Acetate kinase (ACK; EC 2.7.2.12; CH$_3$COO$^-$ + ATP ⇌ CH$_3$COPO$_4^{2-}$ + ADP) is a key enzyme in prokaryotic metabolism for the activation of acetate as a carbon and energy source or for the generation of ATP during fermentative growth. ACK is a member of the ASKHA phosphotransferase superfamily, which includes acetate kinase, hexokinase, and other sugar kinases, as well as the Hsc70 heat shock cognate and actin (5, 6, 14, 15). In 2001, Buss et al. (9) published the first structure for an ACK, that from the archean *Methanosarcina thermophila*, and they suggested that ACK is the urkinase for the ASKHA superfamily.

Several crystal structures have now been solved for the well-characterized *M. thermophila* ACK (9, 13), and the roles of a number of active site residues in substrate binding and catalysis have been examined experimentally (16, 17, 21, 22, 28, 29). Kinetic and structural studies support a direct in-line transfer of the phosphoryl group of ATP to acetate. An MgADP-AlF$_3$-acetate transition state analog resulting in an abortive complex (22) and was found to be in a linear array in the active site (13). Based on analysis of site-altered enzyme variants and structural studies, Gorrell et al. (13) postulated a mechanism detailing the roles of active site residues in catalysis. The active site residues implicated in this mechanism are well conserved among the ACKs, consistent with their key roles in catalysis.

Here we report the biochemical and kinetic characterization of the *Entamoeba histolytica* ACK, the only known member of the ASKHA structural superfamily that utilizes inorganic pyrophosphate (PP$_i$/inorganic phosphate (Pi)) as the sole phosphoryl donor/acceptor. Detection of ACK activity in *E. histolytica* cell extracts in the direction of acetate/PP$_i$, formation but not in the direction of acetate phosphate/Pi, formation suggests that the physiological direction of the reaction is toward acetate/PP$_i$, production. Kinetic parameters determined for each direction of the reaction are consistent with this observation. The *E. histolytica* PP$_i$-forming ACK follows a sequential mechanism, supporting a direct in-line phosphoryl transfer mechanism as previously reported for the well-characterized *Methanosarcina thermophila* ATP-dependent ACK. Characterizations of enzyme variants altered in the putative acetate/acetyl phosphate binding pocket suggested that acetyl phosphate binding is not mediated solely through a hydrophobic interaction but also through the phosphoryl group, as for the *M. thermophila* ACK. However, there are key differences in the roles of certain active site residues between the two enzymes. The absence of known ACK partner enzymes raises the possibility that ACK is part of a novel pathway in *Entamoeba*.

**Materials and Methods**

Cultivation of *E. histolytica* and cell extract preparation. Trophozoites of *E. histolytica* strain HM1:IMSS were cultured under axenic conditions in TYI-S-33 medium (10). *E. histolytica* cell extracts were prepared by resuspending 4 × 10$^6$ cells in 25 mM Tris, 150 mM NaCl (pH 7.4) and vortexing with acid-washed glass beads for 1 min, followed by 1 min on ice, for three cycles. The extract was centrifuged at 5,000 × g for 15 min, and the supernatant was isolated.

Cloning the *E. histolytica* ACK gene. The gene encoding the *E. histolytica* ACK was PCR amplified from *E. histolytica* strain HM1:IMSS genomic DNA (kindly provided by Lesly Temesvari, Clemson University) and cloned into the *Escherichia coli* expression plasmid pQE-30 (Qiagen) in-frame with the N-terminal His$_6$ tag sequence. Constructs were confirmed by sequencing at the Clemson University Genomics Institute.

Received 19 June 2012 Accepted 10 August 2012
Published ahead of print 17 August 2012
Address correspondence to Kerry S. Smith, kssmith@clemson.edu.
This article is technical contribution number 5862 of the Clemson University Experiment Station.
Supplemental material for this article may be found at http://ec.asm.org/.
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doi:10.1128/EC.00169-12
Site-directed alteration of *E. histolytica* ACK. Site-directed mutagenesis of the genes encoding the *E. histolytica* and *M. thermophila* ACKs was performed using the QuikChange site-directed mutagenesis kit (Stratagene). The altered sequences were confirmed by sequencing at the Clemson University Genomics Institute.

Production and purification of recombinant ACKs. The *Entamoeba* ACK expression plasmid was transformed into *Escherichia coli* strain YBS121 ΔackA Δpta (kindly provided by George Bennett, Rice University) along with the *lacI*-containing plasmid pREP-4 (Qiagen) for recombinant protein production. Transformants were grown in LB broth containing 50 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 25 μg/ml kanamycin at 37°C, 200 rpm to an optical density at 600 nm of ~0.9. Recombinant protein production was induced by addition of isopropyl-β-D-thiogalactopyranoside to a 1 mM final concentration. Cultures were shaken overnight at ambient temperature and harvested by centrifugation.

