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Investigations of the PPi-dependent Acetate Kinase from the Parasite Entamoeba histolytica

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INVESTIGATIONS OF THE PP₁-DEPENDENT ACETATE KINASE FROM THE PARASITE ENTAMOEBA HISTOLYTICA

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biochemistry and Molecular Biology

by
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August 2011

Accepted by:
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ABSTRACT

Acetate, a short-chain fatty acid that plays a key role in all domains of life, can be utilized as a carbon source or excreted as a product of metabolism. Acetate kinase (ACK), a member of the acetate and sugar kinase-Hsp70-actin (ASKHA) enzyme superfamily, is responsible for the reversible phosphorylation of acetate to acetyl phosphate utilizing ATP as the phosphoryl donor. Acetate kinases are ubiquitous in the Bacteria, found in one genus of Archaea, and are also present in microbes of the Eukarya. A partially purified ACK which can utilize pyrophosphate (PPi) as the phosphoryl donor in the acetyl phosphate-forming direction was previously identified in Entamoeba histolytica, the causative agent of amoebic dysentery. Here I describe my research on the E. histolytica PPi-dependent ACK.

My biochemical and kinetic characterization of the E. histolytica ACK revealed several properties that are unique to this enzyme. First, the E. histolytica enzyme is capable of utilizing acyl substrates longer than butyrate and can utilize acyl substrates as long as hexanoate in the direction of acetyl phosphate synthesis. Second, this ACK can only utilize PPi as a phosphoryl donor and cannot utilize ATP. The kinetic parameters for substrate affinity indicate that the enzyme prefers both an acetate and propionate in the acetyl phosphate-forming direction; however, the low $k_{cat}$ raises question whether this direction of the reaction is physiological. The enzyme operates preferentially in the direction of acetate/PPi synthesis as the rate of catalysis and overall catalytic efficiency are 1,102-fold and 240,351-fold higher, respectively. Studies of the mechanism of this enzyme indicates that the PPi-ACK follows a sequential mechanism, supporting a direct
in-line phosphoryl transfer mechanism previously reported in the well characterized *Methanosarcina thermophila* ATP-dependent ACK.

A modified hydroxamate assay was developed for measuring ACKs in the direction of acetate formation. A coupled enzyme assay has been used to measure ATP production in the direction for the prokaryotic ACKs, but there was not a method available for measuring a PP\(_i\)-forming ACK in this direction. The assay described here allowed for the determination of kinetic parameters in the acetate-forming direction for the *E. histolytica* ACK and can not only be used to measure ACK activity of both ATP-dependent and PP\(_i\)-dependent acetate kinases in the direction of acetate formation, but could also prove useful for other enzymes that utilize activated acyl substrates such as acetyl adenylate and acetyl-CoA.

Investigation of the residues in the putative PP\(_i\)-ACK active site in *E. histolytica* revealed that while residues conserved across the ACK family are present in the active site, the enzyme has a number of significant active site changes in comparison to its counterpart, the *M. thermophila* ATP-dependent ACK. The residues His\(^{117}\), His\(^{172}\), Val\(^{87}\), Thr\(^{201}\), Thr\(^{15}\), Arg\(^{274}\), and Asp\(^{272}\) appear to be essential for catalysis in the direction of acetate synthesis. Furthermore, His\(^{117}\) appears to be critically important for binding acetyl phosphate and therefore important in acetate/PP\(_i\) synthesis reactions.

RNA interference of *E. histolytica* ACK was unable to be carried out due to problems with transfection of plasmid DNA into the parasite. However, data garnered from cellular extracts indicates that *E. histolytica* has a PP\(_i\)-dependent ACK but not ATP-dependent ACK. Activities of the eukaryotic ACK partner enzymes
phosphotransacetylase and xylulose 5-phosphate/fructose 6-phosphate phosphoketolase were absent in cellular extracts. The absence of bacterial ACK partner enzymes in *Entamoeba* suggests that a novel acetyl phosphate generating enzyme is the partner for the PPi-ACK in this protist. This speculative partner enzyme could generate acetyl phosphate from an intermediate of the pentose phosphate pathway or the end product of a yet to be identified catabolic pathway.
DEDICATION

This work is dedicated to my parents, for their encouragement and support which have made my dreams possible. And to my students, who have provided me with a remarkable experience that I will treasure forever.
ACKNOWLEDGMENTS

First, I would like to express my sincere gratitude to my advisor, Dr. Kerry Smith, who has been a mentor, friend, and father figure through this process. His support, wisdom, and remarkable patience have greatly inspired me, and I am forever grateful.

To Dr. Jim Morris, Dr. William Marcotte, and Dr. Lesly Temesvari, my other committee members, I am especially grateful for your input, support, and assistance.

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CHAPTER I
LITERATURE REVIEW ON ACETATE KINASE AND THE PARASITE

ENTAMOEBA HISTOLYTICA

I. INTRODUCTION

Acetate, a short-chain fatty acid that plays a key role in all domains of life, can be utilized as a carbon source or can be excreted as a product of metabolism. The interconversion of acetate to acetyl-CoA is an important step in a variety of both catabolic and anabolic pathways and can occur via three pathways. The first pathway consisting of the enzymes acetate kinase (ACK) and phosphotransacetylase (PTA), is reversible and nearly ubiquitous in the Bacteria. ACK catalyzes the reversible transfer of the γ-phosphoryl group of ATP to acetate to form acetyl phosphate [EC 2.7.2.1, Eqn. 1], PTA then catalyzes the reversible transfer of coenzyme A (CoA) forming acetyl-CoA [EC 2.3.1.8; Eqn. 2]. A unidirectional ADP-forming acetyl-CoA synthetase (ADP-ACS), found in amitochondriate protists and some microbes in the Bacteria and Archaea, catalyzes the conversion of acetyl-CoA to acetate to generate ATP [EC 6.2.1.1.3, Eqn. 3]. Finally, the AMP-forming acetyl-CoA synthetase (AMP-ACS) is widespread in the Archaea, Bacteria, and Eukarya and is the predominant enzyme for activating acetate to acetyl-CoA [Eqn. 4].

\[
\text{acetate + ATP } \rightleftharpoons \text{acetyl-P}_1 + \text{ADP} \quad \text{[Eqn. 1]}
\]

\[
\text{acetyl-P}_1 + \text{CoA } \rightleftharpoons \text{acetyl-CoA} + \text{P}_i \quad \text{[Eqn. 2]}
\]

\[
\text{acetyl-CoA} + \text{ADP} + \text{P}_i \rightarrow \text{acetate} + \text{ATP} \quad \text{[Eqn. 3]}
\]

\[
\text{acetate} + \text{ATP} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{PP}_i \quad \text{[Eqn. 4]}
\]
ACK, a member of the acetate and sugar kinase-Hsp70-actin (ASKHA) enzyme superfamily, has recently been identified in a number of eukaryotic microbes including algae and oomycetes, non-yeast fungi, and a few protists including *Entamoeba histolytica*. In this chapter, the biochemistry, enzymology, and mechanism of prokaryotic ACKs are introduced including a thorough discussion of the *Methanosarcina thermophila* enzyme, the best characterized prokaryotic ACK. The physiological roles of ACK in the *Bacteria* and *Archaea* are also explored. A brief introduction to *E. histolytica*, which has a novel PPi-dependent ACK, is provided with respect to the metabolic processes of the parasite.
II. ATP-DEPENDENT ACETATE KINASES

1. Physiological Roles of Acetate Kinase in the *Bacteria* and *Archaea*.

ACK, an important enzyme in bacterial metabolism, partners with PTA forming one of three pathways for the interconversion of acetyl-CoA and acetate [1]. ACK and PTA are nearly ubiquitous in bacteria which undergo fermentation though the use of the AK-PTA pathway for energy conservation in the conversion of acetyl-CoA to acetate. In non-fermentative bacteria, the pathway operates to activate acetate to acetyl-CoA for use as a carbon and/or energy source. Some bacteria such as *Escherichia coli* take advantage of the reversibility of the ACK-PTA pathway (Figure 1.1). The ACK-PTA pathway is used for acetate activation at high acetate concentrations while AMP-ACS is favored under low acetate conditions [2, 3]. Under conditions that result in mixed acid fermentation, acetyl-CoA cannot enter the TCA cycle and excess acetyl-CoA is used to produce acetate and ATP via the ACK-PTA pathway [1]. Under aerobic conditions the ACK-PTA pathway is used to excrete acetate and generate ATP.
Figure 1.1. The acetate switch. Bacterial pathways for acetate fermentation and the generation of acetyl-CoA are accomplished though an ACK/PTA pathway. Modified from [1].
Other enzymes that can generate acetyl phosphate include pyruvate oxidase (POX), xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP), sulfoacetaldehyde acetyltransferase (XSC), and glycine reductase (GR). These enzymes have been shown to function with ACK and are found in a group of diverse bacteria, but not in *E. coli* [4]. POX, present in diverse lactobacilli and *Streptococcus pneumoniae*, catalyzes the oxidative decarboxylation of pyruvate to acetyl phosphate, carbon dioxide, and hydrogen peroxide. XFP, present in heterofermentative lactobacilli such as *Bifidobacterium* and *Lactobacillus* species, catalyzes the formation of acetyl phosphate and glyceraldehyde 3-phosphate (or erythrulose 4-phosphate) from xylulose 5-phosphate (or fructose 6-phosphate) and inorganic phosphate. XSC, present in several genera of Proteobacteria, cleaves sulfoacetaldehyde to acetyl phosphate and sulfite. Acetyl phosphate formed by these enzymes has two possible metabolic fates, either conversion to acetyl-CoA by PTA for anabolic pathways or conversion to acetate by ACK for ATP synthesis. GR is an important enzyme involved in the metabolism of glycine in some strictly anaerobic gram-positive bacteria such as *Clostridium* and *Eubacterium* [5]. Sarcosine and betaine reductases, like GR also yield acetyl phosphate as a product. In all three pathways, energy is formed by the ACK-catalyzed conversion of acetyl phosphate to ATP.

Only two genera of methane-producing archaea, *Methanosaeta* and *Methanosarcina*, have been isolated that are capable of converting acetate to methane [6]. *Methanosaeta* is an obligate acetotroph and utilizes AMP-ACS to activate acetate to acetyl-CoA at the expense of two high-energy phosphates in the first step of
methanogenesis. *Methanosarcina*, which can also utilize carbon dioxide, methylated amines and methylated sulfides as energy and growth substrates [7], employs acetate kinase (ACK) and phosphotransacetylase (PTA) to activate acetate to acetyl-CoA [1,2]. *Methanosarcina* is the only genus in the *Archaea* with ACK or PTA. In both genera, acetyl-CoA is then cleaved at the carbon-carbon and carbon-sulfur bond by carbon monoxide dehydrogenase/acetyl-CoA synthetase (CODH). The carbonyl group is oxidized to carbon dioxide [8, 9] and the methyl group reduced to methane [10]. Electrons derived from CODH are transferred via ferrodoxin and an iron-sulfur flavoprotein to a membrane-bound electron transport system which may serve to generate an ion gradient for the synthesis of ATP [10-12].

2. Biochemistry of the *Methanosarcina thermophila* Acetate Kinase

The native ACK from *Methanosarcina thermophila* has been purified and characterized [13]. The presence of high concentrations of acetate kinase in extracts of *M. thermophila* cultured on acetate supported the hypothesis that ACK functioned physiologically in the initial activation of acetate for the conversion to methane and carbon dioxide [13]. An additional supporting argument was the detected presence of PTA [14, 15] which would allow the conversion of ACK-generated acetyl phosphate to be converted to acetyl-CoA [12]. Increased levels of PTA had also been reported when *M. thermophila* was grown on acetate [12]. Furthermore, when *M. thermophila* was cultured in the presence of methanol or acetate, the synthesis of PTA was not inhibited by methanol, but was found to be induced by acetate in the growth medium [15].
The genes encoding the *M. thermophila* ACK and PTA were cloned and sequenced [14], and found to be transcribed as a single transcriptional unit with *pta* located upstream of *ack* and separated by an intergenic sequence [16]. Examples of acyl kinase/PTA genes co-expressed from the same promoter have been previously noted in *E. coli* [3] and *Clostridium acetobutylicum* [17]. The *M. thermophila* *pta-ack* operon was shown to be regulated by growth substrate. Northern blot analyses indicated that the steady-state levels of the *pta-ack* transcript were several fold greater in acetate-grown cells than cells grown on methanol or methylated amines [16]. Consistent with the transcript studies, the activities of PTA and ACK are at least 8- to 11- fold higher in acetate grown cells than in cells grown on other carbon and energy sources [16]. The development of molecular genetics tools such as *in vivo* transposon mutagenesis in *Methanosarcina acetivorans* [18] allowed Rother and Metcalf [19] to demonstrate that the *pta-ack* mutants could not grow on acetate as a carbon and energy source. Thus, the activation of acetate to acetyl-CoA in aceticlastic methanogenesis in *Methanosarcina* occurs through ACK and PTA [15].

