Acetate kinase (ACK; EC 2.7.2.12; \(CH_3COO^- + ATP \rightleftharpoons CH_3COPO_4^{2-} + ADP\)) 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 archaean 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 resulted 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 \(E. histolytica\) ACK, the only known member of the ASKHA structural superfamily that utilizes inorganic pyrophosphate (PP\(_i\)) rather than ATP. In 1975, Reeves and Guthrie (23) identified a PP\(_i\)-dependent ACK activity in \(E. histolytica\), an amitochondriate protist that is the causative agent of human amebiasis. Amebiasis is the third leading cause of morbidity and the fourth leading cause of mortality due to protozoan infections, resulting in approximately 70,000 deaths worldwide (30). \(E. histolytica\) lacks compartmentalized, ATP-generating mitochondria and hydrogenosomes, and glycolysis serves as the major pathway for ATP generation (26). The \(E. histolytica\) glycolytic enzymes phosphofructokinase and pyruvate phosphate dikinase use PP\(_i\), as an alternative to ATP as the phosphoryl donor (24, 26). We show that the \(E. histolytica\) ACK functions primarily in the direction of acetate/PP\(_i\) formation; thus, ACK may play an important role in providing PP\(_i\) for completion of glycolysis. We further demonstrate functional similarities and differences between putative acetate/PP\(_i\) binding pocket residues in the \(E. histolytica\) and \(M. thermophila\) ACKs through kinetic analysis of site-altered enzyme variants.

**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 \(4 \times 10^6\) 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 \(\times g\) 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.

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Address correspondence to Kerry S. Smith, ksmith@clemson.edu.

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**Figure**: Flowchart illustrating the biochemical and kinetic characterization of the \(E. histolytica\) ACK. The enzyme activity is measured in the direction of acetate/PP\(_i\) formation. Kinetic parameters are determined for each direction of the reaction. The results are consistent with the proposed mechanism, which involves a direct in-line transfer of the phosphoryl group of ATP to acetate. The figure also highlights the importance of the active site residues, which are well conserved among the ACKs.

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**Table**: Kinetic parameters for the \(E. histolytica\) ACK activity in the direction of acetate/PP\(_i\) formation. The table includes the Michaelis-Menten constant (K\(_m\)) and the maximum velocity (V\(_{max}\)) for acetate and PP\(_i\).

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**Legend**: Figure 1: The \(E. histolytica\) ACK in action. The enzyme is shown to be active in both directions, acetate/PP\(_i\) and PP\(_i\)/acetate, with distinct kinetic parameters.
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 10% glycerol (pH 7.4). The enzyme was determined to be electrographically pure by SDS-PAGE (see Fig. SI 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 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.

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Kinetic parameters in the acetyl/PP_i-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 determine the enzymatic mechanism, the enzyme was assayed in the direction of acetyl/PP_i 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 diithiothreitol, 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 concentrations 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)-forming directions by monitoring the decrease or increase in absorbance at 233 nm, indicative of the formation or breakage of the thioester bond of acetyl-CoA, respectively. The reaction mixture for the acetyl phosphate-forming assay was prepared as described previously (20), except 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 prepared 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 hydroxymalonic acid 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, Thermo-Fisher Scientific, or VWR Scientific Products.

**RESULTS**

*E. histolytica* has a P₆/PP_i-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 PP_i-dependent ACK in axenically grown *E. histolytica*. In order to confirm the presence of ACK in *Entamoeba* and to determine whether the activity is ATP or PP_i dependent, we assayed for both ATP/ADP- and PP_i/PP_i-dependent ACK activities in cell extracts from axenically grown *E. histolytica*. ACK activity (0.6 μmol min⁻¹ mg⁻¹) was detected in the acetate-forming direction of the reaction, utilizing P_i as the phosphoryl acceptor; however, no activity was observed in this direction when we used ADP. No activity was observed with either PP_i or ATP as the phosphoryl donor in the acetyl phosphate-forming direction.

The deduced amino acid sequence of the *ACK* open reading frame (ORF; EHL_170010; XM 650898.1) 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 PP_i, as the phosphoryl donor and displays high activity in both directions of the reaction (1). To determine whether the encoded enzyme is indeed a PP_i-dependent ACK 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.