Cells were resuspended in breaking buffer (25 mM Tris, 150 mM NaCl, 20 mM imidazole, 10% glycerol; pH 7.4) and lysed by two passages through a French pressure cell at 138 MPA. Cellular debris was removed by ultracentrifugation at 100,000 × g for 1 h, and the supernatant was applied to a 5-ml HisTrap nickel affinity column (GE Healthcare, Piscataway, NJ). Protein was eluted from the column by using a linear gradient from 0 mM to 500 mM imidazole in 25 mM Tris, 150 mM NaCl, 10% glycerol (pH 7.4). Fractions containing active enzyme were pooled and dialyzed against buffer containing 25 mM Tris, 150 mM NaCl, and 0.1% glycerol (pH 7.4). The enzyme was determined to be electrophoretically pure by SDS-PAGE (see Fig. S1 in the supplemental material).

The protein concentrations in cellular extracts and recombinant enzyme preparations were determined by the Bradford method (7), using the Bio-Rad protein assay with bovine serum albumin as standard.

**Determination of kinetic parameters for EhACK.** The hydroxamate assay (1, 19, 25) was used to determine kinetic parameters in the acetyl phosphate/Pi-forming direction with a reaction mixture that contained 100 mM morpholinooethanesulfonic acid (MES), 5 mM MgCl₂, and 600 mM hydroxylamine hydrochloride (pH 7.5) with various concentrations of acyl substrate and sodium PPi. Reactions were performed at 45°C, the optimal temperature determined for this enzyme. Acetyl phosphate formation was determined by comparison to an acetyl phosphate standard curve. Kinetic calculations and progress curves were generated using Kineledgraph (Synergy Software, Reading, PA).

Kinetic parameters in the acetyl/PPi-forming direction of the reaction were determined using a modified reverse hydroxamate assay (12). The reaction mixtures contained 100 mM Tris (pH 7.0), 10 mM MgCl₂, and various concentrations of sodium phosphate and acetyl phosphate. Reactions were performed at 37°C. A standard curve of acetyl phosphate concentrations was used to determine the amount of acetyl phosphate depleted in the reaction.

To examine the enzymatic mechanism, the enzyme was assayed in the direction of acetyl/PPi formation with various concentrations of acetyl phosphate (0.5, 0.7, 1.0, and 1.5 mM) and sodium phosphate (40, 50, 60, and 70 mM) in a four-by-four matrix. All kinetic results are reported as means ± standard deviations of three experiments.

**Determination of kinetic parameters for MtACK variants.** MtACK site-altered enzyme variants were produced and purified as previously described (16, 17). Kinetic parameters in the acetyl/ATP-forming direction of the reaction were determined using a coupled enzyme assay in which ATP formation was coupled to the reduction of NADP to NADPH (1). The reaction mixtures contained 100 mM Tris (pH 7.5), 0.2 mM dithiothreitol, 10 mM MgCl₂, 5.5 mM glucose, 1 mM NADP, 5 mM ADP, and 10 units each of yeast hexokinase and glucose-6-phosphate dehydrogenase, with various concentration of acetyl phosphate. Reactions were initiated by the addition of enzyme, and the change in absorbance at 340 nm was monitored.

**PTA assay.** Phosphotransacetylase (PTA) activity was measured in both the acetyl phosphate-forming and the acetyl coenzyme A (CoA)-formers by monitoring the decrease or increase in absorbance at 233 nm, indicative of the formation of the thioester bond of acetyl-CoA, respectively. The reaction mixture for the acetyl phosphate-forming assay was prepared as described previously (20), except that the standard reaction mixture consisted of 100 mM Tris (pH 7.0), 2 mM dithiothreitol (DTT), 10 mM sodium phosphate, and 120 μg of native *E. histolytica* cell extract in a total volume of 200 μl. The reaction was initiated by the addition of acetyl-CoA to a 0.5 mM final concentration.

The reaction mixture for the acetyl-CoA-forming assay was performed as described previously (20), except that the standard reaction mixture consisted of 100 mM Tris (pH 7.0), 2 mM DTT, 0.5 mM CoA, and 120 μg of native *E. histolytica* extract in a total volume of 200 μl. The reaction was initiated by the addition of acetyl phosphate to a 2 mM final concentration.

**XFP assay.** Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP) activity in *E. histolytica* cell extracts was analyzed in a hydroxamate assay to detect the formation of acetyl phosphate (1, 19, 25). The reaction mix contained 100 mM Tris, 600 mM hydroxylamine hydrochloride, 2 mM DTT, and 100 mM fructose 6-phosphate (pH 7.0) in a total volume of 300 μl. The reaction was initiated by the addition of 120 μg of native *E. histolytica* extract and terminated after 30 min by the addition of 2 volumes of 1.25% FeCl₃, 1 N HCl, 5% trichloroacetic acid. Product formation was determined by the change in absorbance at 540 nm and comparison to an acetyl phosphate standard curve.

