Evidence for a Transition State Analog, MgADP-Aluminum Fluoride-Acetate, in Acetate Kinase from Methanosarcina thermophila*

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Aluminum fluoride has become an important tool for investigating the mechanism of phosphoryl transfer, an essential reaction that controls a host of vital cell functions. Planar AlF₃ or AlF₄⁻ molecules are proposed to mimic the phosphoryl group in the catalytic transition state. Acetate kinase catalyzes phosphoryl transfer of the ATP γ-phosphate to acetate. Here we describe the inhibition of acetate kinase from Methanosarcina thermophila by preincubation with MgCl₂, ADP, AlCl₃, NaF, and acetate. Preincubation with butyrate in place of acetate did not significantly inhibit the enzyme. Several NTPs can substitute for ATP in the reaction, and the corresponding NDPs, in conjunction with MgCl₂, AlCl₃, NaF, and acetate, inhibit acetate kinase activity. Fluorescence quenching experiments indicated an increase in binding affinity of acetate kinase for MgADP in the presence of AlCl₃, NaF, and acetate. These and other characteristics of the inhibition indicate that the transition state analog, MgADP-aluminum fluoride-acetate, forms an abortive complex in the active site. The protection from inhibition by a non-hydrolyzable ATP analog or acetylsphosphate, in conjunction with the strict dependence of inhibition on the presence of both ADP and acetate, supports a direct in-line mechanism for acetate kinase.

Acetate kinase, which catalyzes the phosphoryl transfer of the ATP γ-phosphate to acetate, was one of the earliest phosphoryl transfer enzymes to be recognized. Since the discovery of acetate kinase in 1944 by Lipmann (1) and isolation in 1954 by Ochoa (2), most research on the catalytic mechanism has been performed with the Escherichia coli enzyme. The early research led to two proposed catalytic mechanisms, a triple-displacement mechanism via a covalent phosphoenzyme intermediate and a direct in-line mechanism. The direct in-line transfer mechanism is consistent with steady state kinetics (3) and also stereochemical evidence (4), which indicates that there are an odd number of in-line phosphoryl transfers (5, 6). The triple displacement mechanism was proposed when a phosphoenzyme was reported after incubation with radiolabeled ATP or acetylsphosphate (7), and this phosphoenzyme was found to be chemically competent to transfer the phosphoryl group to ADP or acetate (7). However, the triple displacement mechanism was challenged when it was shown that phosphorylated acetate kinase from E. coli is a phosphoryl donor to Enzyme I of the bacterial phosphotransferase system, which suggested an alternate function for phosphorylated acetate kinase in sugar transport (8). There has been very little mechanistic work on acetate kinase, since this intriguing triple displacement mechanism was proposed by Spector in 1980 (5).

The thermostable Methanosarcina thermophila acetate kinase has been cloned and hyper-produced in E. coli making it the first available for modern biochemical approaches including site-directed mutagenesis (9–11) and a crystal structure (12). The crystal structure identifies acetate kinase as a member of the superfamily now identified as the acetate and sugar kinases/Hsc70/actin (ASKHA) superfamily (12, 13). Glucose binding to hexokinase results in partial closure of the active site cleft and the subsequent binding of ATP is proposed to result in even greater closure of the cleft (14, 15). When glucose is absent, the phosphates and metal of the metal-nucleotide complex are proposed to be disordered possibly to reduce the inherent ATPase activity of hexokinase (14–16). Acetate kinase is expected to undergo an analogous conformational change upon substrate binding. Consistent with this hypothesis, the β-phosphate appears to be replaced in the current crystal structure of acetate kinase in complex with ATP (12); hence, identification of residues involved in catalysis from the crystal structure is precluded. Thus, a crystal structure of acetate kinase in the transition state bound to both MgADP and acetate as well as an analog of the γ-phosphoryl group of ATP is key to the identification of residues involved in transition state stabilization and the orientation of the substrates for catalysis.