Noting the high similarity of the *M. thermophila* and *E. coli* ACKs, and the conservation of residues near the active site of those ACKs purified from bacteria, Latimer and Ferry [14] initiated a preliminary investigation of the active site of the *M. thermophila* ACK using a number of amino acid modifying agents. Using diethylpyrocarbonate (DEPC) and phenylglyoxal, modifiers of histidine and arginine respectively, they suggested that one or more arginine residues and possibly one histidine residue were implicated in the active site of the *M. thermophila* ACK [14]. This
hypothesis was partially supported by previous evidence of an essential arginine residue in the active site of the *E. coli* ACK which could be inactivated by chemical modification [20]. Further evidence was garnered by Latimer and Ferry [14] using thermal stability assays by comparing the optimal activity temperatures of the commercially available ACK from *E. coli* and the recombinantly produced *M. thermophila* ACK in order to further highlight the presence of an essential arginine residues by substrate protection. Latimer and Ferry [14] were also able to show that binding of ATP induced a conformational change in the ACK, thereby making the enzyme more stable. When pre-incubated with 10 mM ATP for 5 minutes, the enzyme retained full activity up to 70°C. In Tris buffer alone, the enzyme was completely inactive after incubation for 5 minutes at 55°C [14]. Although 10 mM phenylglyoxal resulted in 95% inactivation of enzyme, pre-incubation with ATP resulted in only 61% inhibition indicating some protection from inactivation. However, the presence of either acetyl phosphate or acetate did not prevent the inactivation by phenylglyoxal [14]. This data is consistent with at least one arginine residue being present in the ATP active site of the ACK [14].

Singh-Wissmann et al. [21] investigated five arginine residues (R91, R175, R241, R285, and R340) in the *M. thermophila* ACK which were identified as being highly conserved among other ACKs. R91 and R241 alanine variants displayed extremely low specific activities which precluded obtaining kinetic data [21]. Leucine and lysine alteration to these two amino acid positions resulted in specific activity ranges similar to the recombinant wild-type, with exception of the R91K variant which was reported to have a specific activity at the lower limit of detection at 0.5 µmol min⁻¹ mg⁻¹ [21]. While
considered a conservative replacement for arginine, the R91K variant displayed a $k_{cat}$ of over 100-fold lower and a $K_m$ for acetate nearly 14-fold greater than the wild-type (Table 1.1) which implicated R91 as important for catalysis and binding of acetate [21]. A 100-fold decrease in the $k_{cat}$ for ATP and more than 400-fold decrease in catalytic efficiency by the R91K variant in comparison to the wild-type further implicated the importance of this residue in catalysis. Replacement of R285 with alanine, lysine, or leucine, resulted in 1% catalytic efficiency of the wild-type. However, an increase of nearly 10-fold in the $K_m$ for acetate for R285 indicated that this residue may be important for acetate binding [21]. Variants of R175 and R340 displayed low activity, but their respective kinetic parameters were not significantly altered to implicate them in substrate binding or catalysis. Thus, R91 and R241 are important for catalysis in the *M. thermophila* ACK and may be important for acetate binding. With the assistance of crystal structures, Gorrell et al. [22] later confirmed the importance of R91 and R241 in catalysis.
Table 1.1. Kinetic Constants of Recombinant Wild-Type and Arginine Variants of \textit{M. thermophila} ACK. Taken from [21].

<table>
<thead>
<tr>
<th>enzyme</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) (mM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (mM(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>19 ± 3</td>
<td>1596 ± 140</td>
<td>84</td>
<td>1.0 ± 0.3</td>
<td>1669 ± 288</td>
<td>1669</td>
</tr>
<tr>
<td>R91K</td>
<td>260 ± 35</td>
<td>13.5 ± 0.3</td>
<td>0.1</td>
<td>3.9 ± 0.2(^a)</td>
<td>16.7 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>R175K</td>
<td>4 ± 2</td>
<td>70 ± 18</td>
<td>18</td>
<td>1.0 ± 0.5</td>
<td>73 ± 17</td>
<td>73</td>
</tr>
<tr>
<td>R285A</td>
<td>206 ± 25</td>
<td>216 ± 25</td>
<td>1</td>
<td>6.0 ± 1.0(^a)</td>
<td>302 ± 38</td>
<td>50</td>
</tr>
<tr>
<td>R285K</td>
<td>219 ± 148</td>
<td>234 ± 41</td>
<td>1</td>
<td>5.0 ± 1.0(^a)</td>
<td>262 ± 22</td>
<td>87</td>
</tr>
<tr>
<td>R285L</td>
<td>129 ± 7</td>
<td>186 ± 17</td>
<td>1</td>
<td>3.0 ± 1.0(^a)</td>
<td>224 ± 48</td>
<td>75</td>
</tr>
<tr>
<td>R340K</td>
<td>42 ± 12</td>
<td>1014 ± 15</td>
<td>24</td>
<td>1.3 ± 0.2(^a)</td>
<td>1179 ± 182</td>
<td>907</td>
</tr>
<tr>
<td>R340L</td>
<td>20 ± 3</td>
<td>631 ± 158</td>
<td>32</td>
<td>0.9 ± 0.2</td>
<td>584 ± 63</td>
<td>649</td>
</tr>
</tbody>
</table>

\(^a\) Determined at 1.5 M potassium acetate.
Previous research on the *E. coli* ACK [23] had suggested that the mechanism for the ACK involved a phosphoryl-enzyme intermediate, with an unspecified glutamate residue phosphorylated. E384, E385, and E97 in the *M. thermophila* ACK were identified as having a role in either substrate binding or catalysis (Table 1.2) [24]. E384 variants had very low or no detectable activity indicating an important role in catalysis. Replacement of E385 with alanine or aspartate did not significantly alter the $k_{cat}$, but did significantly increase the $K_m$ values for both acetate and ATP, indicating that E385 is important for substrate binding, but not catalysis (Table 1.2) [24]. Changes to E97 resulted in decreases in the $K_m$ for acetate corresponding decreases in $k_{cat}$ indicating that this residue may influence acetate binding.
Table 1.2. Kinetic constants of unaltered and altered ACKs from *M. thermophila*.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic$^b$</td>
<td>22</td>
<td>1,050</td>
<td>47</td>
<td>2.8</td>
<td>1,221</td>
<td>436</td>
</tr>
<tr>
<td>Unaltered</td>
<td>19 ± 3</td>
<td>1,596 ± 140</td>
<td>84</td>
<td>1.0 ± 0.3</td>
<td>1,669 ± 288</td>
<td>1,669</td>
</tr>
<tr>
<td>E32A</td>
<td>26 ± 2</td>
<td>1,059 ± 97</td>
<td>40</td>
<td>1.1 ± 0.2</td>
<td>1,012 ± 262</td>
<td>920</td>
</tr>
<tr>
<td>E97A</td>
<td>2.3 ± 0.5</td>
<td>165 ± 3</td>
<td>72</td>
<td>1.4 ± 0.1</td>
<td>382 ± 49</td>
<td>216</td>
</tr>
<tr>
<td>E97D</td>
<td>7.2 ± 3.5</td>
<td>430 ± 60</td>
<td>60</td>
<td>1.4 ± 0.6</td>
<td>567 ± 173</td>
<td>405</td>
</tr>
<tr>
<td>E97Q</td>
<td>4.0 ± 0.4</td>
<td>273 ± 56</td>
<td>68</td>
<td>1.4 ± 0.7</td>
<td>275 ± 59</td>
<td>197</td>
</tr>
<tr>
<td>E334A</td>
<td>15 ± 6</td>
<td>951 ± 35</td>
<td>62</td>
<td>0.7 ± 0.2</td>
<td>1,198 ± 62</td>
<td>1,711</td>
</tr>
<tr>
<td>E385A</td>
<td>86 ± 30$^e$</td>
<td>636 ± 158$^e$</td>
<td>7</td>
<td>6.9 ± 0.5</td>
<td>1,308 ± 112</td>
<td>190</td>
</tr>
<tr>
<td>E385C</td>
<td>17 ± 4</td>
<td>637 ± 139</td>
<td>37</td>
<td>1.6 ± 0.5</td>
<td>813 ± 214</td>
<td>508</td>
</tr>
<tr>
<td>E385D</td>
<td>337 ± 81$^e$</td>
<td>456 ± 132$^e$</td>
<td>1</td>
<td>4.5 ± 0.5</td>
<td>671 ± 34</td>
<td>149</td>
</tr>
<tr>
<td>E385Q</td>
<td>44 ± 19</td>
<td>1,869 ± 479</td>
<td>42</td>
<td>2.0 ± 0.6</td>
<td>1,711 ± 231</td>
<td>856</td>
</tr>
</tbody>
</table>

$^a$ Unaltered and altered ACKs were produced in *E. coli*.  

$b$ Published values for the ACK purified from *M. thermophila* (1) are given.  

$c$ Apparent value.
In 2001, Buss et al. [25] solved the structure of the *M. thermophila* ACK and indicated that it may be the ancestral enzyme of the ASKHA (acetate and sugar kinases/Hsc70/actin) superfamily. The enzyme contained two domains with a duplicated $\beta\beta\alpha\beta\alpha\beta\alpha$ core with insertions of subdomains between elements of the $\beta$-sheet [25]. Of note were the numbers of highly conserved residues known to play a role in the active sites of other ACKs as well as other members of the ASKHA family. In the active site, Buss et al. [25] noted the presence of a hydrophobic pocket, composed of R285, I332, I339. Furthermore, the structure also confirmed the presence of H180 and H123 and the importance of these residues in binding the phosphate of acetyl phosphate, along with R91, R241, and G212 [25]. The presence of a sulfate ion in the active site from crystallization conditions is believed to occupy the site of the phosphate of acetyl phosphate. The sulfate ion ligands binding to these side chains are conserved within the acetate kinase-butyrate kinase family [25]. The importance of E384, a residue conserved among all acetate and butyrate kinases, was shown to be moved into the active site upon cleft closure, participating in catalysis. Known to be essential for enzyme function [24], Buss et al. [25] hypothesized that this residue is likely a phosphorylation site for a direct in-line phosphoryl transfer mechanism.

The role of histidines in the *M. thermophila* ACK was investigated around the same time using DEPC inactivation and structure-function studies. When incubated with increasing concentrations of DEPC, the *M. thermophila* ACK displayed both a time and concentration-dependent loss of enzymatic activity [26]. These results suggested the presence of a number of histidine residues in the active site with at least one residue
located directly in the active site, as previously suggested by Latimer and Ferry [14].

Ingram-Smith et al. [26] identified a conserved histidine residue H180 as essential for catalysis and hypothesized that this residue could potentially function as a hydrogen bond donor or positive charge stabilizer for a pentacoordination transition state for a direct, in-line mechanism proposed earlier [27].

Miles et al. [28] further confirmed the importance of E384 on catalysis in the *M. thermophila* ACK. A 30-fold increase in the magnesium concentration was required for half-maximum activity of the E384A variant, implicating this residue in magnesium binding. Furthermore, D148 and N7 variants resulted in catalytic efficiencies of less than 1% of the native *M. thermophila* ACK. Miles et al. [28] proposed that the third stabilization site, required for a direct, in-line phosphoryl transfer mechanism as proposed by Blättler and Knowles [27], may be a magnesium ion stabilized by E384 and N7, with R91 and R241 serving as the other two stabilization sites.