**Chemicals.** Chemicals were obtained from Sigma-Aldrich, ThermoFisher Scientific, or VWR Scientific Products.

**RESULTS**

*E. histolytica* has a P/P₈₆-dependent ACK. In 1962, Bragg and Reeves (8) reported an ATP-dependent ACK in the nonpathogenic *E. histolytica* strain Laredo (now *Entamoeba moshkovskii*). However, this strain was grown in the presence of bacteria, raising the possibility that this activity was of bacterial origin. Thirteen years later, Reeves and Guthrie (23) identified a PP₈₆-dependent ACK in axenically grown *E. histolytica*. In order to confirm the presence of ACK in *Entamoeba* and to allow kinetic and biochemical characterization, recombinant *E. histolytica* ACK was produced in *Escherichia coli* and purified by nickel affinity chromatography to electrophoretic homogeneity.

The deduced amino acid sequence of the ACK open reading frame (ORF; EHL_170010; XM 650898.3) is identified in the *E. histolytica* genome shares 34% identity and 53% similarity to the well-characterized *M. thermophila* ACK, which utilizes ATP and other nucleotide triphosphates (NTPs) but not PP₈₆ as the phosphoryl donor and displays high activity in both directions of the reaction (1). To determine whether the encoded enzyme is indeed a PP₈₆-dependent ACK and to allow kinetic and biochemical characterizations, recombinant *E. histolytica* ACK was produced in *Escherichia coli* and purified by nickel affinity chromatography to electrophoretic homogeneity.

Unlike all other characterized ACKs, EhACK showed only PP₈₆-dependent activity in the direction of acetyl phosphate formation (Fig. 1A). ATP did not serve as a phosphoryl donor (Fig. 1A), nor did other NTPs (CTP, GTP, TTP, UTP, and ITP) or ADP. In the acetyl-forming direction of the reaction, only inorganic phosphate could serve as the phosphoryl acceptor, and no activity was observed with ADP, AMP, or PP₆ (Fig. 1B).

**REFERENCES**

The observation that ACK activity in *E. histolytica* cell extracts was detected only in the acetate-forming direction of the reaction suggests that the physiological direction of the reaction is acetate/PPᵢ formation. Consistent with this, the purified recombinant EhACK had an over-1,000-fold-higher $k_{cat}$ in the acetate versus acetyl phosphate-forming direction (Table 1). The greater-than-200-fold-lower $K_m$ for acetyl phosphate versus acetate also supported this supposition (Table 1).

In the acetyl phosphate/PPᵢ-forming direction of the reaction, EhACK was found to have a broad acyl substrate range, utilizing substrates as long as octanoate (C₈). Although the apparent $K_m$ value decreased with increasing acyl chain length, the turnover rate, $k_{cat}$, also decreased (Table 1). The catalytic efficiency, $k_{cat}/K_m$, with acetate was similar to that observed with propionate, and both values were significantly higher than observed with any other acyl substrate (Table 1). The apparent $K_m$ values for PPᵢ remained relatively unchanged with different acyl substrates, with the exception of hexanoate (Table 1). Activities with heptanoate and octanoate were too low for determination of kinetic parameters, and the enzyme was not able to use the branched-chain acyl substrates 2-methylpropionate, 2-methylbutyrate, 3-methylbutyrate, 2-methylvalerate, 3-methylvalerate, or 4-methylvalerate. Other acyl phosphates are not commercially available, and therefore acetyl phosphate was the only substrate tested in the acetate/PPᵢ-forming direction.

The enzyme utilizes the cofactors Mg²⁺ ($K_m$ 2.1 ± 0.2 mM) and Co²⁺ ($K_m$ 5.5 ± 0.4 mM), but no activity was observed with Ca²⁺, Cu²⁺, Ni²⁺, or Zn²⁺. Use of Mn²⁺ resulted in less than 10% activity compared to Mg²⁺. The temperature optimum was determined to be 37°C in the acetate-forming direction of the reaction, but it was slightly higher (45°C) in the acetyl phosphate-forming direction. This higher thermal stability in the less-favored direction was most likely due to the much higher concentration of enzyme required for determination of kinetic parameters. Determination of kinetic parameters at the lower temperature or at lower enzyme concentrations in the acetyl phosphate-forming direction was not possible, as the activity was too low.