Aluminum fluoride has recently become a powerful tool in the study of phosphoryl transfer enzymes. In several phosphorylases and kinases, AlF₃₄⁻ mimics the planar phosphoryl group in the catalytic transition state (17–19). Here we present evidence for a transition state analog, MgADP-aluminum fluoride-acetate, for the M. thermophila acetate kinase. To our knowledge, this work represents the first use of aluminum fluoride to mimic a transition state in an acetate kinase or in any kinase from the ASKHA superfamily. In conjunction with recent site-directed replacement studies, our results favor a direct in-line transfer of the ATP-γ-phosphoryl group to acetate and provide a new direction in which to investigate other unanswered questions regarding the mechanism of acetate kinase.

EXPERIMENTAL PROCEDURES

Chemicals—The following chemicals were of the highest purity commercially available. Aluminum chloride hydrate, magnesium chloride hexahydrate, sodium fluoride, and potassium hydroxide (for pH adjustment) were purchased from Aldrich. The monopotassium salt of adenosine 5’-diphosphate was purchased from Roche Molecular Biochemi-
Acetate after preincubation for 30 min with: complete inhibitory mixture (\(\text{Gall components except buffer}\)), minus NaF and AlCl\(_3\) (\(\text{Gall components except buffer}\)). Protein concentrations were determined by the Bradford method.

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Potassium acetate (25 mM) at ambient temperature for 30 min (200 m\(\text{dimer}\)). The enzyme activity was then assayed and plotted relative to 100% activity (600 \(\mu\text{mol}\) of acetylphosphate/min/mg of protein) obtained from a control mixture in which AlCl\(_3\), NaF, and Na\(\text{H}_2\text{PO}_4\) were omitted. Inset, the time course of the assay after preincubation for 30 min with: complete inhibitory mixture (\(\bullet\)), minus NaF and AlCl\(_3\) (\(\square\)), minus MgCl\(_2\), ADP and acetate (\(\bullet\)), minus all components except buffer (\(\Delta\)).

**RESULTS**

**Inhibition of M. thermophila Acetate Kinase by MgCl\(_2\), ADP, AlCl\(_3\), NaF, and Acetate**—Preincubation with a mixture of MgCl\(_2\), ADP, AlCl\(_3\), NaF, and acetate inhibited the activity of acetate kinase from *M. thermophila*. The inhibition was dependent on the preincubation time with a first-order rate constant of 0.24 ± 0.01 min\(^{-1}\) (Fig. 1). While this inhibition rate is slower than expected for an enzyme-ligand interaction, the rate can be attributed to the slow formation of aluminum fluoride complexes as investigated previously (22). When MgCl\(_2\), ADP, and acetate were omitted from the preincubation mixture, there was no detectable loss of activity, indicating that AlCl\(_3\) and NaF alone are not inhibitory (Fig. 1, inset). The results indicate that MgCl\(_2\), ADP, AlCl\(_3\), NaF, and acetate are required...
for maximum inhibition (Table I). There was a corresponding decrease in enzyme activity as the concentration of each component of the preincubation mixture was increased in the presence of fixed concentrations of all other components (Fig. 2). Preincubation of acetate kinase with MgCl₂, ADP, acetylphosphate, and AlCl₃ (100 μM) failed to inhibit enzyme activity (data not shown) verifying fluoride-dependent inhibition and demonstrating AlCl₃ is not inhibitory to the enzyme at the concentrations tested. Replacement of AlCl₃ with BeCl₂ (10 μM) in the preincubation mix did not inhibit acetate kinase activity (data not shown). The data for Fig. 2 were fit to an analog of the Hill equation (Equation 1) (23),

\[ i = \frac{i_{\text{max}}}[K_{i}^{\text{app}} + [I]] \]  

(Eq. 1)

where \( i \) is percent inhibition, \( i_{\text{max}} \) is the maximum percent inhibition, \( K_{i}^{\text{app}} \) is the apparent inhibition constant, \([I]\) is the concentration of inhibitor, and \( n \) is the Hill coefficient (plots not shown). The apparent \( K_{i}^{\text{app}} \) was determined to be 2.9 ± 0.8 mM, 7-fold less than the previously reported \( K_{i} \) value of 20 mM (21), and an \( n \) value equal to 1.0 ± 0.1 was determined for acetate. The apparent \( K_{i}^{\text{app}} \) value was determined to be 18.4 ± 0.7 mM, 130-fold less than the experimental \( K_{i} \) value of 2.4 mM (21). The \( n \) value for NaF, was found to be 3.0 ± 0.8, suggesting three fluoride atoms are required for inhibition. The apparent \( K_{i} \) values for magnesium and AlCl₃ were found to be 0.95 ± 0.02 mM and 3.4 ± 1.1 mM, respectively.