Using the solved structure of the *M. thermophila* ACK, Ingram-Smith et al. [29] identified a hydrophobic pocket formed by the residues V93, L122, F179, and P232 in the active site cleft of the enzyme which is hypothesized to serve as the acetate-binding site (Figure 1.2). Changes to these residues resulted in significant increases in the *K*_m* for acetate (Table 1.3) highlighting the contribution of these residues in acetate binding [29]. Alteration of V93 to alanine and glycine resulted in 14-fold and 26 fold respective increases in the *K*_m* for acetate. The P232A variant displayed a 21-fold increase in acetate affinity compared to the wild type. Specifically, the identification of the F179 is important with respect to other members of the ASKHA enzyme family, as this residue is
conserved in all acetate, propionate, and butyrate kinases [25]. Members of this family undergo a catalytically-required domain closure [30-32], characterized by the presence of a glycine residue forming a phenylalanine-glycine pair. This structural architecture, which has been observed in glycerol kinase [32, 33], hexokinase [34], and phosphoglycerate kinase [35] was also observed by these researchers [29] in acetate and propionate kinases. The 233-fold and 99-fold decrease in respective turnover and catalytic efficiency for acetyl phosphate observed for the F179A variant also highlights the importance of this residue in the active site [29]. F179 is located at the N terminus of the helix α3, a bridge between domains I and II with respect to ASKHA superfamily structures [25].
Figure 1.2. Structure of the ACK dimer from *M. thermophila*. Areas in red represent clefts of the binding pockets. Taken from [25].
Table 1.3. Kinetic parameters of wild-type and variant ACKs assayed in the direction of acetyl phosphate synthesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>Acetate $K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
<th>ATP $K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.055 ± 57</td>
<td>1.5 ± 0.16</td>
<td>703 ± 84</td>
<td>71.3 ± 7.0</td>
<td>14.8 ± 1.7</td>
</tr>
<tr>
<td>Val$^{19}$ Ala</td>
<td>644 ± 34</td>
<td>21.4 ± 1.8</td>
<td>39 ± 2.0</td>
<td>49.9 ± 3.3</td>
<td>16.9 ± 1.3</td>
</tr>
<tr>
<td>Val$^{19}$ Gly</td>
<td>347 ± 21</td>
<td>39.5 ± 1.7</td>
<td>8.8 ± 0.7</td>
<td>68.7 ± 1.9</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Leu$^{126}$ Ala</td>
<td>150 ± 12</td>
<td>19.5 ± 1.8</td>
<td>7.7 ± 0.9</td>
<td>57.6 ± 2.7</td>
<td>2.6 ± 0.24</td>
</tr>
<tr>
<td>Phe$^{176}$ Ala</td>
<td>2.2 ± 0.3</td>
<td>10.4 ± 1.1</td>
<td>0.21 ± 0.04</td>
<td>50.5 ± 0.6</td>
<td>0.043 ± 0.006</td>
</tr>
<tr>
<td>Pro$^{226}$ Ala</td>
<td>132 ± 2</td>
<td>32.0 ± 2.6</td>
<td>4.1 ± 0.3</td>
<td>54.2 ± 1.5</td>
<td>2.4 ± 0.08</td>
</tr>
</tbody>
</table>
3. The Catalytic Mechanism of Acetate Kinase

The studies of the catalytic mechanisms of ACKs have been limited to the *E. coli* and *M. thermophila* enzymes. The *E. coli* ACK was first described by Lipmann in 1944 and finally isolated by Rose [36] in 1954. The majority of research performed on the *E. coli* enzyme has been limited to mechanistic studies with the earliest information concerning the mechanism in 1972. A phosphoenzyme intermediate was generated by incubating the enzyme with either $\gamma^{32}\text{P-ATP}$ or acetyl $^{32}\text{P-phosphate}$ [37]. This result was consistent with the enzyme functioning through a ping-pong mechanism where the enzyme and ATP reacted to form a phosphoenzyme, and then a phosphoryl transfer from the phosphoenzyme to acetate to form acetyl phosphate. The phosphoenzyme intermediate was shown to be capable of phosphoryl transfer to either ADP or acetate [37, 38]. A caveat to this proposed mechanism was that the substrate exchange rates for ADP/ATP and acetate/acetyl phosphate were less than the rate of catalysis. In fact, one report indicated a 7-fold faster ADP/ATP exchange reaction than the reported rate of catalysis [39]. In the same report, the acetate/acyetyl phosphate rate of exchange was 64-fold slower than the rate of catalysis [39], and a second report indicated no detectable exchange of acetate/acyetyl phosphate [36]. Furthermore, reported slower exchange rates for acetate/acyetyl phosphate in the presence of ATP, culminated in these researchers inability to exclude that reactions were occurring in both the acetate/ATP and the acetyl phosphate/ADP directions; and therefore, may not be limited to one particular direction [40].
Since the initial mechanistic studies two phosphoryl transfer mechanisms have been proposed for ACK [22, 41]. In a single displacement mechanism, a direct phosphoryl transfer occurs from the gamma phosphate of ATP to acetate. In 1980, Spector [41], based on the evidence of the phosphoenzyme intermediate, proposed a triple-displacement mechanism where the gamma phosphate of ATP is transferred over two separate enzyme sites before final transfer to acetate [27]. The reaction would be characterized by a net inversion of configuration of the phosphorus atom during transfer from ATP to acetate. While the presence of the phosphoenzyme intermediate had been previously demonstrated [37], Spector [41] noted that a direct transfer was sterically problematic; as experimental evidence on tetra- and penta-coordinate phosphorus compounds had resulted in evidence to suggest one or more $S_N2$-type displacements; with mechanisms involving steric inversions during each initial and subsequent reaction(s) [27, 42, 43]. He therefore proposed a triple displacement mechanism for ACK involving two phosphoenzyme intermediates, and three inversions of the phosphorus (Figure 1.3) to accommodate the steric and special requirements between the substrate binding sites in the enzyme [41].
Figure 1.3. Representation of the active center of ACK. Associated with the nucleotide-binding region is the catalytic carboxyl that has ATP as its immediate phosphoryl donor. Associated with the acetate (and acetyl-P) binding region is an unknown group (X) that has acetyl-P as its immediate phosphoryl donor. The phosphoryl group is reversibly transferable between X and the carboxyl group. All phosphoryl transfers among substrates and the catalytic groups proceed with steric inversion on the phosphorus. It is conjectured that the intermediate E-X-PO$_3$ is at a higher energy level than E-COOPO$_3$, because only the latter is isolated when the enzyme is phosphorylated by ATP or acetyl-P. Taken from [41].
More recent research on the *M. thermophila* ACK suggests that the enzyme functions through a single displacement or direct, in-line phosphoryl transfer mechanism. A number of phosphoryl transfer enzymes, both phosphatases and kinases, have been mechanistically studied using aluminium fluoride, which mimics the planar phosphoryl group in the transition state [44-46]. Pre-incubating the *M. thermophila* ACK with magnesium chloride, ADP, aluminium chloride, and sodium fluoride inhibited the activity of the enzyme by forming an abortive active site complex (Figure 1.4) [47]. Miles et al. [47] reported that the rate of observed inhibition was slow for enzyme-ligand interactions, but could be attributed to the sluggish rate of aluminium fluoride complex formation which had been previously observed [48]. In the absence of ADP and magnesium chloride (Figure 1.3, inset) no detectable loss of activity was observed [48]. This evidence indicated that the presence of magnesium chloride, ADP, acetate, and aluminium fluoride are required for maximum inhibition of the *M. thermophila* ACK (Table 1.4) [47].
Figure 1.4. Inhibition of *M. thermophila* ACK by MgCl₂, ADP, AlCl₃, NaF, and acetate. ACK (0.4 µM dimer) was pre-incubated for the indicated times in a mixture containing MgCl₂ (10 mM), ADP (10 mM), AlCl₃ (0.1 mM), NaF (0.5 mM), and potassium acetate (25 mM) in 100 mM BisTris buffer (pH 5.5). The enzyme activity was then assayed and plotted relative to 100% activity (600 µmol of acetyl phosphate/min/mg of protein) obtained from a control mixture in which AlCl₃ and NaF were omitted. Inset, the time course of the activity assay after pre-incubation for 30 min with: complete inhibitory mixture (●), minus NaF and AlCl₃ (■), minus MgCl₂, ADP and acetate (♦), minus all components except buffer (▲). Taken from [47].
Table 1.4. Inhibition of the ACK from *E. coli* or *M. thermophila*. Taken from [47].

<table>
<thead>
<tr>
<th>Preincubation mixture components$^a$</th>
<th><em>M. thermophila</em>$^b$</th>
<th><em>E. coli</em>$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>% activity</td>
<td>3.0 ± 0.8</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>MgADP, aluminum fluoride, acetate</td>
<td>90 ± 2</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>ADP, aluminum fluoride, acetate</td>
<td>95 ± 1</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>Mg, aluminum fluoride, acetate</td>
<td>77 ± 4</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>MgADP, fluoride, acetate</td>
<td>95 ± 4</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>MgADP, aluminum, acetate</td>
<td>89 ± 8</td>
<td>98 ± 2</td>
</tr>
</tbody>
</table>

$^a$ The enzyme (0.4 µM dimer) was pre-incubated for 30 min with MgCl$_2$ (10 mM), ADP (10 mM), AlCl$_3$ (0.1 mM), NaF (0.5 mM), and acetate (25 mM) unless otherwise noted before the activity was assayed.

$^b$ 100% (approximately 600 µmol of acetyl phosphate/min/mg of protein) is the activity of the enzyme pre-incubated for 30 min with the indicated concentrations of MgCl$_2$, ADP, and acetate.

$^c$ 100% (approximately 630 µmol of acetyl phosphate/min/mg of protein) is the activity of the enzyme pre-incubated for 30 min with the indicated concentrations of MgCl$_2$, ADP, and acetate.
Gorrell et al. [22] solved structures of the *M. thermophila* ACK with the ADP-aluminium fluoride-acetate transition state analog which allowed additional examination of the R91 and R241, residues previously implicated in acetate binding and catalysis [21]. A structure with the non-hydrolyzable ATP analog ATPγS supported the evidence presented by Miles et al. [47] that ATPγS is bound equivalently to the binding of ATP in the active site displacing the β-phosphate and theoretically the γ-phosphate of ATP [22]. Kinetic parameters in both the acetate-forming and acetyl phosphate-forming directions for all of the R91 and R241 variants indicated these residues were essential for catalysis in the acetyl phosphate-forming direction (Table 1.5). Significant decreases in $k_{cat}$ values for R91 and R241 variants in the acetate-forming direction were also observed. (Table 1.6) [22].
Table 1.5. Kinetic parameters of wild-type and variant ACKs in the forward (ADP/AcP synthesis) direction. Taken from [22].

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$</th>
<th>$K_{m(ATP)}$</th>
<th>$K_{m(AcP)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>913 ± 52</td>
<td>80 ± 9</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>R91A</td>
<td>0.11 ± 0.04</td>
<td>16 ± 0.6</td>
<td>250 ± 38</td>
</tr>
<tr>
<td>R91L</td>
<td>0.22 ± 0.04</td>
<td>145 ± 9</td>
<td>429 ± 40</td>
</tr>
<tr>
<td>R91K</td>
<td>3.7 ± 0.2</td>
<td>70 ± 3</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>R241A</td>
<td>0.68 ± 0.10</td>
<td>297 ± 27</td>
<td>710 ± 130</td>
</tr>
<tr>
<td>R241L</td>
<td>1.38 ± 0.10</td>
<td>17,000 ± 2800</td>
<td>270 ± 67</td>
</tr>
<tr>
<td>R241K</td>
<td>1.3 ± 0.06</td>
<td>11,400 ± 1900</td>
<td>77 ± 7</td>
</tr>
</tbody>
</table>
Table 1.6. Kinetic parameters of wild-type and variant ACKs in the reverse (ATP/acetate synthesis) direction. Taken from [22].

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$</th>
<th>$K_{cat,ADP}$</th>
<th>$K_{cat,DP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$2360 \pm 60$</td>
<td>$98 \pm 7$</td>
<td>$0.47 \pm 0.05$</td>
</tr>
<tr>
<td>R01A</td>
<td>$5.5 \pm 0.8$</td>
<td>$63 \pm 6$</td>
<td>$1.36 \pm 0.20$</td>
</tr>
<tr>
<td>R01L</td>
<td>$7.7 \pm 0.4$</td>
<td>$63 \pm 5$</td>
<td>$2.3 \pm 0.1$</td>
</tr>
<tr>
<td>R01K</td>
<td>$53 \pm 6.2$</td>
<td>$168 \pm 6$</td>
<td>$0.61 \pm 0.08$</td>
</tr>
<tr>
<td>R241A</td>
<td>$4.5 \pm 0.4$</td>
<td>$210 \pm 28$</td>
<td>$0.84 \pm 0.02$</td>
</tr>
<tr>
<td>R241L</td>
<td>$0.42 \pm 0.06$</td>
<td>$1260 \pm 89$</td>
<td>$1.64 \pm 0.40$</td>
</tr>
<tr>
<td>R241R</td>
<td>$4.5 \pm 0.4$</td>
<td>$578 \pm 21$</td>
<td>$0.92 \pm 0.03$</td>
</tr>
</tbody>
</table>
R91 and R241 were previously postulated to interact with the phosphoryl group of acetyl phosphate and the carbonyl oxygen of acetate [21]; however, Gorrell et al. [22] noted that the negligible changes in the $K_m$ values for acetyl phosphate for the R91 and R241 variants did not provide sufficient evidence to implicate either residue in binding the phosphoryl group of acetyl phosphate. Conversely, the significant changes in the $K_m$ values for acetate for the R91 and R241 variants supported previous evidence for these two residues in acetate binding [21]. The increases in the $K_m$ values for acetate for the R91K and R241K variants were much less than for variants replaced with alanine and leucine (Table 1.5), confirming a requirement for a positive charge at these positions which would interact with the carboxyl group of acetate [22].

Gorrell et al. [22] proposed a catalytic mechanism (Figure 1.5) in which additional residues that are important in substrate binding and catalysis were identified. A role for H180 in stabilizing the transition state was previously proposed based on variant studies [21, 26]. In the analysis of the aluminium fluoride crystal structures, Gorrell et al. [22] discussed the close proximity of H180 along with the R241 residue to the aluminium fluoride in the structure. They concluded that this proximity is consistent with stabilization of the transition state and that H180 also interacted with $\gamma$-phosphate of ATP. Furthermore, evidence was provided by the new structure that supported enzyme catalysis involving E384, and D148 which have been implicated in magnesium binding [28].

