Identification of Essential Arginines in the Acetate Kinase from *Methanosarcina thermophila*†

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ABSTRACT: Site-directed mutagenesis is a powerful tool for identifying active-site residues essential for catalysis; however, this approach has only recently become available for acetate kinase. The enzyme from *Methanosarcina thermophila* has been cloned and hyper-produced in a highly active form in *Escherichia coli* (recombinant wild-type). The role of arginines in this acetate kinase was investigated. Five arginines (R91, R175, R241, R285, and R340) in the *M. thermophila* enzyme were selected for individual replacement based on their high conservation among sequences of acetate kinase homologues. Replacement of R91 or R241 with alanine or leucine produced variants with specific activities less than 0.1% of the recombinant wild-type enzyme. The circular dichroism spectra and other properties of these variants were comparable to those of recombinant wild-type, indicating no global conformational changes. These results indicate that R91 and R241 are essential for activity, consistent with roles in catalysis. The variant produced by conservative replacement of R91 with lysine had approximately 2% of recombinant wild-type activity, suggesting a positive charge is important in this position. The K_m value for acetate of the R91K variant increased greater than 10-fold relative to recombinant wild-type, suggesting an additional role for R91 in binding this substrate. Activities of both the R91A and R241A variants were rescued 20-fold when guanidine or derivatives were added to the reaction mixture. The K_m values for ATP of the rescued variants were similar to those of recombinant wild-type, suggesting that the rescued activities are the consequence of replacement of important functional groups and not changes in the catalytic mechanism. These results further support roles for R91 and R241 in catalysis. Replacement of R285 with alanine, leucine, or lysine had no significant effect on activity; however, the K_m values for acetate increased 6−10-fold, suggesting R285 influences the binding of this substrate. Phenylglyoxal inhibition and substrate protection experiments with the recombinant wild-type enzyme and variants were consistent with the presence of one or more essential arginine residues in the active site as well as with roles for R91 and R241 in catalysis. It is proposed that R91 and R241 function to stabilize the previously proposed pentacoordinate transition state during direct in-line transfer of the γ-phosphate of ATP to acetate. The kinetic characterization of variants produced by replacement of R175 and R340 with alanine, leucine, or lysine indicated that these residues are not involved in catalysis but fulfill important structural roles.

Phosphoryl transfer is one of the most common enzymatic functions and is essential for biological processes such as signal transduction as well as energy storage and utilization. Acetate kinase, which catalyzes the transfer of the γ-phosphate of ATP to acetate, exemplifies the importance of this reaction in nature. In the anaerobic decomposition of biomass, fermentative microbes first convert complex organics to acetate, which is then utilized by methane-producing microbes (*1*).

$$CH_3CO_2PO_3^{2-} + ADP \rightleftarrows CH_3COO^- + ATP \quad (1)$$

Fermentatives convert the metabolic intermediate acetyl phosphate to acetate, catalyzed by acetate kinase (reaction 1), which generates the majority of ATP for biosynthesis. In the reversal of this reaction, the genus *Methanosarcina* utilizes acetate kinase to activate acetate which is then cleaved into methyl and carbonyl groups (*2*). The electrons gained by oxidation of the carbonyl group to carbon dioxide are used to reduce the methyl group to methane. Energy for ATP synthesis is derived from the coupling of this reduction through a membrane-bound electron transport chain to an electrochemical ion gradient. Thus, acetate kinase is at the interface of energy-yielding metabolism between fermentatives and methane-producing microbes. Acetate kinase plays

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other key roles in the physiology of procaryotes. The phosphoryl-enzyme intermediate of acetate kinase is a phosphoryl donor to enzyme I of the phosphoenolpyruvate: glucose phosphotransferase system of *Escherichia coli* and *Salmonella typhimurium* (*3*). Furthermore, it has been proposed that acetyl phosphate is a global regulatory signal in *E. coli* (*4*).

Although most fermentatives are classified in the Bacteria domain, their acetate kinases have high identity with the enzyme from *Methanosarcina thermophila* (Archaea domain), suggesting similar catalytic mechanisms (*5*).

