of the Arg²⁴¹Ala/Gln⁴³Trp variant vs. the Gln⁴³Trp variant. The most straightforward explanation is that the Gln⁴³Trp substitution, together with the additional space created by substituting alanine, rearranged residues affecting the binding of ATP and acetate. The Arg²⁴¹Lys/Gln⁴³Trp variant also had a very high $K_m^{\text{ATP:Mg}}$ vs. the Gln⁴³Trp variant, a trend consistent with the Arg²⁴¹Lys vs. wild-type that was previously attributed to the larger lysine side chain sterically hindering ATP binding (11). Thus, the overall differences in $K_m^{\text{ATP:Mg}}$ values for the double variants vs. the Gln⁴³Trp variant do not contradict the previous proposal that neither arginine is important for binding ATP. Finally, with the exception of the Arg²⁴¹Ala/Gln⁴³Trp variant, the differences in K_m^{Ac} values for the double variants vs. the Gln⁴³Trp variant are consistent with the previously proposed role for both arginines in binding acetate based on previously published K_m^{Ac} values for the single variants vs. wild-type (11).

In the direction of ATP/acetate synthesis (Table 2), all the double variants showed large decreases in k_{cat} vs. the Gln⁴³Trp variant that were of similar magnitude as published values for the single variants vs. wild-type that support the previously proposed roles for these arginines in catalysis (11). The differences in K_m values for all the double variants vs. the Gln⁴³Trp variant were in accord with published differences between the cognate single variants vs. wild-type which lead to the previous conclusion that Arg⁹¹ and Arg²⁴¹ have no influence on binding of ADP or acetyl phosphate (11).

Determination of Nucleotide Binding Constants

The utility of the fluorescent Gln⁴³Trp variant to probe the catalytic mechanism was assessed by determining the ability of ATP:Mg, ADP:Mg and acetate to induce fluorescence quenching of the variant. The nucleotides alone caused quenching (data not shown) while acetate and acetyl phosphate was unable to cause quenching (data not shown).

Two possible schemes for binding of the nucleotides to the dimeric enzyme are shown in Figure 3. Scheme 1 indicates 2 equal independent binding sites per dimer each with the same affinity for the ligand, a version of the Stern-Volmer equation (25) (Eqn. 1),

$$\frac{\Delta F}{F_o} = \frac{(\Delta F_{\max}/F_o)L^n}{K_d + L^n} \quad \text{Eqn. 1}$$

Where F_o is the intrinsic fluorescence, ΔF is the change in fluorescence in presence of ligand, K_d is the dissociation constant, and L is concentration of ligand, or quencher. Initial fit of fluorescence data to the equation of two independent sites (Eqn. 1) showed that Scheme 1 did not fit the experimental data (data not shown). Scheme 2 indicates that binding to one site on the dimer influences ligand binding to the second site on the dimer, an expression analogous to the Hill equation (25). Note that Schemes 1 and 2 (Fig. 3) are for ligand binding, without enzymatic catalysis occurring. Thus, two binding sites with dissimilar equilibrium binding constants (K_{d1} and K_{d2} ; Fig. 3 Scheme 2) were considered for the dimeric enzyme.

Consequently, the equation to fit the fluorescence data was modified to incorporate the dissimilar binding constants. In the equations that follow, F_o is the total intrinsic fluorescence, $F_{o(1)}$ and $F_{o(2)}$ are the total intrinsic fluorescence intensities attributable to each site, F is the resultant fluorescence for the whole protein, $F_{(1)}$ and $F_{(2)}$ are the resultant fluorescence for sites 1 and 2, and L is the concentration of free ligand, or quencher (25). The expression for dissociation of ligand at binding site 1 is given in (i), with the expressions for total observed fluorescence (ii), total intrinsic fluorescence (iii) and observed fluorescence from each site (iv) and (v). With rearrangement of (ii) and substitution into (i) results in the expression for fluorescence from site 1 represented by (vi);

