The Role of Histidines in the Acetate Kinase from *Methanosarcina thermophila*

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Cheryl Ingram-Smith, Robert D. Barber, and James G. Ferry‡

From the Department of Biochemistry and Molecular Biology, Eberly College of Science, Pennsylvania State University, University Park, Pennsylvania 16802-4500

Acetate kinase is of major importance in the metabolism of prokaryotes, especially in the energy-yielding metabolism of anaerobes. In fermentative microbes the catabolic intermediate acetyl-CoA is converted to acetate by phosphotransacetylase (Equation 1) and acetate kinase (Equation 2) with the production of ATP, which provides the majority of energy.

\[
\text{CH}_3\text{COSCoA} + \text{P}_i = \text{CH}_3\text{COPO}_2^\text{2-} + \text{CoA} \quad (\text{Eq. 1})
\]

\[
\text{CH}_3\text{COPO}_2^\text{2-} + \text{ADP} = \text{CH}_3\text{COO}^- + \text{ATP} \quad (\text{Eq. 2})
\]

In methane-producing Archaea of the genus *Methanosarcina*, acetate kinase and phosphotransacetylase act in the opposite direction to activate acetate to acetyl-CoA, which is the substrate for carbon monoxide dehydrogenase/acetyl-CoA synthase (1). The synthase cleaves acetyl-CoA into a methyl group, a carbonyl group, and CoA. In subsequent steps of the pathway, the methyl group is reduced to methane with electrons gained from oxidation of the carbonyl group to carbon dioxide. ATP synthesis is coupled to methane production through an electrochemical ion gradient generated by a membrane-bound electron transport chain.

Acetate kinase has been purified from numerous prokaryotes (2–9); however, although the *Escherichia coli* enzyme has been studied biochemically and kinetically, the catalytic mechanism is unclear. The *E. coli* acetate kinase is phosphorylated *in vitro* by ATP or acetyl phosphate, and the phosphoenzyme is competent to transfer its phosphate group to either ADP or acetate, suggesting a covalent mechanism involving a phosphoenzyme intermediate (2, 10–14). The phosphoenzyme intermediate is a phosphoryl donor to enzyme I of the phosphoenolpyruvate: sugar phosphotransferase system of both *E. coli* and *Salmonella typhimurium* (2); thus, phosphorylation of acetate kinase may not be a consequence of the kinase mechanism but may instead play a role in other cellular processes. Furthermore, stereochemical studies reveal that the reaction catalyzed by acetate kinase proceeds with net steric inversion of the phosphate group (15). Because participation of a phosphoenzyme intermediate would result in net retention of the phosphate configuration, this result suggests a direct in-line transfer of the phosphoryl group from the donor to the acceptor. Alternatively, a covalent triple displacement mechanism involving two phosphoenzyme intermediates and three in-line phosphate transfers has been proposed to reconcile the apparent conflicting observations of a phosphoenzyme and net steric inversion of the phosphate group (16). Histidine residues have a high probability of serving as phosphorylation sites in the triple displacement mechanism; thus, the identification of active site histidines and the role they play in catalysis is important for distinguishing between the proposed direct in-line and covalent triple displacement mechanisms.

It was concluded that an unspecified histidine in the *Acinetobacter calcoaceticus* acetate kinase (4) is essential for catalysis based only on DEP2 inactivation studies. Preliminary chemical modification studies have suggested that one or more unspecified histidines play a role in the enzymatic mechanism of the acetate kinase from *Methanosarcina thermophila* (17). We combined chemical modification and site-directed mutagenesis approaches to further investigate the role of histidine residues in the catalytic mechanism of the *M. thermophila* enzyme. We identified two active site histidines (His-123 and His-180) whose modification by DEP inactivates the enzyme.

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‡ To whom correspondence should be addressed. Tel.: 814-863-5721; Fax: 814-863-6217; E-mail: jgf3@psu.edu.

1 The abbreviations used are: DEP, diethylpyrocarbonate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
The results show that His-123 has no role in catalysis and that phosphorylation of His-180 is not essential for catalysis. The results presented here, combined with recently published results, favor a direct in-line mechanism.

**EXPERIMENTAL PROCEDURES**

**Purification of Acetate Kinase Overproduced in E. coli**—The wild type and variant acetate kinases were overproduced in *E. coli* BL21(DE3) (F−*dcm ompT hsdS (rB− mB−) gal (DE3)) and purified as described previously (17). Protein purity was examined by SDS-polyacrylamide gel electrophoresis (18). Protein concentrations were determined by the Bradford method (19) using Bio-Rad dye reagent and bovine serum albumin as the standard.