Despite a significant amount of research on the *E. coli* acetate kinase, the catalytic mechanism is still unknown, although two diverse mechanisms have been proposed. The first involves a direct in-line transfer of the γ-phosphate of ATP to the phosphoryl acceptor acetate (*6*). The second is a triple displacement mechanism involving two covalent phosphoryl-enzyme intermediates (*7*). In this mechanism, the γ-phosphate of ATP is successively transferred to two separate sites on the enzyme before final transfer to acetate. As for phosphoryl transfer enzymes in general, it is also unknown if phosphate transfer is associative or dissociative for acetate kinase. The associative mechanism involves a pentacoordinate transition state that bears a total of three negative charges, one on each equatorial oxygen (*8*). In the dissociative mechanism, bonds are broken before new bonds are formed, and only one negative charge is distributed among the oxygens of the metaphosphate intermediate in addition to a negative charge on the ATP β-phosphate (*8*). The locations of positively charged residues in the active site are expected to reflect the charge distribution during transfer of the phosphoryl group favoring either the associative or the dissociative mechanism. Thus, identifying essential amino acid residues involved in catalysis and transition state stabilization is a first step in understanding phosphoryl transfer in acetate kinase. Irrespective of the mechanism, inhibition studies suggest at least one unspecified arginine residue is essential for the acetate kinase from *E. coli* (*9*). Acetate kinase is a member of the sugar kinase/hsc70 protein/actin superfamily (*10*). In only one member of this superfamily, human brain hexokinase, has a specific arginine (R539) been identified as essential for catalysis. Arginine 539 is proposed to stabilize the transition state product, ADP-hexokinase (*11*). Understanding the residues essential for the acetate kinase catalytic mechanism will have a broad impact on understanding phosphoryl transfer in this superfamily.

Site-directed mutagenesis is a powerful tool for identifying active-site residues essential for catalysis; however, this approach has only recently been available for any acetate kinase. The enzyme from *M. thermophila* has been cloned and hyper-produced in a highly active form in *E. coli* (*12*). This development has led to site-directed mutagenesis studies to identify glutamate residues with catalytic functions (*5*). Here we identify two essential active-site arginines (R91 and R241) and propose roles for these residues in transition state stabilization and binding of substrate in a catalytically competent manner.

EXPERIMENTAL PROCEDURES

Protein Sequence Analyses. Databases were searched at the National Center for Biotechnology Information using the BLAST network server (*13*). Clustal X (*14*) was used for multiple protein sequence alignment.

Site-Directed Mutagenesis. Mutagenesis was performed according to manufacturer's instructions using the Muta-Gene phagemid mutagenesis kit (Bio-Rad) which employs the oligonucleotide-directed in vitro mutagenesis method (*15*). The *M. thermophila* acetate kinase gene was excised from pUC19/*ack* (*12*) by *Kpn*I and *Bam*HI digestion and ligated into pTZ18U (Bio-Rad) to generate pTZ*ack*. The mutations were verified by double-stranded sequence analysis using the dideoxy chain termination method (*16*) and Sequenase version 2.0 (United States Biochemicals).

Heterologous Production and Purification of Acetate Kinase. The wild-type and mutant acetate kinase genes were subcloned into the T7-based expression vector pET15b (Novagen). In these plasmids, a 60-nucleotide leader sequence with 6 tandem histidine codons was fused in-frame to the 5′ end of the wild-type and mutant *ack* genes. *E. coli* BL21(DE3) was transformed with the expression vectors, inoculated into 50 mL of Luria−Bertani medium containing 100 μg/mL ampicillin, and grown at 37 °C to an $A_{600\ nm}$ of 0.6−0.9 at which time IPTG was added to a final concentration of 1 mM. After 1.5−2.0 h induction, the cells were harvested and stored at −70 °C. The recombinant wild-type and variant acetate kinases were purified using a Ni−nitrilotriacetic acid silica spin kit (QIAGEN) according to manufacturer's instructions. The enzymes were eluted in buffer (pH 7.0) containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole. For large-scale purification of the R91A and R241A variants, 2 L cultures were grown as described above, and the enzymes were purified as previously described (*12*). Protein concentrations were determined by the Bradford method (*17*), using protein dye reagent (Bio-Rad) and bovine serum albumin as the standard.

Enzyme Activity Assays. Acetate kinase activity was determined by the previously described (*18*) hydroxamate assay which detects the formation of acetyl phosphate from acetate and ATP. When determining the K_m for acetate, the concentration of ATP was 10 mM. When determining the K_m for ATP, the acetate concentration was 200 mM unless the K_m for acetate was determined to be higher than for the recombinant wild-type enzyme in which case the concentration of acetate was increased to 1.5 M.