Gorrell et al. [22] described the mechanism as a $S_N$2 nucleophilic attack by the carboxyl group of acetate upon the $\gamma$-phosphate of ATP through a trigonal bipyramidal
transition state (Figure 1.4B), with stabilization of the equatorial oxygen atoms through an interaction with H180 and R241. In addition, H180 is proposed to interact with the phosphoryl group of acetyl phosphate similar to its interaction with acetate, which may implicate this residue in orientating acetyl phosphate for attack by ADP [22].
Figure 1.5. Postulated mechanism of ACK from *M. thermophila* for the forward (AcP-producing) reaction direction. *A*, ATP and acetate substrate interactions with ACK; *B*, interactions for the proposed direct in-line mechanism transition state; *C*, interactions of the products ADP and AcP with ACK. The *arrows* indicate direction of electron movement. Taken from [22].
III. ACETATE KINASE IN THE EUKARYA

Although previously thought to be present in bacteria and one genus of archaea, ACK is now known to be present in all three domains of life [49]. Very little is currently known about the eukaryotic ACKs except knowledge gained from bioinformatic and phylogenetic analyses. Through BLAST searches, ACKs have been identified in three of the four fungal phyla: Ascomycota, Basidiomycota, and Chytridiomycota [49]. In addition, ACK sequences have been identified in oomycetes (Phytophthora spp.), unicellular green alga (Chlamydomonas reinhardtii), and a protozoan parasite (Entamoeba histolytica) (Figure 1.6) [49]. Fungi lack PTA, and ACK has been proposed to instead partner with xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP) allowing sugar phosphates to be utilized to generate ATP (Figure 1.7a) [49]. Interestingly, eukaryotic ACKs from Phytophthora and Chlamydomonas utilize phosphotransacetylase (PTA) as a partner (Figure 1.7b) [49]. Furthermore, it has been hypothesized that the presence of two ACK ORFs and two PTA ORFs in C. reinhardtii may suggest the two ACK-PTA pathways operating in opposing directions which may be influenced by varying growth conditions [49]. Because C. reinhardtii can utilize acetate as a carbon growth source, the ACK-PTA pathway could allow for the generation of acetyl-CoA and eventually triose via the glyoxylate cycle [50]. This would be a second pathway for acetate activation as AMP-ACS is present in C. reinhardtii. Inversely, a suggested PTA-ACK pathway functioning in the direction of acetate formation would
allow for the generation of ATP via acetate fermentation [51].
Phytophthora–Chlamydomonas AKs

Putative propionate kinases

Rhodospinulata balica 1

Entamoeba histolytica

Fungal AKs

Cryptococcus neoformans

Ustilago maydis

Cochliobolus sativus

Phytophthora infestans

Lycopersicon esculentum

Fusarium oxysporum

Aspergillus fumigatus

Magnaporthe grisea

Fusarium oxysporum f. sp. lycopersici

Trichoderma reesei

Chaetomium globosum

Neurospora crassa
Figure 1.6. Phylogeny of ACK. The deduced amino acid sequences of acetate kinases (AKs) and the closely related propionate kinases (PKs) and butyrate kinases (BKs) from the draft and completed genome sequences available at NCBI (www.ncbi.nlm.nih.gov), the Broad Institute (http://www.broad.mit.edu/annotation/fgi/) and the U.S. Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/euk_cur1.html). Sequences were aligned with ClustalX [21] and phylogenetic analysis was performed with MEGA [22] using the neighbor-joining algorithm. The data were analyzed using the complete deletion option and the tree was constructed using a gamma distance $\alpha=2$. The reliability of each tree was examined with 1000 bootstrap replicates with values $>80\%$ shown. For brevity, only a representative set of bacterial sequences were used for the ACK tree presented here and the clade containing the BKs is not shown. AK sequences from bacteria are shown in black, archaea in blue and eukaryotes in red. Taken from [49].
Figure 1.7. Proposed pathways for ACK in Eukaryotic microbes. (a) In *Chlamydomonas reinhardtii* and *Phytophthora* species, the ACK–PTA pathway (blue) could be used in the assimilation of acetate in the presence of high extracellular acetate concentrations or could operate in the reverse direction to generate ATP from excess acetyl-CoA. The presence of two ACK ORFs and two PTA ORFs in *C. reinhardtii* raises the possibility that two ACK–PTA pathways operate in opposite directions under different growth conditions. (b) In fungi, the ACK–XFP pathway (red) could function during growth in the breakdown of sugars to produce acetate and ATP. The acetate produced could then be converted to acetyl-CoA through the action of ACS. Taken and modified from [49]. A PTA (grey) is absent in fungi.
IV. THE PARASITE *ENTAMOEBA HISTOLYTICA*

*Entamoeba histolytica* is a protozoan parasite of the phylum Amoebozoa. The parasite was first noted in 1875, but not fully described until 1903 as the causative agent of amoebiasis. An intestinal disease of humans, which is primarily present in Mexico, India, sub-Saharan Africa, and developing countries throughout the world, amoebiasis is a waterborne illness, causing dehydration, diarrhea, and sometimes extreme dysentery. Due to the limited resources of modern medicine in the remote locations where *E. histolytica* dominates, the protozoan has become a considerable source of morbidity and mortality, estimated to infect more than 50 million people worldwide [52].

The life cycle of the parasite begins with ingestion of an infectious cyst. The cyst form is non-dividing, and consists of a quadrinucleated structure consisting of a chitinous cell wall. This chitin-containing cell wall protects the cyst from the acidic environment of the stomach [53, 54]. Once the cyst enters the small intestine, excystation occurs, producing the active trophozoite. The trophozoite binds to the mucosal walls of the host’s colon and begins colonization. Penetration of the mucosal barrier results in disease in the host either by direct destruction of the intestinal epithelium, or by more hostile invasion of the host. Motile trophozoites of *E. histolytica* are capable of disseminating into other body tissues where abscesses can form if left untreated (Figure 1.8).
Figure 1.8. Life cycle of *E. histolytica*. The mature cyst is ingested by the human host.

Excystation of the cyst occurs in the small intestine where the active trophozoite is produced. The trophozoite can then replicate by binary fission to form additional trophozoites, or can undergo encystation to produce a number of cysts. Cysts are passed into the host’s feces where water-borne transmission allows for continuation of the parasite’s life cycle. Taken from [55].
*E. histolytica* has a functionally tetraploid genome; however, it has been noted that the ploidy of individuals varies from cell to cell [56] and in an assortment of growth conditions [57]. The genome consists of both linear chromosomes and circular, plasmid-like molecules containing a low G+C content of 22.4%; with nearly all genes lacking introns [58, 59]. Because the genome has an unknown ploidy and complex variations in both cell cycles and cell structure nucleation, a consistent complete assembly of the genome has not been accomplished.

Pathogenesis in *E. histolytica* is primarily dependent on adherence of the organism to the intestinal mucosa [60]. This process is accomplished by the presence of galactose/N-acetyl galactosamine binding receptors on the surface of the parasite and has been implicated in both virulence and cellular differentiation [61]. The protein has also been shown to play an important role in a number of other processes, including vesicle trafficking [62] and participation in dynamic cytoskeleton reorganization [63].

The production of a cytotoxic amoebapore protein has been shown to be essential in host invasion and nutrient sequestering in the host’s intestinal environment. Strains of *E. histolytica* lacking amoebapore-A are avirulent, implicating this protein as a critical factor in pathogenesis [64, 65]. The protein is responsible for the liberation of host cell components by the creation of these ion channels resulting in a loss of membrane integrality and failure, and ultimately leading to a significant loss of intestinal fluid; a hallmark of amoebic dysentery [66].

The parasite also produces a number of cystine proteases which are thought to degrade proteins and enzymes secreted by the host immune system [67], selectively
damage enteric cells [68], and participate in processing of intracellular host proteins [69] liberated by amoebapore.

Treatment for *E. histolytica* infection typically involves the administration of two drugs. Metronidazole (Flagyl), a nitromidazole which interacts with protozoal ferredoxin of PFOR to produce toxic byproducts is used to treat invasive trophozoites. In addition, paromomycin (Humatin) an aminoglycoside, is used as a luminal amoebicide [70]. Problems arising from observed *in vitro* amoebic resistance to metronidazole and *in vivo* cytotoxic and nephrotoxic effects from paromomycin indicate a need for the identification of novel drug targets in this parasite [52].

ATP synthesis in *E. histolytica* is primarily limited to substrate-level phosphorylation. The parasite lacks a mitochondrion, but does have a complete pentose phosphate pathway capable of metabolising various carbohydrate substrates (e.g. galactose, fructose, and other pentoses) [71]. Pathways for ATP synthesis via amino acid degradation have also been identified in the genome sequence, but glycolysis remains a staple pathway for ATP synthesis in the parasite [59, 72]. An extended glycolytic pathway is present in which pyruvate is converted to acetyl-CoA by PFOR (Figure 1.9). Acetyl-CoA is then converted to acetate by an ADP-forming ACS to generate ATP [73].

Amino acid catabolism by *Entamoeba* can lead to the generation of pyruvate in the absence of glucose. Pyruvate or other ketoacids can be formed by the metabolism of alanine, aspartic acid, asparagine, cystine, glutamine, glutamic acid, methionine, serine, threonine, and tryptophan [74, 75]. PFOR has been shown to have broad substrate specificity with the ability to oxidatively decarboxylate 2-ketobutanoate, oxaloacetate,
and 2-ketoglutarate. 2-ketobutanoate, a product of threonine and methionine catabolism, is converted to propionyl-CoA, which can be utilized by ADP-ACS as a substrate for ATP generation. *Entamoeba* has also been shown to grow at normal rates under restricted glucose and in the presence of the small chain fatty acids acetate, propionate, and butyrate [76].
Figure 1.9. Proposed metabolic pathway(s) for acetate synthesis and utilization in \textit{E. histolytica}. An extended glycolytic pathway is present in \textit{E. histolytica} in which pyruvate is converted to acetyl-CoA by the PFOR. Acetyl-CoA can then be converted to acetate by and ADP-ACS. HK, hexokinase; 3-PGK, 3-phosphoglycerate kinase; PPDK, phosphoenolpyruvate dikinase; HYD, hydrogenase; PFOR, pyruvate ferrodoxin oxidoreductase; ADP-ACS, ADP-forming acetyl-CoA synthetase.
V. REMAINING QUESTIONS CONCERNING ACETATE KINASE

In 1975, Reeves and Guthrie first described a PP\(_i\)-dependent ACK activity in *E. histolytica* [77]. At the time of the publication, modern molecular biology and biochemistry techniques for the cloning and recombinant production of enzymes were not possible. Due to the incomplete purification as well as limited quantity and concentration of the native enzyme, a complete profile of the biochemistry, kinetics, and mechanism of the *E. histolytica* ACK was not accomplished.

Within this dissertation, I have shown that the annotated ACK gene in the *E. histolytica* genome encodes for a PP\(_i\)-dependent not ATP-dependent ACK. I have completed both biochemical and kinetic characterization of the recombinant *E. histolytica* PP\(_i\)-dependent ACK. This work has resulted in identifying commonalities and differences between the ATP-dependent ACK found in *M. thermophila* and this unique PP\(_i\)-dependent ACK from *E. histolytica*. Modification of the ACK assay first developed by Lipmann [78], has made possible the kinetic characterization of the enzyme in the acetate-forming direction of the ACK reaction, the physiological reaction of the enzyme. Furthermore, the kinetic characterization of a number of enzyme variants has resulted in a better understanding of the essential amino acid residues that comprise the active site of this ACK.

Characterization of this novel enzyme is significant in gaining better understanding of eukaryotic amitochondrial metabolism. In addition, the new assay described in this dissertation may serve as a basis for generating new methods for examining some of the other metabolic enzymes found in *E. histolytica*. 
VI. REFERENCES


9. Jablonski, P.E., W.P. Lu, S.W. Ragsdale, and J.G. Ferry, *Characterization of the metal centers of the corrinoid/iron-sulfur component of the CO dehydrogenase*


CHAPTER II
DIRECT DETECTION OF THE ACETATE-FORMING ACTIVITY OF THE
ENZYME ACETATE KINASE

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ABSTRACT

A method for the determination of acetate kinase activity is described. This assay utilizes a direct reaction for determining enzyme activity and kinetics of acetate kinase in the acetate-forming direction with different phosphoryl acceptors. Furthermore, this method can be utilized for assaying other acetyl phosphate or acetyl-CoA utilizing enzymes.
I. INTRODUCTION

Acetate kinase, a member of the acetate and sugar kinase-Hsp70-actin (ASKHA) enzyme superfamily [1-5], is responsible for the reversible phosphorylation of acetate to acetyl phosphate utilizing ATP as a substrate. Acetate kinases are ubiquitous in the Bacteria, found in one genus of Archaea, and are also present in microbes of the Eukarya [6]. The most well characterized acetate kinase is that from the methane-producing archaeon Methanosarcina thermophila [7-14]. An acetate kinase which can only utilize PPi but not ATP in the acetyl phosphate-forming direction has been isolated from Entamoeba histolytica, the causative agent of amoebic dysentery, and has thus far only been found in this genus [15, 16].