Unlike all other characterized ACKs, EhACK showed only PP_i-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 acetate-forming direction of the reaction, only inorganic phosphate could serve as the phosphoryl acceptor, and no activity was observed with ADP, AMP, or PP_i (Fig. 1B).
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/PP\(_i\) formation. Consistent with this, the purified recombinant EhACK had an over-1,000-fold-higher \(k_{\text{cat}}\) 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/PP\(_i\)-forming direction of the reaction, EhACK was found to have a broad acyl substrate range, utilizing substrates as long as octanoate (C\(_8\)). Although the apparent \(K_m\) value decreased with increasing acyl chain length, the turnover rate, \(k_{\text{cat}}\), also decreased (Table 1). The catalytic efficiency, \(k_{\text{cat}}/K_m\), 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 PP\(_i\) 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/PP\(_i\)-forming direction.

The enzyme utilizes the cofactors Mg\(^{2+}\) (\(K_m\) 2.1 \pm 0.2 mM) and Co\(^{2+}\) (\(K_m\) 5.5 \pm 0.4 mM), but no activity was observed with Ca\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), or Zn\(^{2+}\). Use of Mn\(^{2+}\) resulted in less than 10% activity compared to Mg\(^{2+}\). 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/PP\(_i\)-forming direction in an array of reaction mixtures in which the acetyl phosphate and P\(_i\) 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 P\(_i\) 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 Val\(^{95}\), Leu\(^{122}\), and Pro\(^{232}\) of MtACK (17) for site-directed alteration and determination of kinetic parameters in both directions. ACK sequence alignment indicated that Val\(^{95}\) of MtACK is conserved in EhACK.
(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/PP 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 acetic acid; however, the Ile^{116}Leu variant did have a 26-fold-decreased $k_{cat}$ (Table 3). Neither the Ile^{116}Ala nor Ile^{116}Leu variant had activity with longer acyl substrates. In the direction of acetate formation, the Ile^{116}Ala and Ile^{116}Leu variants both displayed a mild increase in 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).

### Table 2 Kinetic parameters for the EhACK Thr^{223}Pro variant

| Acyl substrate | $K_m$ (mM) | $k_{cat}/K_m$ (mM^{-1} s^{-1}) | $K_m$ $PP_i$ (mM) |
|----------------|------------|-------------------------------|-------------------|
| Acetate        | 169.5 ± 0.06 | 1.3 ± 0.01                    | 0.0077 ± 0.01     | 6.3 ± 0.1         |
| Propionate     | 98.7 ± 0.2  | 0.61 ± 0.01                   | 0.0062 ± 0.0001   | 4.0 ± 0.1         |
| Butyrate       | 56.5 ± 0.5  | 0.18 ± 0.01                   | 0.0032 ± 0.0001   | 4.9 ± 0.1         |
| Valerate       | 41.6 ± 0.3  | 0.21 ± 0.01                   | 0.0049 ± 0.0001   | 3.0 ± 0.1         |
| Hexanoate      | 20.4 ± 0.5  | 0.065 ± 0.001                 | 0.0032 ± 0.0001   | 1.9 ± 0.1         |

*Kinetic parameters were determined at 45°C using 100 mM MES, 5 mM MgCl₂, and 600 mM hydroxyamine hydrochloride (pH 7.5) with various concentrations of acyl substrate and sodium PP_i.*
Alteration of active site His residues. Chemical modification studies with MtACK have indicated that two active site His residues, His^{123} and His^{180}, 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 γ-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 acetyl-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 $k_{cat}$ 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^{117} 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 $\rightarrow$ acetyl-CoA + P$_i$) or XFP (xylulose 5-phosphate/fructose 6-phosphate + P$_i$ $\rightarrow$ acetyl phosphate + glyceraldehyde 3-phosphate/erythrose 4-phosphate) (18) (Fig. 4). In the green algae 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/PP$_i$ instead of ADP/ATP as the phosphoryl acceptor/donor and is the only member of the AKS family of phosphotransferases 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 200:NIH) 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-