Inhibition by Phenylglyoxal. Acetate kinase was incubated at 37 °C in 50 mM triethanolamine buffer (pH 7.6) with phenylglyoxal (final concentration, 10 mM) in a final volume of 100 μL. Aliquots (10 μL) were removed at the indicated times and assayed for kinase activity. Substrate protection experiments were performed by preincubating the enzyme for 5 min at 37 °C with (final concentrations) ATP (10 mM) or potassium acetate (200 mM) prior to addition of phenylglyoxal. The decrease in activity during incubation at 37 °C (a control in which no phenylglyoxal was added) was taken into account when the inactivation at each concentration of phenylglyoxal was determined.

Chemical Rescue by Guanidine and Derivatives. The previously described hydroxamate assay reaction mix (*18*) was modified to include the indicated concentrations of guanidine hydrochloride or derivatives. Enzyme was added to the reaction mix and assayed at 37 °C. When determining the K_m for ATP in the presence of 300 mM guanidine, the

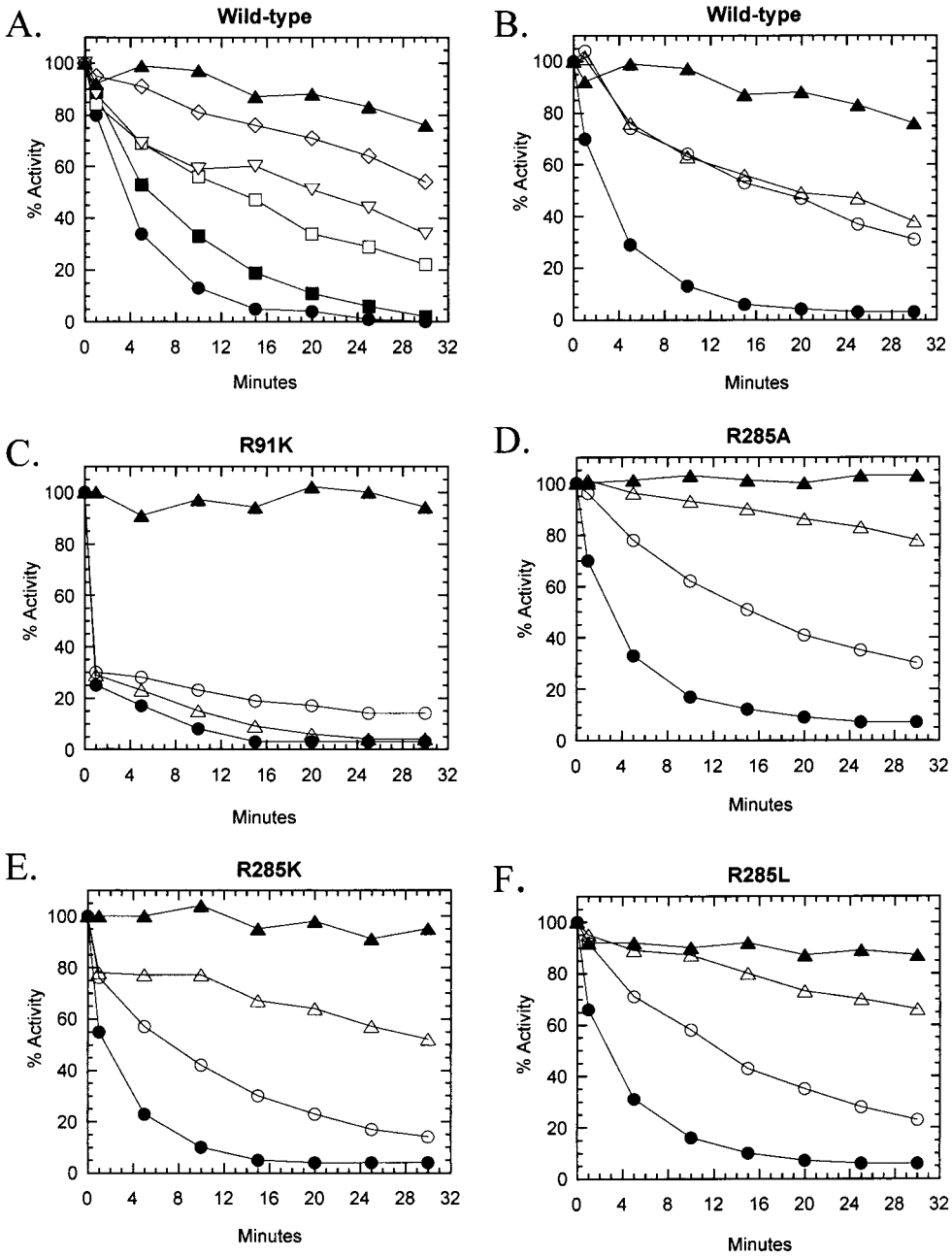

FIGURE 1: Inactivation of recombinant wild-type and variant acetate kinases by phenylglyoxal. Panel A: The recombinant wild-type acetate kinase was incubated at 37 °C with various concentrations of phenylglyoxal: none (▲), 0.5 mM (◇), 1 mM (▽), 2 mM (□), 4 mM (■), and 8 mM (●). The enzyme activity was determined after addition of phenylglyoxal at the indicated time intervals. Panels B−F: Activity was determined at the indicated times after addition of phenylglyoxal (final concentration 10 mM) to recombinant wild-type and variant acetate kinases preincubated with 10 mM ATP (○), 200 mM potassium acetate (△), or no substrate (●). Control without inhibitor (▲).

concentration of acetate was increased to 500 mM or 1 M as indicated.

Circular Dichroism Spectroscopy. Spectra were acquired at 25 °C with an Aviv circular dichroism spectrometer, model 62DS. Samples (1−10 μM) of acetate kinase in 25 mM Tris (pH 7.2) were placed in a cuvette with a 1 mm path length and data points obtained from 200 to 300 nm in 1.0 nm increments (5 s averaging time). The resulting spectra were normalized for direct comparison.

Molecular Mass. SDS/PAGE was performed as described previously (*19*) using 12% gels. The native molecular mass was determined by gel filtration chromatography, using a Superose 12 gel filtration column (Pharmacia) calibrated with cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). Protein samples (1.0 mL) were loaded onto the column preequilibrated with 145 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl, and the column was developed with a flow rate of 0.5 mL/min.