ATPγS was tested as a substrate of acetate kinase and found to be a non-hydrolyzable inhibitor (data not shown). Acetate kinase was incubated with ATPγS or acetylated phosphate before and during preincubation with MgCl₂, ADP, AlCl₃, NaF, and acetate to determine whether ATPγS or acetylated phosphate protected against the inhibition. A corresponding decrease in the inhibition of enzymatic activity was observed as the concentration of ATPγS (Fig. 3) or acetylated phosphate (Fig. 4) increased. There was an apparent lag in the relief of inhibition by ATPγS, while acetylated phosphate protection leveled off at 30% inhibition. One possibility for the lag is that the ATP analog ATPγS binds the enzyme with a lower affinity than ATP, thus affording less protection from the transition state analog. The residual 30% inhibition can possibly be attributed to turnover of the enzyme during preincubation with ADP and acetylated phosphate, which allowed for formation of the transition state complex.

Effect of Alternative Substrates on the AlCl₃ and NaF-dependent Inhibition—Several NTPs can replace ATP for the M. thermophila enzyme. The reported specific activities (μmol of acetylated phosphate/min/mg of protein) with ATP, ITP, UTP, or CTP are 412, 342, 390, and 218, respectively (21). Preincubation of acetate kinase with MgCl₂, AlCl₃, NaF, acetylphosphate, and either IDP, UDP, or CDP in place of ADP resulted in almost complete inhibition of activity (Table II). To determine the phosphoryl acceptor requirements for formation of the inhibitory complex, Acetate was substituted with propionate or butyrate. Activity of the M. thermophila enzyme with propionate is 60% of that with acetate, whereas butyrate is not a substrate (21). Preincubation with MgCl₂, AlCl₃, NaF, and propionate resulted in almost complete inhibition of activity; however, preincubation with butyrate in place of acetate did not significantly inhibit the enzyme (Table II).

Fluorescence Monitoring of MgADP Binding—Buffered solutions of either acetate kinase or bis-ANS had very low intrinsic fluorescence; however, when these two components were incubated together there was an ~20-fold enhancement of fluorescence indicative of bound bis-ANS. The fluorescence quenching dependent on the MgADP concentration was used to examine the MgADP binding to the acetate kinase in the absence or presence of AlCl₃, NaF, and acetate (Fig. 5). When AlCl₃, NaF, and acetate were absent, the pattern of fluorescence quenching was biphasic in response to increasing MgADP concentrations.

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**Table I**

Inhibition of the acetate kinase from E. coli or M. thermophila

| Preincubation mixture components | E. coli | M. thermophila |
|----------------------------------|---------|----------------|
| MgADP, aluminum fluoride, acetate | 3.0 ± 0.8 | 3.7 ± 0.1 |
| ADP, aluminum fluoride, acetate  | 90 ± 2  | 97 ± 1 |
| Mg, aluminum fluoride, acetate   | 95 ± 1  | 78 ± 2 |
| MgADP, fluoride, acetate         | 77 ± 4  | 90 ± 1 |
| MgADP, aluminum, acetate         | 95 ± 4  | 101 ± 2 |
| MgADP, aluminum fluoride         | 99 ± 8  | 98 ± 2 |

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\( ^{a} \) The enzyme (0.4 μM dimer) was preincubated for 30 min with MgCl₂ (10 mM), ADP (10 mM), AlCl₃ (0.1 mM), NaF (0.5 mM), and acetate (25 mM) unless otherwise noted before the activity was assayed. \( ^{b} \) 100% (approximately 600 μmol of acetylated phosphate/min/mg of protein) is the activity of the enzyme preincubated for 30 min with the indicated concentrations of MgCl₂, ADP, and acetate. \( ^{c} \) 100% (approximately 630 μmol of acetylated phosphate/min/mg of protein) is the activity of the enzyme preincubated for 30 min with the indicated concentrations of MgCl₂, ADP, and acetate.
A Transition State Analog for Acetate Kinase