**Enzymatic Assays**—The hydroxamate assay (9), an adaptation of the method of Lipmann and Tuttle (20) and Rose et al. (21), detects acetyl phosphate formation from acetate and ATP and was routinely used to measure acetate kinase activity. For inactivation studies, enzyme-linked assays in the forward (acetate phosphate and ADP forming) and reverse (acetate and ATP forming) directions were used to measure activity. The forward enzyme-linked assay (22) couples ADP formation to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. The reverse enzyme-linked assay (5) couples ATP formation from acetyl phosphate and ADP to the reduction of NADP using hexokinase and glucose-6-phosphate dehydrogenase.

**Inactivation by Diethylpyrocarbonate**—DEP was diluted in ethanol immediately prior to use. The DEP concentration of the diluted sample was determined by the increase in absorbance at 240 nm after reaction with 10 mM imidazole (pH 7.0) using an extinction coefficient of 3000 M−1 cm−1 (23, 24). Inactivation reactions contained 100 mM triethanolamine HCl (pH 7.0) plus the indicated concentrations of DEP and substrate. All reactions were performed at 37 °C unless otherwise indicated. Aliquots were removed at the indicated times and assayed directly or were diluted with an equal volume of 1 mM imidazole (pH 7.0) to stop the inactivation reaction and then assayed for enzymatic activity. In substrate protection assays, the enzyme was preincubated with substrate at the indicated concentrations for 5 min prior to addition of DEP.

**Protein Sequence Analysis**—The nonredundant sequence data bases and the unfinished genome data base were searched at the National Center for Biotechnology Information using the BLAST network server and the BLASTp and tBLASTn programs (25, 26). The sequences were aligned with ClustalX (27) using a Gonnet PAM 250 weight matrix with termination cycle sequencing (31) using an ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences) at the Nucleic Acid Facility at Pennsylvania State University.

**Thermal Stability**—Each enzyme was suspended at a final concentration of 0.1 µg/µl in 50 mM BisTris (pH 7.0) in the absence or presence of 10 mM ATP or 10 mM acetyl phosphate. Aliquots of 0.5 ml were incubated at the given temperatures and then placed on ice. A control sample of each enzyme was incubated on ice as a reference. Enzymatic activity was determined at 37 °C by the hydroxamate assay.

**Molecular Mass**—The native molecular mass was determined by gel filtration chromatography using a Superose 12 gel filtration column (Amersham Pharmacia Biotech) calibrated with bovine milk α-lactalbumin (14.2 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin (66 kDa; dimer, 132 kDa), urease (trimer, 272 kDa; hexamer, 545 kDa), and blue dextran (2,000 kDa). Protein samples (0.5 ml) were loaded onto the column after equilibration with 50 mM potassium phosphate (pH 6.8) containing 150 mM KCl, and the column was developed at a flow rate of 0.4 ml/min.

**Materials**—Chemicals were purchased from Sigma, VWR Scientific, or Fisher. Radioisotopes were purchased from PerkinElmer Life Sciences. Oligonucleotides for DNA sequencing and site-directed mutagenesis were purchased from Integrated DNA Technologies.

**RESULTS**

**Inactivation of Acetate Kinase by DEP and Substrate Protection**—Incubation of acetate kinase with DEP in 100 mM triethanolamine HCl (pH 7.0) resulted in both time- and concentration-dependent loss of enzymatic activity (Fig. 1A). The enzymatic activity remaining after incubation with DEP is described by Equation 3.

\[
\ln(A/A_0) = -k_2 t (1 - e^{-t/k_1})
\]

where the residual activity is \(A/A_0\) at time \(t\), \(I_0\) is the initial DEP concentration, \(k_2\) is the second order rate constant for the reaction of enzyme with DEP, and \(k_1\) is the pseudo-first order rate constant for the decomposition of DEP in aqueous solution. The value of \(k_1\) for DEP in 100 mM triethanolamine HCl (pH 7.0) was determined to be \(8.9 \times 10^{-3} \text{ min}^{-1}\). Plots of \(\ln(A/A_0)\) versus \((1 - e^{-k_1 t})/k_1\) at several concentrations of DEP yielded straight lines with slopes equal to the
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Fig. 2. Hydroxylamine reactivation of DEP-modified acetate kinase. Enzyme was incubated with 100 μM DEP followed by the addition of hydroxylamine to a final concentration of 200 mM at the time indicated by the arrow.