RESULTS

Inactivation of Recombinant Wild-Type Acetate Kinase by Phenylglyoxal. The recombinant wild-type acetate kinase from *M. thermophila* was rapidly inactivated ($t_{1/2}$ = 3.0 min, at 8 mM) in a concentration-dependent manner by phenylglyoxal (Figure 1A) which is highly specific for arginine (*20*). Preincubation with ATP or acetate protected against inactivation (Figure 1B). These results indicate that one or

Table 1: Kinetic Constants of Recombinant Wild-Type and Variant *M. thermophila* Acetate Kinases

	acetate			ATP		
enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
wild-type	19 ± 3	1596 ± 140	84	1.0 ± 0.3	1669 ± 288	1669
R91K	260 ± 35	13.5 ± 0.3	0.1	3.9 ± 0.2[a]	16.7 ± 0.2	4
R175K	4 ± 2	70 ± 18	18	1.0 ± 0.5	73 ± 17	73
R285A	206 ± 25	216 ± 25	1	6.0 ± 1.0[a]	302 ± 38	50
R285K	219 ± 148	234 ± 41	1	3.0 ± 1.0[a]	262 ± 22	87
R285L	129 ± 7	186 ± 17	1	3.0 ± 1.0[a]	224 ± 48	75
R340K	42 ± 12	1014 ± 15	24	1.3 ± 0.2[a]	1179 ± 182	907
R340L	20 ± 3	631 ± 158	32	0.9 ± 0.2	584 ± 63	649

[a] Determined at 1.5 M potassium acetate.

more arginines in the active site are required for catalysis.

Generation of Arginine Variants and Initial Characterizations. A search of the databases identified 49 acetate kinase sequences from diverse prokaryotes with identities to the *M. thermophila* enzyme that range from 37 to 65%. Five arginines in the *M. thermophila* acetate kinase are highly conserved with the homologous sequences. Residue R91 from *M. thermophila* is 100% conserved, R241 and R285 are conserved in all but one of the homologous sequences, and R340 and R175 are conserved in all but two of the homologous sequences. The five conserved arginines in *M. thermophila* were individually replaced with alanine, leucine, or lysine to assess their function. With the exception of R175L and R340A, the variants were produced in *E. coli* and purified by one-step metal-affinity chromatography. Variants R175L and R340A were produced in low amounts and were unstable which precluded purification. All purified variants were judged homogeneous by SDS/PAGE, and the subunit molecular masses were indistinguishable from the recombinant wild-type acetate kinase (data not shown). With respect to the K_m values for acetate or ATP, there were no significant differences between recombinant wild-type (Table 1) and authentic acetate kinase purified directly from *M. thermophila* (*18*). The k_{cat} for recombinant wild-type acetate kinase (Table 1) was significantly greater than that for the authentic enzyme (1050 s^{-1}) (*18*), a result attributed to the rapid one-step purification that preserved activity.