In the direction of acetyl phosphate formation, acetate kinase activity is typically measured using the hydroxamate assay, first described by Lipmann [17-20], a coupled assay in which conversion of ATP to ADP is coupled to oxidation of NADH to NAD\(^+\) by the enzymes pyruvate kinase and lactate dehydrogenase [21, 22], or an assay measuring release of inorganic phosphate after reaction of the acetyl phosphate product with hydroxylamine [23]. Activity in the opposite, acetate-forming direction is measured by coupling ATP formation from ADP to the reduction of NADP\(^+\) to NADPH by the enzymes hexokinase and glucose 6-phosphate dehydrogenase [24].

Here we describe a method for the detection of acetate kinase activity in the direction of acetate formation that does not require coupling enzymes, but is instead based on direct determination of acetyl phosphate consumption. After the enzymatic reaction, remaining acetyl phosphate is converted to a ferric hydroxamate complex that
can be measured spectrophotometrically, as for the hydroxamate assay. Thus, unlike the standard coupled assay for this direction that is dependent on the production of ATP from ADP, this direct assay can be used for acetate kinases that produce ATP or PPi.
II. MATERIALS AND METHODS

The overall scheme of this protocol is outlined in Figure 2.1.

1) Solution Preparation for Standard Curves and Assays

1.1) Prepare 100 mL of a 2 mol/L solution of hydroxylamine-HCl. Weigh out 13.9 g of hydroxylamine hydrochloride (MW 69.49 g/mol) and dissolve in approximately 50 mL distilled-deionized water (ddH₂O). Adjust the pH to 7.0 using potassium hydroxide pellets or a concentrated solution. Bring the final volume to 100 mL. The solution can be stored at room temperature for up to 30 days or at 4°C for up to 90 days.

1.2) Prepare 100 mL of a ferric chloride/ hydrochloric acid solution. Weigh out 13.5 g ferric chloride (MW 270.32 g/mol) and dissolve in approximately 50 mL ddH₂O. Add 41.3 mL concentrated hydrochloric acid (12.1 mol/L) and bring to a final volume of 100 mL. The final concentration of ferric chloride will be 0.5 mol/L and the final concentration of hydrochloric acid will be 5 mol/L. The solution is stored at room temperature.

1.3) Prepare 100 mL of a 1.84 mol/L solution of trichloroacetic acid. Weigh out 30.0 g of trichloroacetic acid (MW 163.39 g/mol), dissolve in ddH₂O and bring the final volume to 100 mL. The solution is stored at room temperature.
Protocol Scheme

1. Generate an acetyl phosphate standard curve
2. Dilute enzyme to desired concentration
3. Prepare the reaction mixture and aliquot 300 µl to microcentrifuge tubes
4. Preincubate reactions at 37°C for 1 minute
5. Start reactions by adding enzyme at specific time intervals
6. Add hydroxylamine at the same intervals to stop the reactions and incubate at 60°C for 5 minutes
7. Add ferric chloride/trichloroacetic acid solution for color development
8. Centrifuge tubes at 18,000 x g for 1 minute
9. Measure absorbance at 540 nm
10. Determine amount of acetyl phosphate consumed by comparison to the standard curve

Figure 2.1. Protocol Scheme.
1.4) Prepare 5 mL of a 0.1 mol/L acetyl phosphate solution. Weigh out 0.092 g acetyl phosphate (MW 184.06 g/mol), dissolve in ddH₂O and bring the final volume to 5 mL. Aliquot the solution to microcentrifuge tubes (0.25 mL per tube) and freeze at -20°C until use. Thawed solution should be placed on ice and can be used for approximately 1 hour. Acetyl phosphate solution that has been thawed should not be refrozen and re-used.

1.5) Prepare 5 mL of a 0.1 mol/L solution of ADP. Weigh out 0.24 g of ADP (MW 472.5 g/mol), dissolve in ddH₂O, and bring the final volume to 5 mL. Aliquot the solution in microcentrifuge tubes (0.5 mL per tube) and freeze at -20°C until use. Keep thawed solution on ice until use.

1.6) Prepare 50 mL of a 1 mol/L solution of Tris. Weigh out 6.06 g Tris (MW 121.14 g/mol), and dissolve in 40 mL ddH₂O. Adjust the pH of the solution to 7.0 using hydrochloric acid and bring final volume to 50 mL. Store solution at room temperature.

1.7) Prepare 10 mL of a 1 mol/L magnesium chloride solution. Weigh out 2.03 g magnesium chloride (MW 203.31 g/mol), dissolve in ddH₂O, and bring final volume to 10 mL. Store the solution at room temperature.

1.8) Prepare 25 mL development solution by mixing equal volumes of the ferric chloride/hydrochloric acid solution and the trichloroacetic acid solution. Development solution should be prepared fresh each day and stored at room temperature.
2) Preparation of an Acetyl Phosphate Standard Curve

2.1) An acetyl phosphate standard curve is generated using known amounts of acetyl phosphate. A standard curve of six to eight points is suitable. The standards should range from 0.03 µmoles to 0.9 µmoles per 300 µL, which correlates to final concentrations of 0.1 to 3 mmol/L. Dilute the acetyl phosphate stock solution to the appropriate concentrations in 100 mM Tris solution in a final volume of 300 µL. A control sample without acetyl phosphate should also be prepared.

2.2) Incubate each standard and the control at 37°C in a heat block for 1 minute.

2.3) Add 50 µL of the hydroxylamine hydrochloride solution to each standard and the control and mix by shaking three times. Place the standard into a 60°C heat block and incubate for five minutes.

2.4) Add 100 µL of development solution to the standards and the control, mix by shaking three times, and allow color to develop for at least one minute at room temperature.

2.5) Centrifuge all standards and the control in a microcentrifuge at 18,000 x g for 1 minute.
2.6) Measure the samples spectrophotometrically at 540 nm using 1mL plastic cuvettes, using the control as the blank for spectrophotometry.

2.7) Generate a standard curve using appropriate data analysis and graphing software (e.g. Kaleidagraph, Synergy Software). \( R^2 \) values of 0.99 or greater are desirable. The data is plotted as absorbance at 540 nm versus \( \mu \)moles of acetyl phosphate present.

3) Assay for Acetate Kinase Activity

3.1) Using the solutions described, prepare a reaction mix containing a final concentration of 0.1 mol/L Tris buffer, 10 mmol/L magnesium chloride, 5 mmol/L ADP, and 2 mmol/L acetyl phosphate. A 10 mL reaction mixture will require 8.2 mL ddH\(_2\)O, 1.0 mL Tris solution, 0.1 mL magnesium chloride solution, 0.5 mL ADP solution, and 0.2 mL acetyl phosphate solution. Distribute the reaction mix in 300 \( \mu \)l aliquots to microcentrifuge tubes. For determination of kinetic parameters and optimization of substrate concentrations, one substrate can be varied and the other held at a constant concentration in the reactions.

3.2) Pre-incubate the reactions for 1 minute at 37°C in a heat block.

3.3) Add a desired amount of enzyme to the reaction and immediately return the tube to the 37°C heat block. Also include a control reaction to which no enzyme is added. If several reactions are performed, add enzyme to the tubes at specific time intervals. Be
sure to keep track of time. (NOTE: The amount of enzyme to be used will need to be optimized to ensure that all of the acetyl phosphate or ADP substrate will not be depleted - see Discussion section)

3.4) After 5 minutes, add 50 µL of the hydroxylamine hydrochloride solution to each reaction and the control, mix, and place the tubes in a 60°C heat block. If several reactions are being performed, this should be done at the same time interval as the enzyme was added.

3.5) Incubate the reactions and the control for 5 minutes at 60°C.

3.6) Add 100 µL of the development solution to each reaction and the control, mix, and place in a tube rack on the lab bench. Allow color to develop for at least 1 minute.

3.7) Centrifuge all tubes in a microcentrifuge at 18,000 x g for 1 minute.

3.8) Measure samples spectrophotometrically at 540 nm using 1mL plastic cuvettes and determine the amount of acetyl phosphate present by comparison to the acetyl phosphate standard curve.

3.9) The amount of acetyl phosphate substrate depleted can be determined by subtracting the amount of acetyl phosphate remaining after the enzymatic reaction from the amount
of acetyl phosphate present in the control lacking enzyme. Perform data analysis using appropriate data and graphing software (e.g. Kaleidagraph, Synergy Software). $R^2$ values of greater than 0.97 are desirable.
III. RESULTS

The purpose of this assay is to measure acetate kinase activity in the direction of acetate formation. This is done by measuring consumption of the acetyl phosphate substrate, as there is no easy, direct measurement for acetate production. Acetyl phosphate remaining after a given time during the enzymatic reaction is converted to acetyl hydroxamate and subsequently to ferric hydroxamate complex, which has a reddish color (Figure 2.2) that can be measured at 540 nm. The standard curve plotting absorbance versus µmoles acetyl phosphate present in the standards is used to determine the amount of acetyl phosphate remaining in each reaction. The standard curve should be linear through the zero point. A representative standard curve shown in Figure 2.3 has a slope of 1.2332 absorbance units per µmole acetyl phosphate present and an R² value of 0.99, indicating the data fits the linear equation well. The equation for the linear fit to the standard curve data is used for calculating the quantity of acetyl phosphate remaining in the reaction. The µmoles of acetyl phosphate consumed is determined as the difference between the µmoles of acetyl phosphate present in the control reaction lacking enzyme and the µmoles of acetyl phosphate remaining after the enzymatic reaction.
Figure 2.2. Acetyl phosphate standards. Increasing concentrations of acetyl phosphate were reacted with hydroxylamine and color was developed with the ferric chloride/trichloroacetic acid solution as described. The control lacking acetyl phosphate is bright yellow, and reactions containing increasing amounts of acetyl phosphate are successively darker in color. This color change is measured at 540 nm.
For the experiment shown in Figure 2.4, the concentration of ADP was held constant at 5 mM and the concentration of acetyl phosphate in the reaction was varied to determine the $K_m$ for acetyl phosphate. As expected, this experiment demonstrates that the enzyme follows standard Michaelis-Menten-like kinetics for each substrate and produced a hyperbolic saturation curve when plotting activity versus acetyl phosphate concentration. The apparent $K_m$ value for acetyl phosphate was $0.27 \pm 0.01$ mM, which compares favorably to the published $K_m$ value of $0.47 \pm 0.01$ mM using the coupled assay [13].
Figure 2.3. Sample acetyl phosphate standard curve. Varying concentrations of acetyl phosphate were reacted with hydroxylamine and ferric chloride/trichloroacetic acid and color development was measured at 540 nm. The absorbance at 540 nm versus mol acetyl phosphate was plotted and a linear fit to the data was applied.
Purified, recombinant *M. thermophila* acetate kinase was assayed using the described protocol. For the experiment shown in Figure 2.4, the concentration of ADP was held constant at 5 mmol/L and the concentration of acetyl phosphate in the reaction was varied to determine the $K_m$ for acetyl phosphate. As expected, this experiment demonstrates that the enzyme follows standard Michaelis-Menten-like kinetics for each substrate and produced a hyperbolic saturation curve when plotting activity versus acetyl phosphate concentration. The apparent $K_m$ value for acetyl phosphate was $0.27 \pm 0.01$ mmol/L, which compares favorably to the published $K_m$ value of $0.47 \pm 0.01$ mmol/L using the coupled assay [13].

The direct assay can also be used to measure activity of acetate kinases that utilize substrates other than ATP, which is not possible using the standard coupled assay. Purified recombinant *E. histolytica* acetate kinase [16], which produces PPi rather than ATP in the acetate-forming direction, was subjected to this assay using sodium phosphate in place of ADP. The concentration of sodium phosphate in the reaction was held constant at 0.2 mol/L and the acetyl phosphate concentration was varied. As for the *M. thermophila* acetate kinase, the *E. histolytica* acetate kinase followed Michaelis-Menten-like kinetics for acetyl phosphate and a hyperbolic saturation curve was observed (Figure 2.5). The apparent $K_m$ value for acetyl phosphate was $0.50 \pm 0.006$ mmol/L. Reeves and Guthrie [15] determined a $K_m$ value of 0.06 mmol/L for acetyl phosphate for the native enzyme; however, their enzyme was only partially purified and their assay involved four coupling enzymes which were also only partially purified. Thus, it is difficult to directly compare these values.
Figure 2.4. Utilization of acetyl phosphate by the ATP-producing *M. thermophila* ACK. Enzymatic activity was determined in the direction of acetate formation in the presence of 5 mM ADP with varying concentrations of acetyl phosphate. Assays were performed in triplicate with error bars shown.
Figure 2.5. Utilization of acetyl phosphate by the PP\textsubscript{i}-producing *E. histolytica* ACK.

Enzymatic activity in the direction of acetate formation was determined in the presence of 200 mM sodium phosphate with varying concentrations of acetyl phosphate. Assays were performed in triplicate with error bars shown.
IV. DISCUSSION

The detection of acetyl phosphate in this assay is dependent upon a sufficient concentration of hydroxylamine hydrochloride and the concentration and acidity of the ferric chloride solution. Alteration of the assay volume will require reconsideration of both of these components. The enzymatic reactions described here were performed at 37°C with the hydroxylamine termination performed at 60°C for 5 minutes. This higher temperature is critical to allow for rapid conversion of the remaining acetyl phosphate to acetyl hydroxamate. The timing of the color development step and measurement of absorbance is less critical, and can be performed in as little as 5 minutes and up to 15 minutes following the addition of the hydroxylamine solution. The samples should be centrifuged before reading the absorbance, as the acidity of the sample may lead to precipitation of the enzyme and can produce a falsely high reading due to turbidity rather than actual color change. Breakdown of the developed reactions will begin to occur after 10-15 minutes.