Fig. 3. Spectroscopic analysis of DEP-modified acetate kinase. Enzyme was incubated at 25 °C for 6 min in the presence or absence of 250 μM DEP. The difference spectrum between DEP-treated and untreated enzyme is shown.

pseudo-first order rate constant \( (k_{\text{obs}}) \) for inactivation at each DEP concentration. The plot of \( k_{\text{obs}} \) values versus DEP concentration was linear (Fig. 1B), suggesting a simple bimolecular reaction between the enzyme and DEP. The calculated second order rate constant \( k_2 \) was found to be 4720 M\(^{-1}\) min\(^{-1}\). A reaction order of 0.96 was calculated from a double logarithmic plot of the reciprocal of the half-time of inactivation versus DEP concentration (Fig. 1C).

Substrates for acetate kinase in both the forward and reverse reactions were examined for their ability to protect the enzyme from inactivation by DEP. All four substrates protected the enzyme from inactivation with acetyl phosphate affording complete protection (data not shown).

Amino Acids Modified by DEP—Although DEP primarily modifies histidine, DEP can also modify lysine and tyrosine (23, 32, 33). Treatment of DEP-modified protein with hydroxylamine results in removal of the ethoxycarbonyl group from modified histidines and tyrosines but does not reverse the modification of histidines. As shown in Fig. 2, treatment of DEP-inactivated acetate kinase with hydroxylamine (0.2 mM final concentration) restored 95% activity.

Carbethoxylation of histidine by DEP results in an increase in absorbance at 240 nm, whereas modification of tyrosine results in a decrease in absorbance at 280 nm. The difference spectrum of DEP-treated acetate kinase versus untreated enzyme (Fig. 3) was obtained to determine whether both histidines and tyrosines were modified by DEP. The inactivation of acetate kinase by DEP was accompanied by an increase in absorbance at 240 nm, whereas no decrease in absorbance at 280 nm was observed.

Number of Residues Modified by DEP and Protected by Substrate—Modification of histidine by DEP leads to an increase in absorbance at 240 nm, allowing the number of modified histidines to be estimated and correlated with the rate of enzyme inactivation. Using an extinction coefficient of 3000 M\(^{-1}\) cm\(^{-1}\) for carbethoxylated histidine (23, 24), a value of 2.1 was calculated from the absorbance at 240 nm in the difference spectrum shown in Fig. 3, suggesting that at least two histidines are modified.

The rate of enzyme inactivation compared with the number of modified histidines per subunit is shown in Fig. 4A. A plot of the number of histidines modified by DEP per subunit versus the residual activity (Fig. 4B) was biphasic. Extrapolation of the plot to zero activity indicated modification of either two or three histidines. The number of histidines per subunit essential for inactivation was determined by the statistical equation, \( m = n(1 - (A/A_0)^i) \), where \( m \) is the number of histidines modified at a given time, \( n \) is the number of modifiable histidines, \( A/A_0 \) is the residual activity, and \( i \) is the number of histidines essential for inactivation (34). Values of \( n = 3 \) and \( i = 2 \) provided the best fit to the data (Fig. 4C).

Because acetyl phosphate completely protects acetate kinase from DEP inactivation, the number of histidines modified in the presence of 10 mM acetyl phosphate was measured to determine how many are protected (Fig. 4A). The results indicated that the protection of one histidine per subunit correlated to 100% protection of activity. The results also indicate that one histidine that was modified was incompetent to inactivate the enzyme.

Identification of Histidines Important for Enzymatic Activity—Alignment of 56 acetate kinase sequences revealed that His-123, His-180, and His-208 were completely conserved, whereas His-60, His-90, His-94, His-152, and His-184 were highly conserved (>70%). Each of these histidines was individually changed to alanine. The wild type and variant acetate kinases were produced in E. coli and, with the exception of the H152A variant, purified to apparent homogeneity as judged by SDS-polyacrylamide gel electrophoresis. The yields of the variants were similar to that of the wild type enzyme (data not shown). The subunit molecular masses of the variants, as determined by SDS-polyacrylamide gel electrophoresis, were indistinguishable from that of the wild type. Native gel filtration chromatography indicated that the variants were dimeric in accordance with the wild type enzyme (data not shown). Thermal stabilities of the wild type and variant enzymes were also identical (data not shown). These results indicate that no major structural changes occurred from substitution of a given histidine with alanine.