Kinetic Analyses of R91, R241, and R285 Variants. Replacement of R91 and R241 with alanine produced variants with specific activities of approximately 0.5 μmol min^{-1} (mg of protein)$^{-1}$ that were near the limit of detection and less than 0.1% of recombinant wild-type [740 ± 80 μmol min^{-1} (mg of protein)$^{-1}$]. This low activity precluded reliable estimates of kinetic constants and studies on inhibition by phenylglyoxal, even at high protein concentrations.

The R91A and R241A variants were stable during purification, and the yields were consistently within the range obtained for recombinant wild-type (2−3 mg). Additional experiments to verify the structural integrity of the R91A and R241A variants were performed. Gel filtration indicated that the R91A and R241A variants were dimeric in accordance with recombinant wild-type and authentic acetate kinase. The circular dichroism spectra of the R241A variant and recombinant wild-type were superimposable (data not shown) while the spectra of the R91A variant and recombinant wild-type varied only slightly (Figure 2). The ellipticity value at 220 nm for the R91A variant was 7% more negative than that of recombinant wild-type. These results

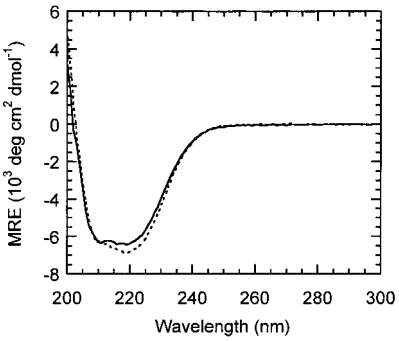

FIGURE 2: Circular dichroism spectra of acetate kinases from *M. thermophila*. Recombinant wild-type (solid line) and R91A (dotted line).

indicate no global conformational changes occurred in the R91A and R241A variants compared to recombinant wild-type. Thus, the low specific activities of the variants suggested that R91 and R241 are essential for catalysis.

Replacement of R91 and R241 with leucine or lysine also produced variants that were stable during purification and with yields within the range obtained for recombinant wild-type. Except for R91K, these variants also had specific activities of approximately 0.5 μmol min^{-1} (mg of protein)$^{-1}$, consistent with essential roles for R91 and R241 in catalysis. Although lysine is a conservative replacement for arginine, the k_{cat} values for variant R91K (Table 1) were still less than 2% of those for recombinant wild-type, further supporting an essential role for R91 in catalysis. The ability of lysine to partially replace R91 indicates that a positive charge in this position is important for activity. The K_m value for acetate for the R91K variant increased greater than 10-fold relative to that of recombinant wild-type, suggesting an additional role for R91 in binding acetate. The K_m for ATP also increased but not to an extent that convincingly supports a role for R91 in binding this substrate.

Replacement of R285 with any of the three residues resulted in enzymes with low but significant activity (Table 1), indicating this arginine is important but nonessential for catalysis. However, the K_m values for acetate increased up to 10-fold relative to recombinant wild-type, suggesting R285 is involved in binding this substrate. The K_m values for ATP also increased (Table 1) but not to an extent that supports an essential role for R285 in binding ATP.

Chemical Rescue of R91A and R241A Variants. Chemical rescue of the R91A and R241A variants was attempted to investigate the roles for the guanido functional groups of essential residues R91 and R241. Addition of guanidine or