**DISCUSSION**

Studies on several kinases including F$_1$-ATPase and UMP kinase indicate that AlF$_{3-4}$ mimics the planar phosphoryl group in the catalytic transition state and, with the substrates, forms an abortive active site complex (17, 19). Here we report the inhibition of _M. thermophila_ acetate kinase activity by MgCl$_2$, ADP, AlCl$_3$, NaF, and acetate. The characteristics of the inhibition and other experiments indicate that a transition state analog, MgADP-aluminum fluoride-acetate, forms an abortive complex in the active site of acetate kinase.

The requirements for formation of a transition state analog should mimic the requirements for catalysis. If MgADP-aluminum fluoride-acetate is a transition state analog for the acetate kinase, then any substrate that substitutes for acetate should form a transition state analog in conjunction with MgCl$_2$, ADP, AlCl$_3$, and NaF. Propionate replaces acetate as a substrate (21), and as shown here inhibited the enzyme when preincubated with MgCl$_2$, ADP, AlCl$_3$, NaF; however, butyrate is not a substrate for acetate kinase (21) and did not form an inhibitory complex with MgCl$_2$, ADP, AlCl$_3$, and NaF. These results support MgADP-aluminum fluoride-acetate as a transition state analog. A distinguishing characteristic of acetate kinase is the ability to utilize various NTPs (21) in place of ATP. Each of the cognate NDFs (in conjunction with MgCl$_2$, ADP, AlCl$_3$, NaF, and acetate) resulted in the inhibition of acetate kinase activity, further supporting the formation of a transition state analog. These results also indicate that inhibition of acetate kinase is not the consequence of a nonspecific interaction of aluminum fluoride with the adenosine moiety of ADP to form an inactive nucleotide complex. Although ADP is not a phosphoryl group donor in the acetate kinase reaction, AMP was able to partially replace ADP in the proposed transition state analog. AMP has previously been shown to be a competitive inhibitor versus ATP in the _E. coli_ acetate kinase ($K_c$ of 7.4 mM) (26). MgAMP, aluminum fluoride, and acetate may form a transition state analog despite the absence of the $\beta$-phosphate.

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**FIG. 3.** Protection by ATP-$\gamma$S from inhibition of _M. thermophila_ acetate kinase activity. The enzyme (0.4 $\mu$m dimer) was incubated with ATP-$\gamma$S in 100 mM BisTris buffer (pH 5.5) for 5 min before addition of MgCl$_2$ (10 mM), ADP (10 mM), AlCl$_3$ (0.1 mM), NaF (0.5 mM), and potassium acetate (25 mM). The enzyme was then incubated for another 30 min at which time acetate kinase activity was measured as described in the legend to Fig. 1.

**FIG. 4.** Protection by acetylphosphate from inhibition of _M. thermophila_ acetate kinase activity. The enzyme (0.4 $\mu$m dimer) was incubated with acetylphosphate in 100 mM BisTris buffer (pH 5.5) for 5 min before addition of MgCl$_2$ (10 mM), ADP (10 mM), AlCl$_3$ (0.1 mM), NaF (0.5 mM), and potassium acetate (25 mM). The enzyme was then incubated for another 30 min at which time acetate kinase activity was measured as described in the legend to Fig. 1.

**TABLE II**

| Preincubation mixture components* | % activity$^b$ |
|----------------------------------|---------------|
| ADP, acetate                     | 2.0 ± 0.8     |
| IDP, acetate                     | 2.0 ± 0.8     |
| UDP, acetate                     | 2.7 ± 0.5     |
| CDP, acetate                     | 7.7 ± 2.0     |
| AMP, acetate                     | 45 ± 3        |
| ADP, propionate                  | 11 ± 1        |
| ADP, butyrate                    | 96 ± 6        |

* The enzyme (0.4 $\mu$m dimer) was preincubated for 30 min with MgCl$_2$ (10 mM), the indicated nucleotide (10 mM), AlCl$_3$ (0.1 mM), NaF (0.5 mM), and acetate, propionate, or butyrate as indicated (25 mM), before the activity was assayed.