Although the H152A variant was produced at high levels in E. coli, multiple attempts to purify this variant failed. No enzymatic activity could be detected in extracts of E. coli, producing the H152A variant, and fractions containing the variant from the first purification step also had no detectable activity. This variant was bound irreversibly to the hydrophobic interaction column during the second step of purification, a result suggesting improper folding.

Kinetic constants for the wild type and variant enzymes are shown in Table I. The \( K_m \) and \( K_{cat} \) values for ATP and acetate are similar for the heterologously produced wild type enzyme and the authentic enzyme purified from M. thermophila. The alanine variants had only minor changes in the \( K_m \) for acetate and ATP compared with wild type enzyme, except for the H90A variant, which had an 18-fold higher \( K_m \) for acetate. The \( k_{cat} \)
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values for all of the alanine variants ranged from approximately one-half to nearly equal that of the wild type enzyme, except for the H180A variant for which the $k_{\text{cat}}$ was over 100-fold reduced compared with that of the wild type enzyme.

**Kinetic Parameters of His-180 Variant Enzymes**—Because the H180A variant was dramatically decreased in $k_{\text{cat}}$ relative to wild type enzyme, other His-180 variants were generated to investigate the role of this residue in catalysis. His-180 was replaced with both positively and negatively charged residues and amide residues that each conserve one of the functional nitrogens present in the imidazole ring of histidine (Table II). Except for H180R, all other His-180 variants had $k_{\text{cat}}$ values $\geq 50\%$ of the wild type value. Although the lowest $k_{\text{cat}}$ value was observed for H180R, this variant still had 20% of the wild type value. In addition to increased $k_{\text{cat}}$ values relative to that for the H180A variant, all other His-180 variants had reduced $K_m$ values for acetate closer to that for the wild type enzyme. Only slight changes (2–3-fold) were observed for the $K_m$ for ATP.

**DEP Inactivation of Variant Enzymes**—The H60A, H90A, H94A, H184A, and H208A variants showed inactivation patterns identical to that observed for the wild type enzyme (data not shown); however, the H123A and H180A variants were less sensitive to DEP inactivation, retaining 38 and 12% activity, respectively, relative to untreated enzyme, whereas the wild type enzyme retained only 2% activity (Fig. 5). Other His-180 variants retained 1–21% activity after DEP inactivation. The H180A variant was partially protected from inactivation by acetyl phosphate, whereas the H123A variant was completely protected (Fig. 5).

**DISCUSSION**

The identification of active site residues essential for catalysis is critical to resolving which of the two proposed phosphoryl transfer mechanisms, direct in-line transfer or covalent triple displacement, apply to acetate kinase. The triple displacement mechanism requires phosphorylation of two residues. Potential candidates are three histidines (His-123, His-180, and His-208) present in the *M. thermophila* enzyme that are perfectly conserved among 56 acetate kinase homologs; indeed, these residues are located in the vicinity of the active site as determined by the crystal structure of the *M. thermophila* enzyme complexed with ATP. A previous report based only on DEP inactivation studies concluded that an unspecified active site histidine is essential for catalysis by the *A. calcoacetica* acetate kinase (4). However, inactivation studies alone can be misleading; thus, we combined both DEP inactivation and site-directed mutagenesis approaches to determine whether histidine is essential for catalysis by the acetate kinase from *M. thermophila*.

Three lines of evidence support that DEP inactivation of the *M. thermophila* acetate kinase is specific for histidine: (i) a robust second order rate constant for inactivation, (ii) increased absorbance at 240 nm correlated with decreased activity, and (iii) restoration of activity by hydroxylamine. Either two or three histidines are modified as determined by absorbance at 240 nm and correlation of the rate of inactivation to the number of histidines modified. A statistical treatment of these data indicated that three histidines are modified, two of which fully inactivate the enzyme. This conclusion is supported by the biphasic plot of residual activity versus the number of modified histidines and the fact that only two variants (H180A and H123A) were less sensitive to DEP inactivation compared with the wild type enzyme. The plot of modified residues versus residual activity of the wild type enzyme, when protected by acetyl phosphate, suggested that one histidine is modified that is incompetent to inactivate the enzyme. This result further suggests a total of three histidines is modified, assuming that the modification of two histidines is necessary for full inactivation of the unprotected enzyme. Substrate protection of the wild type enzyme from DEP inactivation indicated that at least one of the modified histidines is located in the active site. Indeed, acetyl phosphate protected both the H180A and H123A variants from DEP inactivation, which indicates that His-123 and His-180 are in the active site as suggested by the crystal structure.