To determine whether EhACK follows a sequential mechanism, as shown by Miles et al. (22) for MtACK, or a ping pong mechanism consistent with a phosphoenzyme intermediate, as proposed by Anthony and Spector (2–4), we measured activity in the acetate/PPᵢ-forming direction in an array of reaction mixtures in which the acetyl phosphate and Pi concentrations were varied. The double-reciprocal plots of the activity versus substrate concentration resulted in intersecting lines (Fig. 2A and B), consistent with a ternary (sequential) mechanism and supporting a direct transfer of the phosphoryl group between acetyl phosphate and PPᵢ similar to the in-line ternary mechanism for MtACK (22).

**Identification of the putative acetyl phosphate/acetate binding pocket.** To investigate why EhACK has a much broader acyl substrate range than MtACK and other ACKs in the acyl phosphate-forming direction, we targeted the residues in EhACK that corresponded to the acetate binding pocket residues Val93, Leu122, and Pro232 of MtACK (17) for site-directed alteration and determination of kinetic parameters in both directions. ACK sequence alignment indicated that Val93 of MtACK is conserved in EhACK.

### TABLE 1 Kinetic parameters for EhACK

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (mM⁻¹ s⁻¹)</th>
<th>Phosphoryl substrate</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl phosphate</td>
<td>0.5 ± 0.01</td>
<td>1.939 ± 14</td>
<td>3.846 ± 69</td>
<td>Pᵢ</td>
<td>48.9 ± 0.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>106.8 ± 1.0</td>
<td>1.76 ± 0.01</td>
<td>0.016 ± 0.001</td>
<td>PPᵢ</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Propionate</td>
<td>79.8 ± 1.9</td>
<td>1.16 ± 0.01</td>
<td>0.015 ± 0.001</td>
<td>PPᵢ</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>Butyrate</td>
<td>75.6 ± 1.5</td>
<td>0.33 ± 0.01</td>
<td>0.0043 ± 0.0001</td>
<td>PPᵢ</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Valerate</td>
<td>56.7 ± 0.7</td>
<td>0.19 ± 0.01</td>
<td>0.0034 ± 0.0001</td>
<td>PPᵢ</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>20.5 ± 0.5</td>
<td>0.051 ± 0.001</td>
<td>0.0025 ± 0.0001</td>
<td>PPᵢ</td>
<td>16.3 ± 0.4</td>
</tr>
</tbody>
</table>

*a Kinetic parameters in the direction of acetate/PPᵢ formation were determined at 37°C using 100 mM Tris (pH 7.0), 10 mM MgCl₂, and various concentrations of sodium phosphate and acetyl phosphate. Kinetic parameters in the direction of acyl phosphate/PPᵢ formation were determined at 45°C using 100 mM MES, 5 mM MgCl₂, and 600 mM hydroxylamine hydrochloride (pH 7.5), with various concentrations of acyl substrate and sodium PPᵢ."
(Val^{87}), and Leu^{122} is conservatively replaced by Ile (Ile^{116}). However, Pro^{232} of MtACK is not conserved and is replaced by Thr (Thr^{223}) in EhACK (Fig. 3). This Pro is strictly conserved among all ACKs with the exception of those from Entamoeba, and we therefore speculated that this difference may play a role in the expanded acyl substrate range and/or in the preference for acetate/PPi formation.

Alteration of Thr^{223} to Pro in EhACK had little effect on enzyme activity in the direction of acyl phosphate formation (Table 2). The enzyme remained capable of utilizing substrates as long as hexanoate, and the $K_m$ and $k_{cat}$ values for each substrate were comparable to those observed for the unaltered enzyme. In the direction of acetate formation, the $K_m$ for acetyl phosphate increased 4-fold; however, the $k_{cat}$ also showed a 4-fold increase, and thus the catalytic efficiency, $k_{cat}/K_m$, was unchanged (Table 3).

Butyrate kinases, which utilize longer acyl substrates, have Gly at the equivalent position to Pro^{232} of MtACK and Thr^{223} of EhACK. Thus, we also examined a Thr^{223}Gly variant to determine whether this replacement altered the acyl substrate range. This variant showed significant activity in the acetate-forming direction of the assay but was not saturable for P_i and did not display activity in the acyl phosphate-forming direction with any acyl substrate.

For further comparison, we analyzed Pro^{232}Thr and Pro^{232}Gly MtACK variants in the direction of acetate formation, and these variants displayed 3.9-fold and 4.5-fold increased $K_m$ values for acetyl phosphate, respectively, relative to the unaltered enzyme (Table 3). The $k_{cat}$ value for the Pro^{232}Gly variant was reduced 22-fold, but the $k_{cat}$ value observed for the Pro^{232}Thr variant was unchanged (Table 3).