$$K_{d1} = \frac{[F_{(1)}L]}{[F][L]} \quad \text{(i)}$$

$$F_o = F_{o(1)} + F_{o(2)} \quad \text{(ii)}$$

$$F = F_{(1)} + F_{(2)} \quad \text{(iii)}$$

$$F_{o(1)} = F_{(1)} + F_{(1)}L \quad \text{(iv)}$$

$$F_{o(2)} = F_{(2)} + F_{(2)}L_2 \quad \text{(v)}$$

$$F_{(1)} = \frac{F_{o(1)}}{1 + K_{d1}L} \quad \text{(vi)}$$

Binding to the opposite monomer is represented by expression (vii),

$$K_{d2} = \frac{[F_{(2)}L_2]}{[F_{(1)}L][L]} \quad \text{(vii)}$$

and substitution of (iv) and the fluorescence equivalents (ii to v) into the equation for the second binding site results in the expression of fluorescence from site 2 as (viii);

$$F_{(2)} = F_{o(2)} - K_{d1}K_{d2}L^2F_{(1)} \quad \text{(viii)}$$

Attributing the two fluorescence values (vi) and (viii) into the equation for fluorescence (ii) and solving for the change in fluorescence yields the following relationship:

$$\frac{\Delta F}{F_0} = f_1 \left(\frac{K_{d1}L + K_{d1}K_{d2}L^2}{1 + K_{d1}L} \right) \quad \text{Eqn. 2}$$

where f_1 is the fraction of enzyme which has ligand bound to site 1. If ligand does not bind site 2, or if the second site is indistinguishable from F_1 , then Eqn. 2 reduces to the equivalent of a single binding site (Eqn. 1). Fluorescence quenching data were fit with Eqn. 2 using nonlinear regression analysis (Kaleidagraph, Synergy Software) and fits for all variant acetate kinases are shown in Fig. 4 (A-E), with good agreement between Eqn. 2 and the experimental data. The K_d values for ATP:Mg, ADP:Mg determined from the fit for all double variants are tabulated in Tables 3 and 4. All variants exhibited two dissociation constants (K_{d1} and K_{d2}) for the nucleotides, designated 1 for the tight binding site (K_{d1}) and 2 for the loose binding site (K_{d2}), and a f_1 value indicating the fraction of enzyme with ligand bound to the first site. The results indicate that the two binding sites in the dimeric acetate kinase can be distinguished. Since the enzyme is a homodimer, the results suggest that binding of substrate to one active site of the enzyme affects the ability of substrate to bind the opposing identical monomer, matching the description for a half-the-sites active enzyme.

The K_{d1} determined for both ATP and ADP (Tables 3 and 4) for the Gln⁴³Trp variant are in agreement with the Michaelis constants for each nucleotide (Tables 1 and 2). The $K_{d1}^{\text{ATP:Mg}}$ and $K_{d1}^{\text{ADP:Mg}}$ values for both Arg²⁴¹ double variants vs. the Gln⁴³Trp variant (Table 3) are not significantly different indicating Arg²⁴¹ has no role in binding either nucleotide.

Arg⁹¹ was previously postulated to have roles in stabilization of the *meta*-phosphoryl transition state and orientation of acetate for catalysis with no role for binding ATP (11). Neither Arg⁹¹ double variant vs. the Gln⁴³Trp variant showed significant differences in the $K_{d1}^{\text{ADP:Mg}}$ values (Table 4). The Arg⁹¹Ala/Gln⁴³Trp variant showed relatively little difference in $K_{d1}^{\text{ATP:Mg}}$ values vs. the Gln⁴³Trp variant (Table 3) consistent with no role in binding ATP. However, the $K_{d1}^{\text{ATP:Mg}}$ for the Arg⁹¹Lys/Gln⁴³Trp variant was considerably larger than the Gln⁴³Trp variant which contradicts the existing evidence. One possible explanation is that replacement of Arg⁹¹ with Lys interferes with domain closure which prevents fluorescence quenching.

Discussion

Domain motion

The analysis of nucleotide binding constants indicated that binding to one site affects binding to the other, suggesting a half-the-sites mechanism. Half-the-sites activity was first defined by Koshland as proteins of identical subunits where only one-half of the available sites are occupied in the presence of ligand (26), and theoretically described by Hill as concerted isologous dimers, where the two conformations of the dimer are always different, and the subunits change conformation in concert (27). Thus, the results presented here further suggest domain motion during catalysis by acetate kinase.