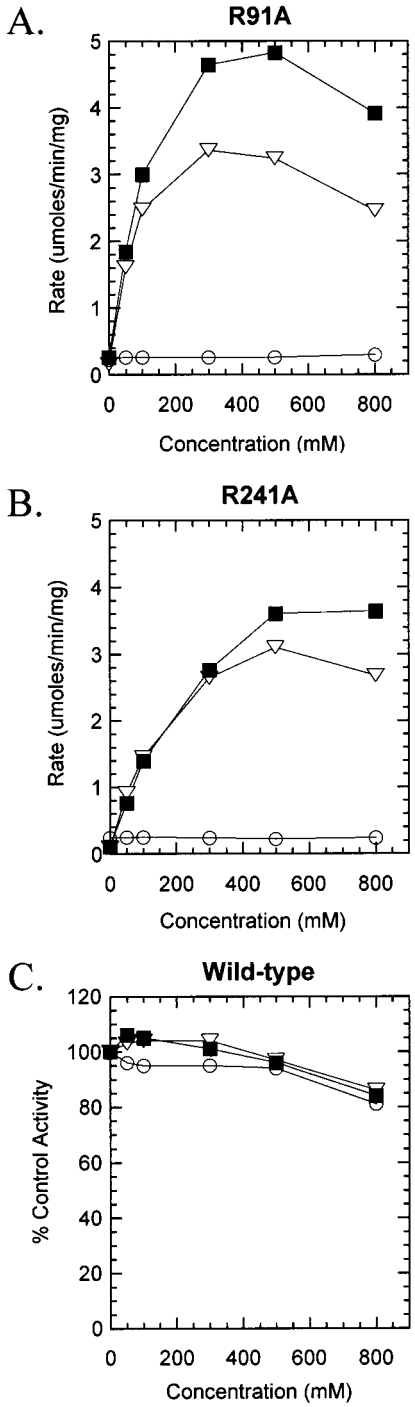

FIGURE 3: Effect of guanidine derivatives on the activity of variant and recombinant wild-type acetate kinases. Activity was measured in the presence of the indicated concentrations of guanidine (▽), methylguanidine (■), or methylamine (○).

methylguanidine to the assay reaction mixture restored activity to both variants (Figure 3A,B). Maximal recoveries occurred between 300 and 500 mM guanidine or methylguanidine, which represented greater than 20-fold increases in activities compared to those of the unrescued variants. Control experiments with recombinant wild-type acetate kinase showed a decrease in activity above 300 mM guanidine or guanidine derivatives (Figure 3C). The chemical rescue of variants R91A and R241A adds to the evidence that loss of essential functional groups, and not misfolding of the variants, produces the loss of activity. Moreover, chemical rescue further supports essential roles for these residues in catalysis. The inability of methylamine to restore any activity to either the R91A or the R241A variant (Figure 3A,B) indicates that chemical rescue involves the specific functionality of the guanidine molecule and not a nonspecific interaction such as a space-filling steric role. Lack of rescue with ethylguanidine (data not shown) verifies that recovery of activity does not result from productive refolding in the presence of denaturant.

Table 2: Kinetic Constants of Chemically Rescued Recombinant Wild-Type and Variant *M. thermophila* Acetate Kinases

	ATP		
enzyme	K_m^{app} (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
wild-type[a]	2.8 ± 0.1	1240 ± 12	444
R91A[b]	6.0 ± 0.3	6.8 ± 0.2	1
R241A[b]	3.4 ± 0.2	5.0 ± 0.2	2

[a] Data were obtained in the presence of 300 mM guanidine and 500 mM potassium acetate. [b] Data were obtained in the presence of 300 mM guanidine and 1 M potassium acetate. The higher concentration of acetate was used in response to the nonsaturating kinetics observed for the variants; thus, the K_m values for ATP are apparent values.

The kinetic constants of recombinant wild-type and rescued R91A and R241A variants were determined in the presence of 300 mM guanidine (Table 2). Plots of initial velocity versus ATP concentration showed normal Michaelis−Menten kinetics. The results, showing that the K_m^{app} values for ATP of the rescued R91A and R241A variants were similar to those of recombinant wild-type, indicate that the rescued activities are the consequence of replacement of important functional groups and not changes in the catalytic mechanism. However, the rescued variants did not exhibit the same Michaelis−Menten behavior for acetate as did recombinant wild-type. The K_m values for acetate could not be determined due to the absence of saturation kinetics for both R91A and R241A variants. Plots of initial velocity versus acetate concentration remained linear up to 1.5 M acetate in the presence of variable amounts of guanidine (20−300 mM) (data not shown). The simplest interpretation of these results is that R91 and R241 function to bind acetate. The increased K_m value for acetate of the R91K variant (Table 1) further supports a role for R91 in binding acetate.

Kinetic Analyses of R175 and R340 Variants. The R175L and R340A variants were unstable and could not be purified. The R175K variant was purified with a yield nearly identical to recombinant wild-type; however, the yields for the R175A, R340K, and R340L variants were less than 30% of that for recombinant wild-type, suggesting that these variants were unstable. The specific activity of the R175A variant was less than 0.1% of the recombinant wild-type; however, substantial activity was observed for the R175K, R340K, and R340L variants. These results indicate that R175 and R340 are not directly involved in catalysis. The K_m values determined for the R175 and R340 variants suggest that these residues do not significantly influence the binding of acetate or ATP (Table 1).