$^b$ 100% (approximately 600 $\mu$mol of acetylphosphate/min/mg of protein) is the activity of the enzyme preincubated for 30 min with the indicated concentrations of MgCl$_2$, nucleotide, and co-substrate.

A robust first phase of fluorescence quenching below 10 $\mu$m MgADP was followed by a weaker second phase at higher concentrations. There was a similar pattern when AlCl$_3$, NaF, and acetate were present; however, the first phase was more pronounced than in the absence of AlCl$_3$, NaF, and acetate (Fig. 5). Two possible explanations for the first phase of fluorescence quenching are: (i) a MgADP-induced environment change around a bound bis-ANS molecule or (ii) displacement of bound bis-ANS by MgADP. In favor of the latter mechanism, it has been reported that bis-ANS binds to hydrophobic pockets and with particularly high affinity to nucleotide binding sites (24, 25). Although the results do not distinguish between these two possibilities, the first phase of fluorescence quenching most likely reflects nucleotide binding at the high affinity catalytic site. The weaker second phase may be attributed to a nonspecific site that binds bis-ANS and has low affinity for MgADP. Consequently, the first phase of fluorescence quenching was used to examine MgADP binding affinity. The results indicate an increase in binding affinity of acetate kinase for MgADP in the presence of AlCl$_3$, NaF, and acetate. When either acetate or AlCl$_3$ plus NaF were omitted from the reaction mixture, the pattern of fluorescence quenching was similar to that observed in the absence of all three of these components (data not shown). This result suggests that the increase in binding affinity for MgADP is dependent on the presence of AlCl$_3$, NaF, and acetate but not AlCl$_3$ plus NaF alone or acetate alone.

**Inhibition of _E. coli_ Acetate Kinase by MgCl$_2$, ADP, AlCl$_3$, NaF, and Acetate**—_E. coli_ acetate kinase activity was fully inhibited by the mixture of MgCl$_2$, ADP, AlCl$_3$, NaF, and acetate (Table I) using the same experimental conditions as for the _M. thermophila_ enzyme. The _E. coli_ enzyme retained 80% or higher activity when each component was individually omitted from the preincubation mixture (Table I), indicating that all of the components are necessary for maximum inhibition.
Either the nucleotide β-phosphate group is not essential for formation of the active conformation of acetate kinase or aluminum fluoride substitutes for both the nucleotide β- and γ-phosphates in the MgAMP-aluminum fluoride-acetate complex. The ability of MgCl₂, ADP, AlCl₃, NaF, and acetate to inhibit acetates from both E. coli (Bacteria domain) and phylogenetically distant M. thermophila (Archaea domain) indicates that the inhibition is related to the common mechanism of catalysis and not a nonspecific event.

Although not always observed, the propensity of enzymes to maximize protein-substrate interactions in the transition state predicts tighter binding of substrates. The fluorescence quenching results indicate that acetate kinase has a higher binding affinity for MgADP when AlCl₃, NaF, and acetate are present. Consistent with these results are the much lower $K_c$ values determined for ADP and acetate (in the presence of MgCl₂, AlCl₃, NaF, and the co-substrate) relative to the $K_m$ values for ADP and acetate. In conclusion, the fluorescence and $K_c$ data are consistent with the formation of a transition state analog, MgADP-aluminum fluoride-acetate.

The primary metal complexes involved in acetate kinase inhibition were MgADP and aluminum fluoride; however, the 23% inhibition of acetate kinase by the preincubation mixture minus AlCl₃ suggests the formation of magnesium fluoride, which is also inhibitory. In support of this scenario, magnesium fluoride, in addition to aluminum fluoride, is proposed to form in the transition state analog of G proteins (27). Consequently, a portion of the metal fluoride in the acetate kinase transition state analog is probably magnesium fluoride. The dependence of acetate kinase inhibition on the AlCl₃ concentration suggests the predominant metal fluoride species in the transition state analog is aluminum fluoride (28). Beryllium failed to replace aluminum in the inhibitory complex. Beryllium fluoride, in association with a bridging β-phosphate oxygen, is thought to mimic the tetrahedral nucleotide γ-phosphate in the ground state (29, 30). Consequently, beryllium fluoride is not a true analog of the planar phosphoryl group during catalysis. Despite this fact, preincubation with MgADP, BeCl₂, and NaF inhibits several phosphoryl transfer enzymes, including adenylate kinase and F₁-ATPase (17, 31). Acetate kinase may differ from these enzymes in the inability to form a locked active conformation without a phosphoryl analog in the inhibitory complex.