Acetate kinase has been classified as a member of the ASKHA structural superfamily (12) based upon the architectural organization, however a second common feature of the superfamily members for which native and substrate bound structures are available is a domain movement upon substrate binding (13). The best characterized domain motion for catalytic activity is the domain closure of glycerol kinase, in which a sheer/sliding motion for domain closure for the ASKHA family has been defined (28). Domain motion in acetate kinase was postulated based upon the crystal structure (12), and supported by the kinetic characteristics of variants occurring at the domain I to II interface (Phe¹⁷⁹Ala and Leu¹²²Ala) (10). The recent *Salmonella typhimurium* propionate kinase structure (29) again generated neither an open or

closed structure, but an intermediate state in the catalytic mechanism. However, a model of domain motion based upon the small differences in the propionate kinase and the *M. thermophila* acetate kinase structures, suggested a potential change as much as 15 Å (29).

Fluorescence quenching occurs when the environment around a fluorescent moiety undergoes a change, resulting in the loss of the ability to release the absorbed energy as a photon of lower wavelength. Tryptophan fluorescence can be quenched when the environment changes from a hydrophobic to hydrophilic environment. The assumption for the fluorescent acetate kinase is that a fluorescence change would occur upon ligand binding. The quenching observed experimentally is through one of two methodologies, the nucleotide would directly interact with the engineered residue or a change in conformation must occur. Based upon Gln⁴³ side chain location buried in domain I, no direct interaction would be observed with a correctly folded protein. Thus, in the engineered acetate kinase, the tryptophan residue would fluorescence unbound (open) state, and be quenched in the bound (closed) state, as a more hydrophobic environment is generated when domain I closed onto domain II. This was observed with the fluorescence quenching of the Gln⁴³Trp enzyme in the presence of the adenine nucleotides. Thus, the hypothesis that domain II closes onto domain I during acetate kinase activity upon nucleotide binding is supported, and this is the first direct evidence that supports a domain closure in the acetate kinase catalytic mechanism.

Kinetic and Dissociation Constants of the Double Variants

The kinetic and dissociation constants determined for the double variants clarify roles for Arg⁹¹ and Arg²⁴¹. The large decrease in k_{cat} for all double variants relative to Gln⁴³Trp supports the previously proposed roles for Arg⁹¹ and Arg²⁴¹ in stabilization of the transition state by interaction with the *meta*-phosphate based on kinetic analyses of variant enzymes (11).

The results presented here indicate that neither Arg is important for recognition and binding of either ATP or ADP, consistent with the previous proposal that adenine ring interactions are the major contributors to nucleotide recognition and binding (12). The f_1 value indicates that while one monomer is ligand-bound, the other is free, consistent with a half-the-sites mechanism. The results also indicate that while the arginines are not involved in the nucleotide recognition, a portion of the binding energy must contribute to closure of the domain, as the double variant enzymes all have a smaller f_1 than the Gln⁴³Trp variant. From the crystal structure, it is known that a distance of 7.1 Å (11) exists between the location of the Arg⁹¹ side chain and the *meta*-phosphate analog AlF₃ (Fig. 1) which would require a substantial change for Arg⁹¹ to contact this phosphate and stabilize the transition state. The differential K_{d1}^{ADP} values for the Arg⁹¹Ala/Gln⁴³Trp to Arg⁹¹Lys/Gln⁴³Trp variants to the respective K_m^{ATP} and K_m^{ADP} values indicates a role in this conformational change. Thus, another role for Arg⁹¹ may be to facilitate domain closure driven by the energy of nucleotide binding that also positions Arg⁹¹ for interaction with this phosphate. The results suggesting that replacement of Arg⁹¹ with Lys interfered with domain closure is consistent with this proposal.

Implications to acetate kinase catalytic and kinetic mechanism

The nucleotide binding results, in conjunction with the known domain motion in the superfamily, suggests that domain closure occurs when substrate binds the active site which sequesters the substrates into a microenvironment where the arginine residues position the carboxyl group of acetate or the corresponding β -phosphate of ADP for nucleophilic attack on the γ -phosphate of ATP or phosphoryl group of acetyl phosphate. The implications for catalysis are that binding of the nucleotide induces a domain closure in that monomer, and catalysis occurs in the closed active site. With active sites in each monomer, three possibilities exist for catalysis. First, the two active sites are independent of one another, and no co-ordination exists between the active sites of the two monomers. The results presented here indicated two non-