Inhibition of Variants by Phenylglyoxal. Compared to recombinant wild-type (Figure 1A), the R91K variant was inhibited by phenylglyoxal to a similar extent (Figure 1C); however, the rate ($t_{1/2}$ = 0.5 min) was accelerated relative to recombinant wild-type ($t_{1/2}$ = 3.0 min). These results indicate the active site of the R91K variant is altered such

that the target arginine is more exposed to inhibitor. Inhibition of the R91K variant was biphasic, suggesting that two separate residues are modified. Site-specific replacement (Table 1) indicated R285 is nonessential for catalysis, suggesting R241 is the target arginine in the rapid phase of inhibition for the R91K variant. Although phenylglyoxal is highly specific for arginine residues, this reagent can react slowly with the ϵ-amino groups of lysine residues (*20*). Thus, the slower phase for inactivation of the R91K variant may be due to modification of the lysine residue at position 91. Contrary to recombinant wild-type, the R91K variant exhibited minimal acetate protection from phenylglyoxal inactivation, which adds to the results indicating a role for R91 in acetate binding. Variant R91K also exhibited reduced ATP protection from phenylglyoxal inactivation compared to recombinant wild-type; however, this result alone is not enough to support a role for R91 in ATP binding.

The rate and extent of inhibition for the R285 variants (Figure 1D,E,F) were similar to recombinant wild-type, indicating replacement of this residue does not alter the accessibility of phenylglyoxal to the target arginine or arginines. Kinetic analyses of the R91 and R241 variants indicate that these residues are essential for catalysis which suggests either or both are phenylglyoxal targets; however, the inhibition data do not distinguish among these possibilities. Inhibition of the R285 variants was nearly complete (Figure 1D,E,F), a result further supporting essential roles for R91 and R241 in catalysis. Although kinetic analyses indicate R285 influences acetate binding (Table 1), protection of the R285 variants by acetate suggests this residue is not essential for binding this substrate (Figure 1D,E,F). In fact, protection of the R285 variants from inhibition was enhanced relative to recombinant wild-type, indicating that the active site is more accessible to acetate in these variants. Patterns of protection by ATP for the R285 variants were identical to that for recombinant wild-type, indicating that R285 is not involved in binding this substrate, a result consistent with the kinetic analyses (Table 1).

The rate and extent of phenylglyoxal inhibition and the pattern of substrate protection for the R175 and R340 variants were similar to those for recombinant wild-type (data not shown), suggesting replacement of these residues has no influence on the active-site conformation.

DISCUSSION

Phenylglyoxal inhibition and substrate protection studies on the *E. coli* acetate kinase lead to the conclusion that at least one unspecified arginine in the active site is essential for activity (*9*). The high identity (44%) between the deduced sequences of the *E. coli* and *M. thermophila* enzymes suggests a similar catalytic mechanism. The results presented here indicate that *M. thermophila* residues R91 and R241 are essential for catalysis. Indeed, the guanido groups of arginine residues R91 and R241 are located near the polyphosphate group of ATP whereas the guanido group of R285 is located near the adenosine moiety in the crystal structure of the acetate kinase from *M. thermophila* (personal communication, Miriam Hasson). The results also identify roles for R91, and possibly R241, in binding acetate. Although the kinetic characterizations indicate that R175 and R340 are not directly involved in substrate binding or catalysis, the generally low activities and instabilities of the R175 and R340 variants suggest that these residues fulfill important structural roles. Both R175 and R340 are remote from the active site (personal communication, Miriam Hasson), consistent with this proposal.