The combined results indicate that His-123 and His-180 are two active site residues that, when modified, fully inactivate the *M. thermophila* acetate kinase. Two observations for the
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TABLE I

Kinetic parameters of acetate kinase variants obtained by replacement of conserved histidines with alanine

| Enzyme          | Acetate          | ATP             |
|-----------------|------------------|-----------------|
|                 | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ | $k_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|                 | $\text{mM}$  | $\text{s}^{-1}$ | $\text{ms}^{-1} \text{s}^{-1}$ | $\text{mM}$  | $\text{s}^{-1}$ | $\text{ms}^{-1} \text{s}^{-1}$ |
| Unaltered$^a$  | 22     | 1050          | 47             | 2.8     | 1221          | 436          |
| H60A$^b$       | 18 ± 1 | 949 ± 12      | 53 ± 2         | 2.0 ± 0.1 | 1033 ± 3      | 520 ± 13     |
| H90A$^b$       | 20 ± 1 | 1036 ± 3      | 50 ± 2         | 3.0 ± 0.5 | 1164 ± 15     | 392 ± 2      |
| H94A$^b$       | 330 ± 24 | 845 ± 15     | <3             | 1.7 ± 0.1 | 815 ± 6       | 476 ± 15     |
| H123A$^b$      | 66 ± 2  | 780 ± 6       | 12 ± 1         | 5.8 ± 0.1 | 1088 ± 5      | 189 ± 1      |
| H180A$^b$      | 16 ± 1  | 411 ± 3       | 25 ± 1         | 1.5 ± 0.3 | 563 ± 3       | 336 ± 3      |
| H180B$^b$      | 56 ± 3  | 7 ± 1         | <1             | 0.7 ± 0.1 | 9 ± 3         | 14 ± 1       |
| H180D$^b$      | 15 ± 2  | 435 ± 13      | 30 ± 3         | 2.7 ± 0.1 | 528 ± 9       | 194 ± 6      |
| H208A$^b$      | 33 ± 1  | 696 ± 3       | 21 ± 1         | 1.9 ± 0.1 | 1088 ± 6      | 580 ± 20     |

$^a$ Published values for acetate kinase purified from *M. thermophila* (9).
$^b$ Enzymes were produced in *E. coli*.

TABLE II

Kinetic parameters of His-180 variant acetate kinases

| Enzyme          | Acetate          | ATP             |
|-----------------|------------------|-----------------|
|                 | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|                 | $\text{mM}$  | $\text{s}^{-1}$ | $\text{ms}^{-1} \text{s}^{-1}$ | $\text{mM}$  | $\text{s}^{-1}$ | $\text{ms}^{-1} \text{s}^{-1}$ |
| H180A           | 56 ± 3  | 7 ± 1       | <1             | 0.7 ± 0.1 | 9 ± 3       | 14 ± 1       |
| H180D           | 13 ± 1  | 529 ± 11    | 40 ± 1         | 4.7 ± 0.3 | 630 ± 11    | 135 ± 7      |
| H180E           | 17 ± 1  | 702 ± 11    | 42 ± 1         | 6.2 ± 0.1 | 919 ± 3     | 148 ± 2      |
| H180K           | 19 ± 2  | 870 ± 7     | 46 ± 4         | 6.3 ± 0.3 | 1026 ± 8    | 163 ± 6      |
| H180N           | 17 ± 1  | 648 ± 5     | 38 ± 1         | 4.6 ± 0.2 | 761 ± 10    | 166 ± 7      |
| H180Q           | 14 ± 1  | 573 ± 3     | 43 ± 3         | 3.7 ± 0.1 | 653 ± 4     | 176 ± 1      |
| H180R           | 10 ± 1  | 200 ± 2     | 21 ± 1         | 1.4 ± 0.1 | 216 ± 3     | 159 ± 7      |

examined with double and triple variants in which His-123 and His-180 or His-123, His-180, and His-208 were replaced; however, these variants were inactive, which leaves this issue unresolved.