identical binding sites argues against this possibility. Second, the two active sites act in a coordinated fashion where both active sites are in the same state, *i.e.* ligand binding to one active site will cause domain closure in both active sites so both monomers are always in the same conformation (open/open or closed/closed only). The third possibility is that again the sites are coordinated, but when one active site is occupied and closed, the opposing monomer is in an open conformation (open/closed), the definition of the half-the-sites mechanism (26). As products are released from one site, the opposing site can now close, the dimer always having one occupied and one unoccupied site. The coordination can be postulated to occur by two mechanisms. In the first mechanism, once product is formed, substrate binding and subsequent domain closure of the opposite monomer contributes to the domain opening and release of product in the first active site. In the second, substrate is bound to the open active site and the release of product induces the closure of the opposing monomer which is already primed for activity. The highly divergent binding constants (K_{d1} vs. K_{d2}) indicate that nucleotide substrates are not bound to both active sites concurrently, a result that argues against the first coordinated scheme (open/open) and supports the second (open/closed). Further investigation is ongoing to distinguish between the two types of coordination in the acetate kinase mechanism.

Conclusions

The binding constants for the nucleotide substrates indicates that Arg²⁴¹ is involved in transition state stabilization and not directly involved in nucleotide recognition or binding, nor in the domain closure required for catalysis. The binding constants of the nucleotide substrates for Arg⁹¹ suggest that this residue has a role in transition state stabilization. For the first time, evidence for domain motion dependant upon nucleotide ligand binding is presented for acetate kinase, and suggests that Arg⁹¹ is important for closure of domain I onto domain II for catalysis. A scheme for coordinated activity between the two active sites in the dimer has been suggested, indicating that acetate kinase follows a half-the-sites activity and presents an avenue for further investigation into the coordination.

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Abbreviations used are

ASKHA	acetate and sugar kinase/Hsc70/actin
AK	acetate kinase
AcP	acetyl phosphate
Ac	acetate

Figure 1.

A. Structure of the *M. thermophila* acetate kinase (1TUY, (11)). The dimer is shown with monomer A (ribbon representation) in rainbow hue from the blue N-terminus to the red C-terminus. Monomer B (wire representation) with domain I (residues 1-148 and 383-398) in marine and domain II (residues 149-382) in magenta. Shown in ball-and-stick representation in monomer B are the active site Arg⁹¹ and Arg²⁴¹ residues in CPK colors, and transition state analogue components ADP, AlF₃ and acetate in CPK colors except Al in orange and F in cyan. Residue Gln⁴³ is shown in ball-and-stick colored CPK in domain I of monomer B.

Figure 2.

Excitation and emission spectra of acetate kinases. Symbols: wild-type (excitation ○, emission ■); Gln⁴³Trp variant (excitation □, emission ●). Scans were performed with 6.5 μg/ml protein, 50 mM Tris (pH 7.4), and 40 mM NaCl in a 1 cm path-length cuvette. Excitation was monitored at 340 nm and emission at 295 nm. a.u. = arbitrary units.

Figure 3. Schemes for nucleotide binding to acetate kinase. Scheme 1, equivalent binding to either monomer, or independent active sites. Scheme 2, non-equivalent ligand binding where binding to one monomer effects binding to second monomer, or coordinated active sites.

Figure 4. Nucleotide-dependent fluorescence quenching of acetate kinase variants. Panel A, Gln⁴³Trp variant; Panel B, Arg⁹¹Ala/ Gln⁴³Trp variant; Panel C, Arg⁹¹Lys/Gln⁴³Trp variant; Panel D, Arg²⁴¹Ala/ Gln⁴³Trp variant; Panel E, Arg²⁴¹Lys/ Gln⁴³Trp variant. Symbols: ATP:Mg²⁺ (○); ADP:Mg²⁺ (□).

Table 1

Kinetic parameters of wild-type and variant acetate kinases assayed in the direction ADP and acetyl phosphate synthesis.