EhACK Val^{87}Ala and Val^{87}Gly variants displayed 16- to 20-fold decreased turnover rates in the direction of acetate synthesis but no change in the $K_m$ for acetyl phosphate (Table 3), and they were inactive in the acetyl phosphate-forming direction. The Ile^{116}Ala and Ile^{116}Leu variants showed no substantial change in the $K_m$ for acetyl phosphate and decreased $k_{cat}$ values for acetyl phosphate (Table 3). Neither the Ile^{116}Ala nor Ile^{116}Leu variant showed significant activity in the acetate-forming direction, and the $K_m$ for acetyl phosphate and decreased $k_{cat}$, resulting in 22- to 27-fold-decreased catalytic efficiencies, respectively. Alteration of the corresponding Leu residue (Leu^{122}) in MtACK had only a marginal effect on the $K_m$ for acetyl phosphate and a weak reduction in $k_{cat}$ (Table 3).

**FIG 2** Double-reciprocal plot of acetate-forming activity of EhACK. (A) The reciprocal of the sodium phosphate concentration (40 mM, 50 mM, 60 mM, and 70 mM) versus the reciprocal specific activity at acetyl phosphate concentrations of 0.5 mM (●), 0.7 mM (○), 1.0 mM (■), and 1.5 mM (□). (B) The reciprocal of the acetyl phosphate concentration of 0.5 mM, 0.7 mM, 1.0 mM, and 1.5 mM versus the reciprocal specific activity at sodium phosphate concentrations of 40 mM (●), 50 mM (○), 60 mM (■), and 70 mM (□).

**FIG 3** Acetate kinase partial sequence alignment. ACK sequences were aligned using Clustal X. Only those regions surrounding the residues altered in EhACK are shown. Numbers above the alignment indicate the positions of residues in MtACK, and those below indicate the position of residues in EhACK. EcACK, Escherichia coli ACK; PrACK, Phytophthora ramorum ACK; CnACK, Cryptococcus neoformans ACK.

**TABLE 2** Kinetic parameters for the EhACK Thr^{223}Pro variant\(^{a}\)

<table>
<thead>
<tr>
<th>Acyl substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s(^{-1}))</th>
<th>$k_{cat}/K_m$ (mM(^{-1}) s(^{-1}))</th>
<th>$K_m$ PP(_i) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>169.5 ± 0.06</td>
<td>1.3 ± 0.01</td>
<td>0.0077 ± 0.01</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Propionate</td>
<td>98.7 ± 0.2</td>
<td>0.061 ± 0.0001</td>
<td>0.00062 ± 0.00001</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Butyrate</td>
<td>56.5 ± 0.5</td>
<td>0.18 ± 0.001</td>
<td>0.0032 ± 0.0001</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>Valerate</td>
<td>41.6 ± 0.3</td>
<td>0.21 ± 0.001</td>
<td>0.0049 ± 0.0001</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>20.4 ± 0.5</td>
<td>0.065 ± 0.0001</td>
<td>0.0032 ± 0.0001</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

\(^{a}\)Kinetic parameters were determined at 45°C using 100 mM MES, 5 mM MgCl\(_2\), and 600 mM hydroxylamine hydrochloride (pH 7.5) with various concentrations of acyl substrate and sodium PP\(_i\).
TABLE 3 Kinetic parameters for acetyl phosphate for EhACK and MtACK acetyl phosphate binding pocket variants in the direction of acetate formation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alteration</th>
<th>$K_{m}^{acetyl phosphate}$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EhACK</strong></td>
<td>Unaltered</td>
<td>0.50 ± 0.01</td>
<td>1,939 ± 14</td>
</tr>
<tr>
<td></td>
<td>Thr$^{223}$Pro</td>
<td>2.0 ± 0.08</td>
<td>8,333 ± 185</td>
</tr>
<tr>
<td></td>
<td>Thr$^{223}$Gly</td>
<td>ND$^a$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Ile$^{116}$Ala</td>
<td>1.6 ± 0.09</td>
<td>280 ± 2</td>
</tr>
<tr>
<td></td>
<td>Ile$^{116}$Leu</td>
<td>2.4 ± 0.02</td>
<td>344 ± 2</td>
</tr>
<tr>
<td></td>
<td>Val$^{87}$Ala</td>
<td>0.53 ± 0.03</td>
<td>1,218 ± 2</td>
</tr>
<tr>
<td></td>
<td>Val$^{87}$Gly</td>
<td>0.51 ± 0.02</td>
<td>97 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>His$^{117}$Ala</td>
<td>8.0 ± 0.07</td>
<td>21 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>His$^{117}$Ala</td>
<td>6.99 ± 0.07</td>
<td>2.0 ± 0.07</td>
</tr>
<tr>
<td><strong>MtACK</strong></td>
<td>Unaltered</td>
<td>0.34 ± 0.01</td>
<td>6,280 ± 45</td>
</tr>
<tr>
<td></td>
<td>Pro$^{232}$Gly</td>
<td>1.3 ± 0.04</td>
<td>124 ± 2</td>
</tr>
<tr>
<td></td>
<td>Pro$^{232}$Thr</td>
<td>1.5 ± 0.16</td>
<td>2,335 ± 83</td>
</tr>
<tr>
<td></td>
<td>His$^{123}$Ala</td>
<td>0.21 ± 0.01</td>
<td>135 ± 8</td>
</tr>
<tr>
<td></td>
<td>His$^{160}$Ala</td>
<td>2.9 ± 0.40</td>
<td>57 ± 3</td>
</tr>
</tbody>
</table>