Kinetic analysis of variants obtained by alanine replacement of the eight conserved histidines revealed that, except for His-180, no other histidines are essential for catalysis. The $K_m$ values for acetate and ATP were only slightly altered for the H180A variant but the $k_{cat}$ was reduced over 100-fold. The question of a catalytic role for this residue was addressed by replacing it with six residues containing diverse functional groups. Remarkably, all six variants had $k_{cat}$ values that were greater than 50% of the wild type value, except H180R which had a lower, albeit substantial, $k_{cat}$ relative to the wild type enzyme. Although glutamine and asparagine are potential phosphorylation sites in the H180E and H180D variants, the other residues examined at position 180 (Lys, Arg, Gln, and Asn) are unlikely to be phosphorylated; thus, the uniformly robust $k_{cat}$ values for all six variants strongly indicate that phosphorylation of His-180 is not essential for catalysis. Although these results cannot rule out the possibility that either His-123 or His-208 is repositioned in the variants and rescues an essential catalytic function of His-180, it is unlikely that the $k_{cat}$ of all six His-180 variants would be rescued to the extent observed. None of the His-180 variants had $K_m$ values for acetate or ATP that were significantly changed from the wild type value, suggesting that His-180 is not involved in binding these substrates. The $K_m$ values for the H123A and H208A variants were also not significantly changed from the wild type value, suggesting that the active site His-123 and His-208 residues are not involved in binding acetate or ATP.

Singh-Wissmann et al. (35) recently reported the identification of two active site arginine residues (Arg-91 and Arg-241) in the *M. thermophila* acetate kinase that are essential for catalysis. They proposed that these residues play a role in stabilization of a pentacoordinate transition state. The identification of only two catalytically essential arginine residues is more consistent with a direct in-line transfer than a covalent triple displacement mechanism because the latter would presumably

**FIG. 5.** Acetyl phosphate protection of the H123A and H180A variants from DEP inactivation. Wild type (●), H123A (◆), and H180A (▲) were incubated with 250 μM DEP in the absence (open symbols) or presence (closed symbols) of 10 mM acetyl phosphate.

Wild type enzyme are in apparent conflict with this conclusion: (i) the protection of only one histidine by acetyl phosphate correlated with 100% protection of wild type activity and (ii) a reaction order of 0.96 was determined with respect to DEP. The simplest explanation for these observations is that modification of one histidine is dependent on prior modification of the other. This explanation is consistent with the biphasic plot of residual activity versus the number of modified histidines. This explanation is, however, only partly consistent with DEP inactivation patterns obtained for the H123A and H180A variants. It was expected that one of the variants would be insensitive to inactivation whereas the other would be partially inactivated and 100% protected by acetyl phosphate. The H123A variant was partially inactivated by DEP and 100% protected by acetyl phosphate, results suggesting that His-180 is the first residue modified. It was unexpected, however, that H180A was also partially inactivated. Apparently, either replacement of His-180 induces a conformational change such that His-123 becomes susceptible to modification without prior modification of His-180 or His-208 is modified by DEP and repositioned in the active site to inactivate the enzyme. These possibilities were
involve three pentacoordinate transition states and as many as nine residues for their stabilization. Evidence has also been obtained for a transition state analog of MgADP-AlF₃-acetate for the acetate kinase from *M. thermophila*, which further argues for a direct in-line mechanism. His-208, in addition to His-123 and His-180, is located in the vicinity of the active site in the crystal structure of the *M. thermophila* acetate kinase. All three of these histidines are perfectly conserved among the 56 acetate kinase sequences available in the data bases; thus, if the covalent mechanism were operable it is very likely that one or more of these three histidines would be essential for catalysis by serving as sites for phosphoenzyme intermediates. The finding that none of the three active site histidines in the *M. thermophila* enzyme is essential for catalysis is inconsistent with the triple displacement mechanism.

Although the results indicate that phosphorylation of His-180 is not essential for catalysis, it cannot be ruled out that this histidine is essential for other functions. His-180 could potentially function as a hydrogen bond donor or provide a positive charge for stabilization of the pentacoordinate transition state proposed for the direct in-line mechanism (15). Romanik and Eckstein (36) have proposed a similar direct in-line mechanism in which hydrogen bonding interactions and Mg²⁺ play an important role in delocalizing the negative charge of the transition state. Except for H180A, all other His-180 variants had replacement residues that could potentially serve as hydrogen bond donors or provide a positive charge to stabilize the transition state. His-180 may also be essential for spatial packing to maintain the correct conformation of the active site. Spatial packing is of added importance for kinases because exclusion of water in the active site is critical to prevent hydrolysis of phosphate groups (37). Although further experimentation is necessary to draw firm conclusions, the results presented here combined with recent published (35) and unpublished results favor the direct in-line mechanism.

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3 R. D. Miles and J. G. Ferry, manuscript in preparation.

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