Enzyme	k_{cat} (s ⁻¹)	$K_m^{ATP:Mg}$ (μM)	K_m^{Ac} (mM)
Wild-type*	913 ± 52	80 ± 9	2.7 ± 0.3
Gln ⁴³ Trp	699 ± 24	53 ± 3	12.4 ± 0.3
Arg ⁹¹ Ala*	0.11 ± 0.004	16 ± 0.6	250 ± 38
Arg ⁹¹ Ala /Gln ⁴³ Trp	2.07 ^a	539 ± 32 ^b	12,000 ^a
Arg ⁹¹ Lys*	3.7 ± 0.18	76 ± 3	70 ± 3
Arg ⁹¹ Lys /Gln ⁴³ Trp	11.8 ± 0.53	234 ± 21	48 ± 5
Arg ²⁴¹ Ala*	0.68 ± 0.1	297 ± 27	710 ± 130
Arg ²⁴¹ Ala /Gln ⁴³ Trp	1.83 ± 0.3	80.5 ± 14	5.8 ± 0.95
Arg ²⁴¹ Lys*	1.3 ± 0.06	11,400 ± 1,900	77 ± 7
Arg ²⁴¹ Lys /Gln ⁴³ Trp	0.40 ± 0.05	1,300 ± 130	179 ± 46

* previously published (11)

^a From Lineweaver-Burk plots with 10 mM ATP:MgCl₂ as enzyme could not be saturated up to 5 M acetate.

^b From Michaelis-Menten plots at 2M acetate.

Table 2

Kinetic parameters of wild-type and variant acetate kinases assayed in the direction of ATP and acetate synthesis.

Enzyme	$k_{cat}(s^{-1})$	$K_m^{ADP:Mg} (\mu M)$	$K_m^{AcP} (mM)$
Wild-type*	1,255 ± 60	98 ± 7	0.47 ± 0.05
Gln ⁴³ Trp*	2,650 ± 370	173 ± 12	0.26 ± 0.06
Arg ⁹¹ Ala*	3.5 ± 0.8	63 ± 6	1.36 ± 0.2
Arg ⁹¹ Ala /Gln ⁴³ Trp	2.63 ± 0.3	147 ± 26	2.58 ± 0.2
Arg ⁹¹ Lys*	23 ± 0.2	168 ± 6	0.61 ± 0.06
Arg ⁹¹ Lys /Gln ⁴³ Trp	25.7 ± 1.7	164 ± 23	1.08 ± 0.08
Arg ²⁴¹ Ala*	4.5 ± 0.4	210 ± 28	0.84 ± 0.02
Arg ²⁴¹ Ala /Gln ⁴³ Trp	14.6 ± 1.6	239 ± 38	0.33 ± 0.03
Arg ²⁴¹ Lys*	4.5 ± 0.4	578 ± 21	0.92 ± 0.03
Arg ²⁴¹ Lys /Gln ⁴³ Trp	10.5 ± 1.3	692 ± 86	1.02 ± 0.11

* Previously published (11)

Table 3ATP:Mg²⁺ binding constants of acetate kinase variants.

Variant	f_1	K_{d1} (μM)	K_{d2} (μM)
Gln ⁴³ Trp	0.45 ± 0.04	38.6 ± 8.2	5,020 ± 1160
Arg ⁹¹ Ala/Gln ⁴³ Trp	0.21 ± 0.03	167 ± 50	4,506 ± 1200
Arg ⁹¹ Lys/Gln ⁴³ Trp	0.44 ± 0.26	2,190 ± 1,600	35,000 ± 12000
Arg ²⁴¹ Ala/Gln ⁴³ Trp	0.30 ± 0.02	30.7 ± 11	5,400 ± 1100
Arg ²⁴¹ Lys/Gln ⁴³ Trp	0.15 ± 0.04	118 ± 90	2,810 ± 1300

Table 4ADP:Mg²⁺ binding constants of acetate kinase variants.

Variant	f_1	K_{d1} (μ M)	K_{d2} (μ M)
Gln ⁴³ Trp	0.39 $\pm$ 0.02	138 $\pm$ 5	7,470 $\pm$ 1,320
Arg ⁹¹ Ala/Gln ⁴³ Trp	0.11 $\pm$ 0.02	221 $\pm$ 105	1,360 $\pm$ 401
Arg ⁹¹ Lys/Gln ⁴³ Trp	0.38 $\pm$ 0.05	193 $\pm$ 72	9,140 $\pm$ 3,200
Arg ²⁴¹ Ala/Gln ⁴³ Trp	0.25 $\pm$ 0.02	55.6 $\pm$ 13	3,590 $\pm$ 490
Arg ²⁴¹ Lys/Gln ⁴³ Trp	0.19 $\pm$ 0.04	256 $\pm$ 94	4,930 $\pm$ 2,460