In 1979, Blättler and Knowles proposed a direct in-line phosphate transfer mechanism for *E. coli* acetate kinase based on the net inversion of configuration of the transferred phosphate (*6*). This mechanism is consistent with the mechanism proposed for phosphoryl transfer enzymes of the sugar kinase/hsc70 protein/actin superfamily of proteins of which the acetate kinase from *M. thermophila* is a member (*6, 10, 21, 22*). In the proposed associative-type mechanism, a pentacoordinate transition state occurs during in-line transfer of the γ-phosphate of ATP to acetate. The structure of the proposed transition state is trigonal bipyramidal with three negatively charged equatorial oxygens. Blättler and Knowles postulated that three electron-deficient residues (positive charges or hydrogen-bond donors) could stabilize this transition state (*6*). Residues R91 and R241 in the *M. thermophila* acetate kinase are conserved in the *E. coli* enzyme and most other acetate kinase homologues and are candidates for stabilization of the proposed transition state. This role for arginine residues has been proposed for other kinases, although not members of the sugar kinase/hsc70 protein/actin superfamily. Three arginine residues, essential for activity, have been implicated in stabilizing the pentacoordinate transition states of UMP kinase and adenylate kinase (*23, 24*). In addition, the crystal structures of UMP kinase and adenylate kinase indicate that these arginines are in close proximity to the proposed transition states (*23, 24*). As stated above, stabilization of a pentacoordinate transition state could require three positively charged residues. For the *M. thermophila* acetate kinase, our evidence is consistent with R91 and R241 as two potential sites for transition state stabilization. The third site is probably not R285 for which all three replacements restored significant activity. Alternatively, the third stabilization site may be fulfilled by the magnesium ion of the metal−nucleotide complex as proposed for the *E. coli* enzyme (*25*). Consistent with this hypothesis is the fact that the *M. thermophila* acetate kinase, like most nucleotide-utilizing enzymes, has a divalent metal ion requirement (*18*).

In addition to potential roles in transition state stabilization, the results presented here indicate that residues R91 and R241 are also involved in substrate binding. Residue R91 may bind the carboxyl group of acetate in a catalytically competent manner, possibly by orienting the negatively charged oxygen for nucleophilic attack on the γ-phosphate of ATP. The ability of guanidine to rescue variant R241A and the failure of lysine to replace residue R241 suggest that a positive charge alone is not sufficient for maximal activity and that a bidentate interaction is important at this position. The guanido group of R241 may bridge the carboxyl group of acetate and the γ-phosphate of ATP to facilitate in-line phosphoryl transfer analogous to the proposed role for R333 in bridging oxalate and the ATP γ-phosphate in phosphoenolpyruvate carboxykinase (*26*). Dual roles for arginine residues in substrate binding and catalysis have also been proposed for *E. coli* glutamine synthetase which contains two arginines thought to be involved in binding ATP and the carboxyl group of glutamate, as well as reaction

intermediates during transfer of the phosphoryl group between ATP and glutamate (*27*).

In addition to the in-line mechanism, a triple displacement mechanism has been proposed for the acetate kinase of *E. coli* in which the terminal nucleotide phosphate is transferred to two enzyme sites before final transfer to acetate (*7*). Consistent with a triple displacement mechanism is evidence for an unspecified glutamate residue found to be phosphorylated upon incubation of the *E. coli* acetate kinase with acetyl phosphate (*28*). More recently, the triple displacement mechanism has been challenged by the finding that phosphorylated *E. coli* acetate kinase is a phosphoryl donor to enzyme I of the bacterial phosphotransferase system (*3*). Thus, phosphorylation of acetate kinase may function in the transport of sugars and not in the acetate kinase reaction. The results reported here do not directly address this important question of a direct in-line versus covalent triple displacement mechanism. However, three in-line phosphate transfers would presumably involve three pentacoordinate transition states and as many as nine electron-deficient sites on the enzyme involved in transition state stabilization. The identification of only two catalytically essential arginine residues as well as the simplicity of the mechanism supports a direct in-line phosphate transfer for acetate kinase.

We utilized chemical rescue in conjunction with site-directed replacement to investigate the role of arginine residues in catalysis by acetate kinase. The results presented here supplement recent reports that guanidine can compensate for loss of function by side chain substitution (*29*−*32*). The rescued activities of variants R91A and R241A represented approximately 1% of recombinant wild-type specific activity, similar to that reported for guanidine rescue of the R127A variant of carboxypeptidase (*29*). The inability to recover recombinant wild-type activity can be attributed to (i) the greater freedom of motion of the free guanidine molecule versus the covalently attached side chain of arginine resulting in a higher energy transition state (*29*) or (ii) concomitant denaturation of the variants in the presence of guanidine. The inability of ethylguanidine to rescue activity of the R91A or R241A variants suggests that the smaller guanidine and methylguanidine molecules are oriented properly in the active sites of the alanine variants whereas the larger ethylguanidine molecule is sterically inhibited from entering or orienting properly in the active sites.

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