$^a$ Kinetic parameters were determined at 37°C using 100 mM Tris (pH 7.5), 10 mM MgCl$_2$, and various concentrations of sodium phosphate and acetyl phosphate.

$^b$ Kinetic parameters were determined at ambient temperature (22 to 23°C) using 100 mM Tris (pH 7.5), 0.2 mM dithiothreitol, 10 mM MgCl$_2$, 5.5 mM glucose, 1 mM NADP, 5 mM ADP, and 10 units each of yeast hexokinase and glucose-6-phosphate dehydrogenase, with various concentrations of acetyl phosphate.

$^c$ ND, not determined. The enzyme was not saturable for $P_i$.

$^d$ From Ingram-Smith et al. (17).

**Alteration of active site His residues.** Chemical modification studies with MtACK have indicated that two active site His residues, His$^{117}$ and His$^{160}$, are protected by acetyl phosphate binding (16). Only His$^{180}$ has been shown to be critical for catalysis, and structural studies have indicated a likely role in stabilization of the transition state through interaction with the $\gamma$-phosphate group of ATP during transfer (13). We examined the roles of the corresponding His residues in EhACK to further compare the active sites and catalytic mechanism of MtACK and EhACK.

Kinetic parameters for the MtACK His$^{123}$Ala and His$^{180}$Ala variants in the acetate-forming direction of the reaction were not reported by Ingram-Smith et al. (16) and therefore were determined in our study. The His$^{123}$Ala MtACK variant displayed a 20-fold reduction in $k_{cat}$ with no change in the $K_m$ for acetyl phosphate, whereas the His$^{180}$Ala MtACK variant had a 47-fold reduction in $k_{cat}$ accompanied by an 8.5-fold increase in the $K_m$ for acetyl phosphate (Table 3) for a 400-fold-reduced catalytic efficiency.

The corresponding His residues, His$^{172}$ and His$^{117}$, were altered to Ala in EhACK. These variants were inactive in the direction of acetyl phosphate formation. In the direction of acetate formation, the His$^{172}$Ala variant had a similar $K_m$ for acetyl phosphate as the unaltered enzyme (Table 3); however, the $k_{cat}$ value was reduced 970-fold, consistent with a critical role for this residue in catalysis. Alteration of the His$^{117}$ of EhACK resulted in a 16-fold increase in the $K_m$ for acetyl phosphate and a 95-fold decrease in $k_{cat}$ for a nearly 1,500-fold reduction in catalytic efficiency (Table 3), implicating this residue in both substrate binding and catalysis, in strong contrast to MtACK.

**The partner enzyme for EhACK remains unknown.** Other eukaryotes that have ACK also have an ORF encoding one of its typical bacterial partner enzymes, PTA (acetyl phosphate + CoA ↔ acetyl-CoA + $P_i$) or XFP (xylulose 5-phosphate/fructose 6-phosphate + $P_i$ ↔ acetyl phosphate + glyceraldehyde 3-phosphate/erythrose 4-phosphate) (18) (Fig. 4). In the green alga *Chlamydomonas* and the oomycete *Phytophthora*, the presence of a gene encoding PTA suggests the presence of a pathway for the interconversion of acetate to acetyl-CoA. A gene encoding XFP has been identified in all fungi that have ACK, allowing for the conversion of xylulose 5-phosphate and fructose 6-phosphate to acetate in order to produce ATP as part of a modified pentose phosphoketolase pathway, as has been observed in lactic acid bacteria (18). ORFs encoding PTA or XFP are absent in the most recent *E. histolytica* genome sequence assembly; however, the genome is incomplete and gaps remain. To rule out the presence of a PTA or XFP ORF in a gap in the genome sequence or the possibility of a novel class of PTA or XFP, *Entamoeba* cell extracts were prepared and assayed for PTA and XFP activity. PTA activity was not observed in the acetyl phosphate-forming or the acetyl-CoA-forming direction. Likewise, XFP activity was not observed when assayed in the direction of acetyl phosphate formation and using fructose 6-phosphate or xylulose 5-phosphate as the substrate.