The primary complications of this assay for examining enzyme kinetics are determination of the amount of enzyme to use and the length of time to run the first step of the reaction. These must be determined empirically for each enzyme such that the level of activity is not so high that all of the acetyl phosphate substrate is consumed. Careful attention should be given to the percent acetyl phosphate consumption for each reaction point in a kinetic curve. Generally, lower concentrations of the varied component on a kinetic curve should result in acetyl phosphate consumption up to 90 percent or slightly above, while consumption of acetyl phosphate at saturating substrate concentrations
should approach 10 percent or slightly less. Once the enzyme concentration is optimized, the substrate range for determination of kinetic constants can then be determined.

Previous methods for measuring acetate kinase activity in the acetate-forming direction involved the use of coupled assays. These methods are problematic with respect to kinetic calculations due to dependence on the activity of the coupling enzyme(s), whose assay conditions (pH, ionic strength, temperature, etc.) may be incompatible with the optimal assay conditions for the enzyme of interest. In addition, use of a direct assay for studies with inhibitors may avoid issues that could arise from inhibition of the coupling enzymes. Overall, this assay should be useful not only for acetate kinase, but for any enzyme that utilizes acetyl phosphate or other activated short chain acyl substrates such as acetyl- or propionyl-CoA, propionyl phosphate, and acetyl-AMP, all of which are reactive with hydroxylamine. In these cases, the standard curve would be generated with the appropriate acyl substrate instead of with acetyl phosphate.
V. ACKNOWLEDGMENTS

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VI. REFERENCES


CHAPTER III
BIOCHEMICAL AND STRUCTURAL CHARACTERIZATION OF A
NOVEL PP\(_i\)-DEPENDENT ACETATE KINASE FROM THE PARASITE
ENTAMOEBA HISTOLYTICA

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ABSTRACT

Acetate kinase (ACK) catalyzes the reversible magnesium dependent synthesis of
acetyl phosphate by transfer of the γ-phosphate of ATP to acetate. Here we report the
biochemical, kinetic, and structural characterization of the \textit{E. histolytica} ACK, the only
known member of the ASKHA structural superfamily that utilizes PP\(_i\) as the sole
phosphoryl donor. The enzyme is capable of utilizing acyl substrates as long as
hexanoate in the direction of acetyl phosphate synthesis. The kinetic parameters for
substrate affinity indicate that the enzyme prefers both acetate and propionate in the
acetyl phosphate-forming direction; however, the low \(k_{cat}\) raises question whether this
direction of the reaction is physiological. Studies of the mechanism of this enzyme
indicates that the PP\(_i\)-ACK follows a sequential mechanism, supporting a direct in-line
phosphoryl transfer mechanism previously reported in the most well characterized
\textit{Methanosarcina thermophila} ATP-dependent ACK. We determined the crystal structure
of the \textit{E. histolytica} ACK to 2.4Å resolution. The structure contains two monomers
related by pseudo two-fold rotational symmetry to form a dimer. This overall shape is similar to that observed in other members of the ASKHA superfamily. Comparison of the structure of the *E. histolytica* ACK to that of *M. thermophila* ACK determined in the presence of both ADP•AlF$_4^-$ and acetate suggests the presence of one active site in each monomer, with two active sites in each dimer. Docking models indicate that acetate binds to a location structurally analogous to the acetate binding site in the *M. thermophila* ACK, and the pyrophosphate binding to a location structurally analogous to the β-phosphate and AlF$_4^-$ of the ADP-AlF$_4$ binding site in MtACK. Docking simulations could not identify a reasonably binding site for either ATP or ADP within the *E. histolytica* ACK structure. ATP binding is sterically unfeasible in the *E. histolytica* further supporting the role of this enzyme as a PPi-dependent ACK.
I. INTRODUCTION

Acetate kinase (ACK), a key enzyme in energy generation in prokaryotic metabolism, catalyzes the reversible transfer of the γ-phosphoryl group of ATP to acetate to form acetyl phosphate and ADP (equation 1).

\[ \text{CH}_3\text{COO}^- + \text{ATP} \leftrightarrow \text{CH}_3\text{COPO}_4^{2-} + \text{ADP} \quad [1] \]

ACK is a member of the ASKHA phosphotransferase superfamily, which includes acetate kinase, hexokinase and other sugar kinases, as well as the Hsp70 heat shock cognate and actin [1-4]. In 2001, Buss et al. [5] published the first structure for an ACK, that from the archaeon *Methanosarcina thermophila*, and suggested that ACK is the urkinase for the ASKHA superfamily.

Several crystal structures have now been solved for the well characterized *M. thermophila* ACK [5, 6] and the roles of a number of active site residues in substrate binding and catalysis have been examined experimentally [7-12]. Kinetic and structural studies support a direct in-line transfer of the phosphoryl group of ATP to acetate. The positions of the ATP ligand and the proposed acetate binding pocket in the *M. thermophila* ACK structure are consistent with direct in-line transfer of the phosphoryl group [5, 6]. Furthermore, a MgADP-AlF$_3$-acetate transition state analog [12] resulted in an abortive complex and was found to be in a linear array in the active site [6]. Based on analysis of site-altered enzyme variants [7, 11, 13] and structural studies, Gorrell *et al.* [6] have 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.
In 1975, Reeves and Guthrie [14] identified a PP$_i$-dependent ACK activity in *Entamoeba histolytica*, an amitochondriate protist that is the causative agent of amoebic dysentery and is currently listed as the third leading cause of morbidity and mortality due to parasitic disease in humans after malaria and schistosomiasis [15]. Here we report the biochemical, kinetic, and structural characterization of the *E. histolytica* ACK, the only known member of the ASKHA structural superfamily that utilizes PP$_i$ as the sole phosphoryl donor.
II. RESULTS

*E. histolytica has a PP\textsubscript{i}-dependent ACK.* In 1962, Bragg and Reeves [16] reported an ATP-dependent ACK in the non-pathogenic *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 [14] identified a pyrophosphate (PP\textsubscript{i})-dependent ACK in axenically-grown *E. histolytica*. The deduced amino acid sequence of the ACK ORF (EHI_170010; XM 650898.1) identified in the *E. histolytica* genome shares 34% identity and 53% similarity to the well-characterized *M. thermophila* ACK (*MtACK*), which utilizes ATP but not PP\textsubscript{i} as the phosphoryl donor and displays high activity in both directions of the reaction [17]. To determine whether the encoded enzyme is indeed a PP\textsubscript{i}-dependent ACK and to allow kinetic and biochemical characterization, recombinant *E. histolytica* ACK (*EhACK*) was produced in *E. coli* and purified by nickel affinity chromatography to electrophoretic homogeneity.

The purified recombinant enzyme utilized PP\textsubscript{i} as a phosphoryl donor in the acetyl phosphate-forming direction of the reaction (Figure 3.1a) and displayed normal hyperbolic saturation curves for both acetate and PP\textsubscript{i}. Unlike all other characterized ACKs, *EhACK* shows only PP\textsubscript{i}-dependent activity in the direction of acetyl phosphate formation. ATP does not serve as a phosphoryl donor (Figure 3.1a) nor do other NTPs (CTP, GTP, TTP, UTP, and ITP; data not shown) or ADP. In the acetate-forming direction of the reaction, the enzyme displayed a normal hyperbolic saturation curve with
inorganic phosphate as the phosphoryl acceptor, and no activity was observed with ADP, AMP, or PP\textsubscript{i} in the acetate-forming direction (Figure 3.1b).
B.

Figure 3.1. Utilization of different phosphoryl donors and acceptors by EhACK. (A) Enzymatic activity in the acetyl phosphate-forming direction of the reaction in the presence of 1 M acetate with varying concentrations of each phosphoryl donor was measured using the hydroxamate assay. (●) PP$_i$, (○) ADP, and (△) ATP. (B) Enzymatic activity in the direction of acetate formation in the presence of 2 mM acetyl phosphate with varying concentrations of each phosphoryl acceptor was determined using the modified reverse hydroxamate assay. (●) P$_i$, (○) PP$_i$, (△) ADP, and (□) AMP.
**EhACK prefers the acetate/PP\textsubscript{i}-forming direction of the reaction**

In the acetate/ATP-forming direction of the reaction, the apparent \( K_m \) value for acetyl phosphate was determined to be 0.5 ± 0.1 mM and the \( k_{\text{cat}} \) was 1939 ± 14 sec\(^{-1}\) (Table 3.1). In the acetyl phosphate/P\textsubscript{i}-forming direction of the reaction, the \( K_m \) value for acetate was 106.8 ± 1 mM and the \( k_{\text{cat}} \) value was 1.76 ± 0.01 sec\(^{-1}\) (Table 3.1). The significantly higher \( K_m \) value for acetyl phosphate versus acetate combined with the over 1000-fold higher \( k_{\text{cat}} \) observed with acetyl phosphate suggest that the physiological direction of the reaction is acetate/PP\textsubscript{i} formation.

In the acetyl phosphate/P\textsubscript{i}-forming direction of the reaction, *EhACK* was found to have a broad acyl substrate range, utilizing substrates as long as octanoate (C\textsubscript{8}). Although the apparent \( K_m \) value decreased with increasing acyl chain length, the turnover rate \( k_{\text{cat}} \) also decreased (Table 3.1). The catalytic efficiency \( k_{\text{cat}}/K_m \) with acetate was similar to that observed with propionate (Table 3.1), and both values were significantly higher than observed with any other acyl substrate. The apparent \( K_m \) values for PP\textsubscript{i} remained relatively unchanged with different acyl substrates with the exception of hexanoate (Table 3.1). 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\textsubscript{i}-forming direction. The enzyme utilizes the cofactors Mg\(^{2+}\) (\( K_m = 2.1 ± 0.2 \) mM) and Co\(^{2+}\).
(\(K_m=5.5 \pm 0.4 \text{ mM}\)), but no activity was observed with \(\text{Ca}^{2+}, \text{Cu}^{2+}, \text{Ni}^{2+}\) or \(\text{Zn}^{2+}\). Use of \(\text{Mn}^{2+}\) resulted in less than 10\% activity as compared to \(\text{Mg}^{2+}\).

To determine whether \(EhACK\) follows a sequential mechanism as shown by \cite{Miles2016} for \(MtACK\) or a ping pong mechanism consistent with a phosphoenzyme intermediate as proposed by Anthony and Spector \cite{Anthony1995, Anthony1996, Anthony1997}, we measured activity in the acetate/\(\text{PP}_i\)-forming direction in an array of reactions in which the acetyl phosphate and \(\text{P}_i\) concentrations were varied. The double reciprocal plots of the activities versus substrate concentrations resulted in intersecting lines (Figures 2A and B), consistent with a ternary (sequential) mechanism and supporting a direct transfer of the phosphoryl group between \(\text{P}_i\) and acetyl phosphate, similar to the in-line ternary mechanism for \(MtACK\) \cite{Miles2016}.
Table 3.1. Kinetic parameters for *EhACK*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
<th>Phosphoryl substrate</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl phosphate</td>
<td>0.5 ± 0.1</td>
<td>1939 ± 14</td>
<td>3846 ± 69</td>
<td>$P_i$</td>
<td>48.9 ± 0.8</td>
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<tr>
<td>acetate</td>
<td>106.8 ± 1.0</td>
<td>1.76 ± 0.01</td>
<td>0.016 ± 0.001</td>
<td>PP$_i$</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>PP$_i$</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>PP$_i$</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>PP$_i$</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>PP$_i$</td>
<td>16.3 ± 0.4</td>
</tr>
</tbody>
</table>
Figure 3.2. 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.
Structure of E. histolytica ACK

We determined the crystal structure of EhACK to 2.4Å resolution. The structure contains two monomers related by pseudo two-fold rotational symmetry to form a dimer. This overall shape is similar to that observed in other members of the ASKHA superfamily, which have previously been described as resembling the appearance of a bird. In order to form this bird-like structure, the N- and C-termini of each monomer fold into separate domains, with the C-terminal domain forming the “body” of the bird and mediating dimerization, and the N-terminal domain forming the “wing” of the bird (Figures 3.3A and 3.3B). Like other ASKHA family proteins, the C-terminal domain of each EhACK monomer adopts the ribonuclease H-like motif, which is comprised of a mixed β-sheet flanked by α-helical insertions (α/β/α fold) (Supplemental Figure 3.1) [1]. This ribonuclease H-like motif has greater sequence and structural similarity to the M. thermophila ACK (for example, 36% identity, and a RMS deviation of 1.4 Å for 240 Cα atoms) than does the wing domain (for example 31% identity, and a RMS deviation of 1.4 Å for 101 Cα atoms). The lower sequence and structural identity is especially in the first 70 amino acids of the wing domain, which have only 21% sequence identity between EhACK and MtACK.