The stoichiometry of the aluminum fluoride complex that forms in acetate kinase is uncertain. It has been proposed that pH plays a significant role in the aluminum to fluoride ratio of the transition state analog (32). The acetate kinase was inhibited in a preincubation mixture containing MgCl₂, ADP, AlCl₃, NaF, and acetate at a pH value of 5.5. At this pH, AlF₄⁻ is proposed to be the dominant species in the transition state analogs of kinases (32) and thus may be the species formed in acetate kinase. A planar AlF₄⁻ molecule would not strictly mimic the planar phosphoryl group during catalysis. Nevertheless, transition state analogs containing AlF₄⁻ have been shown to be reasonable mimics of the transition state (29, 33, 34). The nature of the nucleophilic attack may also help to determine the aluminum fluoride species in the transition state analog (30). Crystal structures of Gα (33) and Gα, GTPases (34), as well as myosin (29), indicate that an octahedrally coordinated aluminum is present in the transition state analog with four equatorial fluoride atoms, a nucleotide β-phosphate oxygen as one apical ligand, and a water molecule as the second apical ligand. Hydrolytic nucleotidases such as these may have more structural flexibility to accept AlF₄⁻ than would enzymes that utilize a protein functional group as the nucleophile (30). In nucleoside diphosphate kinase for example, in which an active site histidine attacks the γ-phosphate, steric considerations were invoked to explain the presence of AlF₃ instead of AlF₄⁻ in the crystal structure (30). If the above scenario is correct, the requirement for acetate in the proposed acetate kinase transition state analog suggests that this molecule acts as the nucleophile and further suggests that the aluminum fluoride species is AlF₃, not AlF₄⁻. This supposition is supported by a binding coefficient of ~3 that was determined for NaF in formation of the inhibitory complex.

Since the 1970s, there has been dispute over a single versus triple displacement mechanism for acetate kinase. The single displacement mechanism assumes direct in-line phosphoryl transfer from ATP to acetate, whereas the triple displacement mechanism involves phosphoryl transfer to two enzyme sites before transfer to acetate. Here we report the requirement for both ADP and acetate in formation of the transition state analog. The most straightforward interpretation of these results is that the phosphorylation of acetate by ATP occurs by a direct in-line mechanism. The protection from inhibition by non-hydrolyzable ATPγS or acetylphosphate suggests that the terminal phosphate of ATP, the phosphate group of acetylphosphate, and the aluminum fluoride of the transition state analog all share the same binding site. These results are consistent with a direct in-line mechanism. Other recent site-directed mutagenesis studies reported for the M. thermophila enzyme support the direct in-line mechanism. These studies have eliminated the possibility of active site histidine residues acting as phosphorylation sites in the catalytic mechanism (11). In addition, active site residues Arg²¹¹ and Arg²⁴¹ were found to be essential for catalysis (10). These arginines are well positioned...
in the crystal structure to stabilize the transition state in a direct in-line mechanism (12). If the acetate kinase reaction involves a direct in-line phosphoryl transfer, an alternative role must be proposed for active site residue Glu\(^{384}\), previously found to be essential for catalysis and postulated as the phosphorylation site of acetate kinase (9). Instead of acting as a catalytic phosphorylation site, Glu\(^{384}\) may be involved in magnesium binding. In support of this proposal, variant Glu\(^{384} \rightarrow \) Ala requires a 30-fold increase in the concentration of magnesium necessary for half-maximal velocity relative to that of the wild-type enzyme (35). Thus, the results presented here combined with recent reports support a direct in-line mechanism. Finally, the work presented here lays the foundation for further research to elucidate the role of active site residues and the catalytic mechanism for this enzyme.

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