**DISCUSSION**

Whereas ACKs in *Bacteria* and *Archaea* have been extensively studied, almost nothing is known about the biochemistry of eukaryotic ACKs. Here we report the first biochemical and kinetic characterization of an ACK from a eukaryotic microbe. EhACK is unique in that it utilizes $P_i$/$PP_i$ instead of ADP/ATP as the phosphoryl acceptor/donor and is the only member of the ASKA phosphotransferase superfamily with this property.

**A proposed physiological role for ACK.** The ACK activity observed in *E. histolytica* cell homogenates supports previous evidence that the ACK gene is transcribed in trophozoites. Microarray experiments on *E. histolytica* have shown that the ACK transcript is present in genetically distinct laboratory strains (HM-1:IMSS, Rahman, and 200NIH) and in clinical isolates (strains MS75-3544 and 2592100) in a variety of different media (11).

The data presented here and those of Reeves and Guthrie (23) suggest that ACK functions primarily in the direction of acetate/PP$_i$ formation in *Entamoeba*. First, ACK activity was detected only in the acetate/PP$_i$-forming direction in *E. histolytica* cell extracts. Second, activity was more than 3 orders of magnitude higher in the direction of acetate/PP$_i$ formation versus acetyl phosphate/PP$_i$ formation with purified enzyme. Third, the enzyme has a much stronger kinetic affinity for acetyl phosphate than for acetate.

Acetate/PP$_i$, formation catalyzed by ACK is consistent with the physiology of this parasite. *E. histolytica* lacks compartmentalized, ATP-generating mitochondria and hydrogenosomes as well as a functional tricarboxylic acid cycle and oxidative phosphorylation and relies on energy generated by various types of substrate-level phosphorylation (26). Glycolysis, a major pathway for energy generation, deviates from that of most other microbes in that PP$_i$ is used as an alternative to ATP as the phosphoryl donor in the steps involving phosphofructokinase and pyruvate phosphate dikinase (24, 26). The reaction catalyzed by ACK would thus provide a source of PP$_i$ for these glycolytic enzymes. Furthermore, both ACK and ADP-forming acetyl-CoA synthetase, which utilizes ADP for the generation of ATP from acetyl-CoA (27), may be responsible for acetate fermentation observed during growth of *E. histolytica* (Fig. 4). Although it is possible that the acetate produced by ACK is converted to acetyl-CoA by ADP-forming acetyl-
CoA synthetase rather than secreted, this is unlikely, as that enzyme strongly prefers the acetate-forming direction of the reaction it catalyzes (C. Howell and C. Ingram-Smith, personal communication).

Although a physiological role for ACK in acetate and PP_i production can be envisioned, the source of acetyl phosphate as a substrate is unknown. Genes encoding PTA or XFP, ACK’s known eukaryal partner enzymes, or other known acetyl phosphate-producing enzymes (Fig. 4) are absent from the *E. histolytica* genome. Three possibilities to explain this are (i) genes encoding PTA, XFP, or other known acetyl phosphate-producing enzymes are in unfinished regions of the genome, (ii) a novel or evolutionarily distinct class of PTA or XFP exists in *Entamoeba*, or (iii) a previously uncharacterized or undiscovered acetyl phosphate-generating enzyme is present in *Entamoeba* species. Arguing against the first two possibilities are the lack of XFP or PTA activity in *Entamoeba* cell extracts and that neither XFP nor PTA ORFs have been identified in the sequence data available from the comparative sequencing projects for other species, such as *Entamoeba dispar* and *Entamoeba invadens*.

**Acyl substrate binding in EhACK versus MtACK.** The extraordinarily broad acyl substrate range for EhACK and the strong preference for the acetate/PP_i-forming direction suggest differences between EhACK and MtACK in acetate/acyetyl phosphate binding. Acyl substrate binding in MtACK appears to be mediated primarily through hydrophobic interaction between the methyl group of acetate and residues within the acetyl binding pocket, with the side chains of the binding pocket residues also serving to properly position the carboxyl group of acetate in proximity to the γ-phosphate of ATP (13, 17). Our results and those of Ingram-Smith et al. (17) revealed that MtACK variants altered at the acetate binding pocket residues Val93, Leu122, or Pro232 showed significantly increased *K_m* values for acetate in the direction of acetyl phosphate formation, but only a minimal effect on acetyl phosphate binding and catalysis in the direction of acetate formation was observed.

We investigated whether acetate/acyetyl phosphate binding was similarly mediated by EhACK in kinetic characterizations of enzyme variants altered at the corresponding residues Val87, Ile116, and Thr223. Although Val87 was shown to influence the acyl substrate range in MtACK, this residue was conserved in EhACK, and we thus speculated that the lack of conservation between Pro232 in MtACK and Thr223 in EhACK might instead be the source of the expanded acyl substrate range for EhACK. However, the EhACK Thr223Pro variant showed a similar acyl substrate range and kinetic parameters to the unaltered enzyme in the direction of acetyl phosphate formation. Thus, it appears that this speculation is incorrect. The other EhACK variants lacked activity in the unfavored acetyl phosphate-forming direction of the reaction. However, given the low activity of unaltered EhACK in this direction, this finding cannot be assumed to be of major significance, and it is not possible to draw conclusions as to the role of these residues in acetyl binding and positioning.