Active site architecture

Co-crystallization of EhACK with acetate or pyrophosphate did not result in the appearance of new electron density. However, a comparison of the structure of EhACK to that of MtACK (PDB entry 1TUY) [6] determined in the presence of both ADP•AlF4− and acetate suggests the presence of one active site in each monomer, with two active sites in
each dimer. These active sites are positioned on opposite faces of the dimer at interfaces between the wing and body domains, and are separated by 35 Å (Figures 3.3 and 3.4).
Figure 3.3. Overall architecture of *EhACK*. The *E. histolytica* ACK dimer is shown as a ribbon diagram with the N-terminus of the protein (residues 1-142) colored in *green*, and the C-terminus (residues 143-392) colored in *silver-blue*. The active site and location of the predicted binding sites for products are highlighted with a dashed oval indicating the likely binding sites for pyrophosphate and the dashed triangle indicating the likely binding location for acetate. (A) The protein is shown in the orientation that most resembles a bird, with the N-termini of each monomer forming the wings, and the C-termini forming the body and mediating dimerization. The Figure 3.is aligned along the pseudo two-fold of the body, which is represented with a *silver-blue* line. The wing domains are related by a shifted pseudo two-fold axis, which is shown as a *green* line. (B) A view of the *E. histolytica* ACK dimer rotated by 90°.
Figure 3.4. Comparison of active site structure between MtACK and EhACK. (A) Surface of MtACK showing cleft with docked ligands. (B) Surface of EhACK in same orientation. (C) Stereo overlay of MtACK and EhACK showing side chains and docked products.
Docking of acetate and pyrophosphate, the putative products of the physiological reaction catalyzed by *EhACK*, resulted in reasonable poses for each (Figure 3.4). In this docking model, the acetate binds to a location structurally analogous to the acetate binding site in *MtACK*, and the pyrophosphate binding to a location structurally analogous to the β-phosphate and AlF₄ of the ADP-AlF₄ binding site in *MtACK* (Figure 3.4A and 4B). With the exception of the Pro → Thr substitution at position 223, residues immediately surrounding both the acetate and the phosphate binding pockets are completely conserved between *EhACK* and *MtACK* (Figure 3.4 C). In stark contrast, conservative substitutions (Gly → Gln at position 323 and Ile → Met at position 324) occlude the binding cleft for the adenosine ring of ATP in the *EhACK* enzyme (Figure 3.4A and B), rendering the binding of ATP or ADP sterically unfeasible. Not surprisingly, docking simulations could not identify a reasonably scored pose for either ATP or ADP within the *EhACK* structure (data not shown).
IV. DISCUSSION

Whereas ACKs in bacteria and archaea have been extensively studied, significantly less is known about the biochemistry of eukaryotic ACKs. Here we report detailed biochemical and kinetic characterization of the *E. histolytica* ACK, the only known member of the ASHKA superfamily that utilizes PP\textsubscript{i} as sole phosphoryl donor. Most of what is known about substrate binding and catalysis comes from studies on the ACK from *M. thermophila*. Intriguing similarities and differences between the putative active site of *EhACK* and the demonstrated active site of *MtACK* suggest how active site architecture tunes specificity for P\textsubscript{i}/PP\textsubscript{i} versus ADP/ATP.

An immediately notable difference is that *MtACK* contains a binding pocket for the adenosine ring of the substrate ATP that is occluded by side chains in *EhACK* (Figure 3.4B) despite these two enzymes containing reasonable sequence identity in the active site. Two prominent alterations account for the occlusion of the site: Gly to Gln and Ile to Met at positions 323-324 of *EhACK* (positions 331-332 in *MtACK*) (Figure 3.4A). As a result, ATP binding is sterically unfeasible in the *E. histolytica* enzyme (Figure 3.4A).

In addition, *EhACK* contains several unusual substitutions within the acetate-binding region of the active site, including the substitution of a Thr at position 223 in *EhACK* for a conserved Pro at the equivalent position (residue 232) in *MtACK* and a change in loop sequence and conformation between residues 110-125 of EhACK (residues 116-131 of *MtACK*). These differences alter the potential ligands to the acetate/acetyl phosphate substrate at two locations, and both decrease the hydrophobicity
of the binding pocket and add putative hydrogen bonds for acetyl phosphate (Figure 3.4C).

The PP$_i$-dependent ACK activity observed in cell homogenates supports previous evidence suggesting that the enzyme is expressed in trophozoites. Microarray experiments on *E. histolytica* have shown that ACK is expressed in genetically distinct laboratory strains (HM-1:IMSS, Rahman, and 200:NIH) and in clinical isolates of both cysts and trophozoites (strain MS75-3544 and 2592100) in a variety of different media [21]. When taken together, these results indicate that ACK is expressed in both the infective and non-infective stages.

The kinetic data presented here and elsewhere [14] suggests that ACK functions primarily in the direction of acetate/PP$_i$ formation in *Entamoeba*. First, EhACK activity is more than three orders of magnitude higher in the direction of acetate/PP$_i$ formation versus acetyl phosphate/P$_i$ formation; and secondly, the enzyme has a much stronger affinity for acetyl phosphate than for acetate. Acetate/PP$_i$ formation catalyzed by ACK is consistent with the physiology of this parasite. Unlike some eukaryotes, *E. histolytica* lacks compartmentalized, ATP-generating mitochondria and hydrogenosomes and is therefore limited to anaerobic metabolism [22]. Glycolysis 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 during substrate-level phosphorylation [14, 23, 24]. The reaction catalyzed by ACK would thus provide a source of PP$_i$ for these glycolytic enzymes. Furthermore, *E. histolytica* has been shown to ferment acetate during growth [14]. Although acetate is produced in the reaction
catalyzed by ADP-forming acetyl-CoA synthetase, which utilizes ADP for the generation of ATP from acetyl-CoA [25], ACK may also be responsible for acetate fermentation by *E. histolytica*.

Although a physiological role for ACK in acetate and PPi 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 in bacteria are absent in the latest *E. histolytica* genome assembly. 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*; and (iii) a previously uncharacterized or undiscovered acetyl phosphate generating enzyme is present in *Entamoeba* species. Lack of XFP or PTA activity in *Entamoeba* cell extracts argues against the first two possibilities, although these activities could be labile. In bacteria, ACK has been shown to play a role in signaling, generating acetyl phosphate for two component systems or for phosphorylation of CheY and components of the PTS system [26-29]. However, the kinetic studies presented here do not support such a role.

In conclusion, the *Entamoeba* ACK is unique among acetate kinases and the ASKHA enzyme superfamily in its ability to not just utilize PPi/Pi as the phosphoryl donor/acceptor, but to solely use PPi/Pi to the exclusion of ATP/ADP and other nucleotide triphosphates/diphosphates. Further examination of the crystal structure of *EhACK* and analysis of enzyme variants in both *EhACK* and *MtACK* will provide additional information as to the determinants of this distinctive phosphoryl donor
specificity. Identification of possible partners for *E. histolytica* ACK will provide insights into the function of this unique enzyme and parasite biochemistry.
Supplemental Figure 3.1S. Loop and wing motifs of the acetate kinase.
IV. 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 [30]. To prepare E. histolytica cell extracts, 4 x 10^6 trophozoites were resuspended in 25 mM Tris, 150 mM NaCl (pH 7.4) and vortexed with acid-washed glass beads for one minute followed by one minute on ice, for three cycles. The extract was centrifuged at 5000 x g for 15 minutes and the supernatant 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) using the primers 5’-GGAACAGGATTCATGTCTAACGTACTAATA-3’ and 5’-GGAACAAAGCTTTTAAAACTGAAATAATTCTTTTC-3’ and cloned into the E. coli expression plasmid pQE-30 (QIAGEN, Valencia, CA) in-frame with the N-terminal His_6 tag sequence using BamHI and HindIII restriction sites incorporated into the PCR primers. Plasmid DNA was isolated using the Zyppy Plasmid Miniprep Kit (Zymo Research Corp., Orange, CA). Constructs were confirmed by sequencing (Clemson University Genomics Institute, Clemson, SC).

Production and purification of recombinant E. histolytica ACK

The Entamoeba ACK expression plasmid was transformed into Escherichia coli strain YBS121 ΔackApta (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 OD$_{600}$~0.9. Recombinant protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside to 1 mM final concentration. Cultures were incubated overnight at ambient temperature/200 RPM 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 x g for 1 hour and the supernatant was applied to a 5 mL HisTrap® nickel affinity column (GE Healthcare, Piscataway, NJ). Protein was eluted from the column 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 electrophoretically pure by SDS-PAGE and Coomassie blue staining.

Protein concentration in cellular extracts and recombinant enzyme preparations was quantified by the Bradford method [31] using the Bio-Rad Protein Assay with bovine serum albumin as standard.

**Determination of kinetic parameters for EhACK**

The hydroxamate assay [17, 32, 33] was used to determine kinetic parameters in the acetyl phosphate/P$_i$-forming direction as previously described using a reaction mixture containing 100 mM MES, 5 mM MgCl$_2$, and 600 mM hydroxylamine hydrochloride (pH 7.5) with the concentrations of acyl substrate and sodium pyrophosphate were varied. Reactions were performed at 45°C, the optimal temperature
for this enzyme. Acetyl phosphate formation was determined by comparison to an acetyl phosphate standard curve. All assays were performed in triplicate. Kinetic calculations and progress curves were generated using Kaleidagraph (Synergy Software, Reading, PA).

Kinetic parameters in the acetate/PP\(_r\)-forming direction of the reaction were determined using a modified reverse hydroxamate assay [34]. The reactions contained 100 mM Tris (pH 7.0), 10 mM MgCl\(_2\), and varying concentrations of sodium phosphate and acetyl phosphate. Reactions were performed at 37\(^\circ\)C in triplicate. 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 acetate/PP\(_r\) formation with varied concentrations of acetyl phosphate (0.5, 0.7, 1.0, and 1.5 mM) and sodium phosphate (40, 50, 60, and 70 mM) as a 4 x 4 matrix. All assays were performed in triplicate.

**Protein crystallization**

Crystals of \(Eh\)ACK were grown at 20\(^\circ\)C using the hanging drop vapor diffusion method by mixing 1.0 \(\mu\)L of 10 mg/ml protein solution with an equal volume of reservoir solution [50 mM N-(2-acetamido) iminodiacetic acid (ADA) pH 6.6, 0.6 M Na-K Tartrate, and 10 mM FeCl\(_3\)] and equilibrating against 1 ml reservoir solution. Crystals formed in 3 to 5 days and grew to a maximal size of approximately \(0.10 \times 0.10 \times 0.05\) mm. Prior to data collection, the \(Eh\)ACK crystals were quickly dragged through a solution containing 50 mM ADA pH 6.6, 0.8 M Na-K Tartrate, 10 mM FeCl\(_3\), and
30%(w/v) (±)-2-Methyl-2,4-pentanediol (MPD), and were then flash-cooled by plunging in liquid nitrogen.

**X-Ray Data Collection and Processing**

X-ray diffraction data were collected on a MAR 225 CCD detector at the Life Sciences Collaborative Access Team (LS-CAT) beamline 21-ID-G at the Advanced Photon Source (Argonne, IL) using a wavelength of 0.979 Å and a temperature of 100 K. Data were processed and scaled using the HKL suite [35], with the best data set merging to 2.4 Å resolution (Table 3.2). EhACK crystals belonged to the space group I222 with unit cell dimensions \( a = 98.8 \, \text{Å}, \ b = 126.8 \, \text{Å}, \ c = 145.5 \, \text{Å}, \ \alpha = \beta = \gamma = 90^\circ \).

**Structure determination, model building, and refinement**

The EhACK structure was determined by molecular replacement using Phaser 2.1 [36] and the coordinates of the *M. thermophila* acetate kinase dimer [PDB entry 1G99 (ref), 34% sequence similarity] [5] as the search model. Iterative rounds of manual model building in COOT [37] and refinement in Refmac [38] and CNS [39] were performed to improve the quality of the model. The final model contains a dimer of EhACK in each asymmetric unit, which includes residues 2-32 and 39-392 of the A chain, and 2-392 of the B chain. The refinement achieved reasonable R-factors and geometry (Table 3.2).