### Table 3 Kinetic parameters for acetyl phosphate for EhACK and MtACK acetyl phosphate binding pocket variants in the direction of acetate formation

| Enzyme | Alteration | $K_m$ (acetyl phosphate) (mM) | $k_{cat}$ (s$^{-1}$) |
|--------|------------|------------------------------|-------------------|
| EhACK$^a$ | Unaltered | 0.50 ± 0.01 | 1.939 ± 14 |
|         | Thr^{223}Pro | 2.0 ± 0.08 | 8.333 ± 185 |
|         | Thr^{223}Gly | ND$^b$ | ND |
|         | Ile^{116}Ala | 1.6 ± 0.09 | 280 ± 2 |
|         | Ile^{116}Leu | 2.4 ± 0.02 | 344 ± 2 |
|         | Val^{87}Ala | 0.53 ± 0.03 | 2,128 ± 2 |
|         | Val^{87}Gly | 0.51 ± 0.02 | 97 ± 1.0 |
|         | His^{117}Ala | 8.0 ± 0.07 | 21 ± 0.1 |
|         | His^{172}Ala | 0.69 ± 0.07 | 2.0 ± 0.07 |
| MtACK$^b$ | Unaltered$^d$ | 0.34 ± 0.01 | 2,680 ± 45 |
|         | Pro^{232}Gly | 1.3 ± 0.04 | 124 ± 2 |
|         | Pro^{232}Thr | 1.5 ± 0.16 | 2,355 ± 83 |
|         | His^{123}Ala | 0.21 ± 0.01 | 135 ± 8 |
|         | His^{180}Ala | 2.9 ± 0.40 | 57 ± 3 |

$^a$ Kinetic parameters were determined at 37°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.

$^b$ 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).

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**Entamoeba histolytica** PP$_i$-Forming Acetate Kinase

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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 *E. 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/acetyl 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 acetate 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 $\gamma$-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 Val$^{93}$, Leu$^{122}$, or Pro$^{232}$ 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 acetylaceyl phosphate binding was similarly mediated by EhACK in kinetic characterizations of enzyme variants altered at the corresponding residues Val$^{87}$, Ile$^{116}$, and Thr$^{223}$. Although Val$^{93}$ 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 Pro$^{232}$ in MtACK and Thr$^{223}$ in EhACK might instead be the source of the expanded acyl substrate range for EhACK. However, the EhACK Thr$^{223}$Pro 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 acetate 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 $k_{cat}$ relative to the unaltered enzyme. These results are consistent 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 phosphoryl 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 characterization 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 of acetate formation. The His$^{180}$Ala variant had an increased substrate binding. In EhACK, the roles of the two active site His range in the unfavored acyl phosphate/Pi-forming direction. In that it shows a strong preference for the acetate/PPi-forming directionality in the unfavored acyl phosphate/Pi-forming direction. In that it shows a strong preference for the acetate/PPi-forming directionality in the unfavored acyl phosphate/Pi-forming direction.

Kinetic analysis of the EhACK variants altered at these histidines indicated substantial roles for both of these residues. Alteration at His$^{177}$ 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, interactions with the phosphoryl group of acetyl phosphate are important 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 phosphate 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$^{177}$ playing a role in both acetyl phosphate binding and catalysis and His$^{172}$ having a role only in catalysis.

Conclusions. The Entamoeba ACK is unique among acetate kinases and the ASKHA enzyme superfamily, not just in its ability to utilize PP/P as the phosphoryl donor/acceptor, but also in that it solely uses PP/P to the exclusion of ATP/ADP and other nucleotide triphosphates/diphosphates. This enzyme also differs fundamentally from the well-characterized MtACK and other ACKs in that it shows a strong preference for the acetate/PP-forming direction of the reaction and has a much broader acyl substrate range in the unfavored acyl phosphate/P$_2$-forming direction. In addition, analysis of EhACK and MtACK enzyme variants indicated 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 analysis 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 understanding of the evolution of these properties.

The unique PP$_2$-forming property and directionality of EhACK suggests a possible role for this enzyme in providing PP$_2$ for use in glycolysis, a major pathway in Entamoeba for energy generation with two PP$_2$-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.

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