The kinetic parameters determined for each variant in the pre-
ferred acetate-forming direction of the reaction generally showed 
only minor to moderate changes in the $K_m$ for acetyl phosphate and $k_m$, relative to the unaltered enzyme. These results are con-
istent with those observed for MtACK and suggest that, as for 
MtACK, acetyl phosphate binding is not mediated solely 
through hydrophobic interactions but also through the phos-
phoryl group.

Interaction with the phosphoryl group of acetyl phosphate 
differs in EhACK compared to MtACK. Studies with MtACK 
identified two active site His residues, His$^{123}$ and His$^{180}$, involved 
in acetyl phosphate binding and/or catalysis. Kinetic characteriza-
tion of variants individually altered at these positions indicated 
that His$^{180}$ is essential for activity but His$^{123}$ is not (16). These 
results are consistent with the catalytic mechanism proposed 
by Gorrell et al., in which His$^{180}$ acts to stabilize the transition state 
and plays a significant role in acetyl phosphate binding and the 
orientation of the phosphate group for attack by ADP (13). In 
order to fully determine the impact of these active site His residues 
on acetyl phosphate binding, we determined the kinetic param-
ters for the MtACK His$^{123}$Ala and His$^{180}$Ala variants in the direc-
tion of acetyl phosphate formation. The His$^{180}$Ala variant had an increased $K_m$ for acetyl phosphate and decreased catalytic rate as expected, 
consistent with the proposed role for this residue. The His$^{123}$Ala 
variant showed a reduced turnover rate but no change in the $K_m$ 
for acetyl phosphate.

Kinetic analysis of the EhACK variants altered at these his-
tidines indicated substantial roles for both of these residues. Alteration at His$^{171}$ resulted in a substantial increase in the $K_m$ 
for acetyl phosphate and a reduction in turnover, whereas 
alteration at His$^{172}$ showed only a substantial reduction in catalysis 
but no effect on the $K_m$ for acetyl phosphate. Both of these 
variants had overall reductions of catalytic efficiency in the 
range of 1,400-fold versus the unaltered enzyme. Thus, inter-
actions with the phosphoryl group of acetyl phosphate are im-
portant for substrate binding and catalysis in both EhACK and 
MtACK. However, these interactions appear to differ between 
the two enzymes.

In MtACK, His$^{180}$ plays a substantial role in both acetyl phos-
phate binding and catalysis through stabilization of the transition 
state, whereas His$^{123}$ appears to play a role only in catalysis and not 
substrate binding. In EhACK, the roles of the two active site His residues are reversed, with His$^{172}$ playing a role in both acetyl phosphate binding and catalysis and His$^{171}$ having a role only in catalysis.

Conclusions. The Entamoeba ACK is unique among acetate 
kinasases and the ASKHA enzyme superfamily, not just in its ability 
to utilize PP$\_p$/P$\_i$ as the phosphoryl donor/acceptor, but also in that 
it solely uses PP$\_p$/P$\_i$ to the exclusion of ATP/ADP and other nucle-
otide triphosphates/diphosphates. This enzyme also differs fun-
damentally from the well-characterized MtACK and other ACKs 
in that it shows a strong preference for the acetyl/PP$\_p$-forming 
direction of the reaction and has a much broader acyl substrate 
range in the unfa vored acyl phosphate/P$\_i$-forming direction. In 
addition, analysis of EhACK and MtACK enzyme variants indi-
cated that they interact differently with acetyl phosphate. The 
crystal structure of EhACK, currently in refinement (T. Iverson, 
M. Tanabe, and T. Thaker, personal communication), and analy-
sis of enzyme variants will provide additional information as to 
the determinants of the distinctive phosphoryl donor specificity 
and directionality of this enzyme and may provide an understand-
ing of the evolution of these properties.

The unique PP$\_p$-forming property and directionality of EhACK 
suggests a possible role for this enzyme in providing PP$\_p$ for use 
in glycolysis, a major pathway in Entamoeba for energy generation 
with two PP$\_p$-dependent enzymes. However, the source of the 
acetyl phosphate substrate remains unknown. Identification of 
possible partners for E. histolytica ACK will provide insights into 
the function of this unique enzyme in parasite physiology and 
biochemistry.

ACKNOWLEDGMENTS

This work was supported by NSF award 0920274 and South Carolina 
Experiment Station project SC-1700340 to K.S.

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