**Chemicals**

Chemicals were obtained from Sigma-Aldrich, ThermoFisher Scientific, or VWR Scientific Products.
Table 3.2. Crystallographic data collection and refinement statistics

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<th>Data Collection</th>
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<tbody>
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<tr>
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<td>I222</td>
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<tr>
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<tr>
<td>Redundancy</td>
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<td>I/σ</td>
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<td>R_sym (^b) (%)</td>
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<td>R_{cryst}(R_{free})(^c) (%)</td>
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<tr>
<td>No. of protein atoms</td>
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<td>No. of solvent atoms</td>
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<tr>
<td>No. of ligand atoms</td>
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<tr>
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<td>rmsd for bond angles (°)</td>
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<table>
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<th>Ramachandran Plot</th>
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<td>Most favored (%)</td>
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<tr>
<td>Additionally allowed (%)</td>
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<tr>
<td>Generously allowed (%)</td>
<td>0</td>
</tr>
<tr>
<td>Disallowed (%)</td>
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</tbody>
</table>
Values in parentheses are values for the highest resolution shell, representing the resolution range from 2.46 Å to 2.4 Å.

\[ R_{\text{ref}} = \frac{\sum |I - \langle I \rangle|}{\sum I} \], where \( I \) is the intensity and \( \langle I \rangle \) is the weighted mean of \( I \) for \( N \) reflections and common indices \( h, k, l \).

\[ R_{\text{free}} = \frac{\sum |F - \langle F \rangle|}{\sum |F|} \].
V. ACKNOWLEDGEMENTS

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Author Contributions

Biochemical and kinetic analyses of EhACK was performed by M. Fowler, determination, refinement, and analysis of the EhACK structure was performed by M. Tanabe and T. Thaker, C. Ingram-Smith contributed to the writing of this manuscript, T. Iverson and K. Smith designed and guided the research and contributed to the writing of the manuscript.
VII. REFERENCES


CHAPTER IV
SITE-DIRECTED MUTATIONAL ANALYSIS OF ACTIVE SITE RESIDUES IN 
THE ACETATE KINASE FROM ENTAMOEBA HISTOLYTICA

Matthew L. Fowler, Cheryl J. Ingram-Smith, and Kerry S. Smith

ABSTRACT

Acetate kinase (ACK) catalyzes the reversible magnesium-dependent synthesis of acetyl phosphate by transfer of the \(\gamma\)-phosphate of ATP to acetate. A novel PP\(_{i}\)-dependent ACK found in \textit{E. histolytica} catalyzes a similar reaction through use PP\(_{i}\) as the phosphoryl donor. Using a PP\(_{i}\) density generated model of the \textit{E. histolytica} ACK, a structure-function approach was utilized to identify residues important for acetate-binding and catalysis. Investigation of the residues in the putative PP\(_{i}\)-ACK active site revealed that while some of the active site residues in the \textit{M. thermophila} ATP-dependent ACK are conserved in the \textit{E. histolytica} enzyme, a number of significant changes to the active site were found. The residues His\(^{117}\), His\(^{172}\), Val\(^{87}\), Thr\(^{201}\), Thr\(^{15}\), Arg\(^{274}\), and Asp\(^{272}\) appear to be essential for catalysis in the direction of acetate synthesis. Furthermore, His\(^{117}\) appears to be critically important for binding acetyl phosphate and therefore important in acetate/PP\(_{i}\) synthesis reactions. The data supports the hypothesis that the \textit{E. histolytica} ACK operates physiologically in the direction of acetate/PP\(_{i}\) synthesis.
I. INTRODUCTION

Acetate kinase (ACK), an important metabolic enzyme present in all three
domains of life [1], is a member of the acetate and sugar kinase-Hsp70-actin (ASKHA)
enzyme superfamily [2]. This enzyme catalyzes the magnesium-dependent transfer of the
γ-phosphate of ATP to acetate to generate acetyl phosphate. In bacteria and in the
methane-producing archaeon Methanosarcina thermophila, the enzyme partners with
phosphotransacetylase (PTA) in order to generate acetyl-CoA [3]. In fungi, the enzyme
partners with xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP) in a
modified pentose phosphoketolase pathway to catalyze ketose phosphates for the
synthesis of ATP [1]. The enzyme has also been discovered in other eukaryotes such as
Phytophthora spp., Chlamydomonas reinhardtii, and Entamoeba histolytica. In
Phytophthora and C. reinhardtii, ACK partners with PTA to allow for the
interconversion of acetyl phosphate and acetyl-CoA [1, 4].

In 1975, Reeves and Guthrie [5] partially purified a novel pyrophosphate (PPi)-
dependent ACK in Entamoeba histolytica. This enzyme was capable of utilizing acetyl
phosphate and P_i (Eq. 1) for the synthesis of acetate.

\[
\text{CH}_3\text{COPO}_4^{2-} + \text{P}_i \rightleftharpoons \text{CH}_3\text{COO}^- + \text{PP}_i \quad (\text{Eq. 1})
\]

At the time of the publication, only extracts of E. histolytica could be utilized to study the
enzyme as recombinant techniques for generating large quantities of enzyme were not yet
available. As such, a complete characterization of the PP_i-dependent ACK could not be
completed, and only limited information concerning the biochemistry and kinetics of the
enzyme were obtained.
More recently a biochemical and kinetic characterization of the recombinantly produced \textit{E. histolytica} ACK has been completed \cite{6}. Unique among ACKs, \textit{E. histolytica} ACK can utilize PP\(_i\) as a phosphoryl donor but is unable to utilize ATP \cite{6}. The enzyme is also capable of utilizing acyl substrates as long as hexanoate, but prefers acetate and propionate \cite{6}. As previously hypothesized by Reeves and Guthrie \cite{5} the enzyme preferentially operates in the direction of acetate synthesis \cite{6}. The recent structure has also provided additional information on the structural makeup of the active site of the enzyme \cite{6}.

Here we report structure-function analyses of the \textit{E. histolytica} ACK to identify amino residues that play key roles in substrate binding and or catalysis. A number of important residues in the active site of the well-characterized \textit{M. thermophila} ACK are not conserved in the \textit{E. histolytica} ACK. Furthermore, additional residues that play an important role in acetate binding and catalysis have been discovered. Residues that may be responsible for phosphoryl donor specificity are noted.
II. METHODS

Protein sequence analyses

Databases were searched at the National Center for Biotechnology Information using the BLAST network server [7]. ClustalX was used for multiple protein sequence alignments [8].

Crystal structure modifications and modeling of variant structures

Structures of the variants were generated using Discovery Studio Modeler® (Accelrys) from coordinates [6]. Modified structures were modeled using Discovery Studio ViewerPro® (Accelrys).

Site-directed mutagenesis of the E. histolytica ACK

Site-directed mutagenesis of the genes encoding the E. histolytica and ACK was performed using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. All mutagenic primers were 33-35 nucleotides in length with the altered base(s) located at the middle of the sequence. The altered sequences were confirmed by Sanger-style sequencing at the Clemson University Genomics Institute (CUGI).

Production and purification of recombinant E. histolytica ACK variants.

Wild-type Entamoeba ACK and its variants were heterologously produced in Escherichia coli strain YBS121 ΔackApta (kindly provided by George Bennett, Rice University) along with the lacI-containing plasmid pREP-4 (Qiagen) as described previously [6]. 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 OD$_{600}$≈0.9.
Recombinant protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside to 1 mM final concentration. Cultures were incubated overnight at ambient temperature/200 RPM 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 x g for 1 hour and the supernatant was applied to a 5 mL HisTrap® nickel affinity column (GE Healthcare, Piscataway, NJ). Protein was eluted from the column 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 enzymes were purified to apparent homogeneity as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was quantified by the Bradford method [9] using the Bio-Rad Protein Assay with bovine serum albumin as standard.

**Determination of kinetic parameters for EhACK**

The hydroxamate assay [10-12] was used to determine kinetic parameters in the acetyl phosphate/P₁-forming direction as previously described using a reaction mixture containing 100 mM MES, 5 mM MgCl₂, and 600 mM hydroxylamine hydrochloride (pH 7.5) with the concentrations of acyl substrate and sodium pyrophosphate were varied. Reactions were performed at 45°C, the optimal temperature for this enzyme. Acetyl phosphate formation was determined by comparison to an acetyl phosphate standard.
curve. All assays were performed in triplicate. Kinetic calculations and progress curves were generated using Kaleidagraph (Synergy Software, Reading, PA).

Kinetic parameters in the acetate-forming direction of the reaction were determined using a modified reverse hydroxamate assay [13]. The reactions (300 µL) contained 100 mM Tris (pH 7.0), 10 mM MgCl₂, and varying concentrations of sodium phosphate and acetyl phosphate. Reactions were pre-incubated for 1 minute at 37°C, initiated by the addition of purified enzyme or native *E. histolytica* extract and terminated by the addition of 50 µL 2 M hydroxylamine hydrochloride (pH 7.0). Following incubation at 60°C for 5 minutes, 100 µL 500 mM FeCl₃, 3 N HCl, 30 % trichloroacetic acid or 500 mM FeCl₃, 7 N HCl, 50 % trichloroacetic acid was added for color development and the absorbance at 540 nm was measured spectrophotometrically. All assays were performed in triplicate. A standard curve of acetyl phosphate concentrations was used to determine the amount of acetyl phosphate consumed in the reaction.
III. RESULTS AND DISCUSSION

Production and purification of variant acetate kinases.

Val^{87}, Thr^{223}, Ile^{116}, Ser^{230}, Ser^{271}, Thr^{15}, Thr^{201}, Asp^{272}, Arg^{274}, His^{117}, His^{172} and Glu^{323} in the *E. histolytica* ACK were subjected to site-specific replacement to test the hypothesis that these residues are involved in substrate binding and/or catalysis (Figure 4.1). The residues selected were identified from multiple amino acid sequence alignments of ACKs from *M. thermophila* and other prokaryotic and eukaryotic microbes, and from preliminary *E. histolytica* structures and models.

Kinetic characterization of putative acyl pocket residues.

The most prominent difference between residues found in the acetate pocket of the well-characterized *M. thermophila* ACK to that of *E. histolytica* ACK is the presence of a threonine at position 223, corresponding to a proline at position 232 in the *M. thermophila* enzyme (Figure 4.1). Pro^{232} is involved in substrate positioning and catalysis in the acetate binding pocket of the *M. thermophila* ACK [14]. Alteration of Thr^{223} to proline for the *E. histolytica* ACK had little effect on the kinetic parameters as the enzyme remained capable of utilizing substrates as long as hexanoate (Table 4.1). Furthermore, kinetic parameters for PP_{i} were also not significantly altered (Table 4.2). In the direction of acetate formation, no significant changes in the kinetic parameters for acetyl phosphate were observed for the Thr^{223}Pro variant (Table 4.3). The Thr^{223}Gly variant did not display activity for any acyl substrate in the acetyl phosphate-forming direction. A specific activity of $285.9 \pm 0.1 \, \mu\text{mol min}^{-1} \, \text{mg}^{-1}$ was determined for the Thr^{223}Gly variant in the acetate-forming direction at 0.6 mM acetyl phosphate in
comparison to the wild type specific activity of $1368.9 \pm 0.1 \text{ µmol min}^{-1} \text{ mg}^{-1}$, but kinetic parameters could not be determined as the enzyme could not be saturated with pyrophosphate.

Variant Thr$^{223}$Ala was produced as an inactive and insoluble enzyme. Changes to this residue likely resulted in the addition of a side chain residue that disrupted proper folding of the enzyme. The Thr$^{223}$Gly variant lacks a true side chain, and the resulting enzyme structure properly folded.
Figure 4.1. Sequence alignment of important residues in ACK from *Eukarya*, *Bacteria*, and *Archaea*. Residues that have been identified as being important for either acetate-binding (red) or catalysis (blue) in the *M. thermophila* ACK are highlighted. Differences in the *E. histolytica* active site are noted in green. Residue numbers above the alignments are of *E. histolytica* ACK and below the alignments are of *M. thermophila* ACK.

**Entamoeba histolytica**, *Ehistolytica*; *Entamoeba invadens*, *Einvadens*; *Phytophthora ramorum*, *Pramorum*; *Phaeosphaeria nodorum*, *Pnodorum*; *Cryptococcus neoformans*, *Cneoformans*; *Escherichia coli*, *Ecoli*; *Methanosarcina thermophila*, *Mthermophila*. 
Table 4.1. Kinetic parameters for the wild type and Thr^{223}Pro variant for acetate in the direction of acetyl phosphate synthesis.

<table>
<thead>
<tr>
<th>Enzyme/Substrate</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Valerate</th>
<th>Hexanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wild type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetate</td>
<td>106.8 ± 1.00</td>
<td>1.8 ± 0.01</td>
<td>0.016 ± 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>propionate</td>
<td>79.8 ± 1.94</td>
<td>1.2 ± 0.01</td>
<td>0.015 ± 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>butyrate</td>
<td>75.6 ± 1.49</td>
<td>0.33 ± 0.01</td>
<td>0.0043 ± 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>valerate</td>
<td>56.7 ± 0.67</td>
<td>0.19 ± 0.01</td>
<td>0.0034 ± 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexanoate</td>
<td>20.5 ± 0.49</td>
<td>0.051 ± 0.001</td>
<td>0.0025 ± 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thr^{223}Pro</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetate</td>
<td>169.5 ± 0.06</td>
<td>1.3 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>propionate</td>
<td>98.7 ± 0.18</td>
<td>0.61 ± 0.01</td>
<td>0.0062 ± 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>butyrate</td>
<td>56.5 ± 0.46</td>
<td>0.18 ± 0.01</td>
<td>0.0032 ± 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>valerate</td>
<td>41.6 ± 0.25</td>
<td>0.21 ± 0.01</td>
<td>0.0049 ± 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexanoate</td>
<td>20.4 ± 0.52</td>
<td>0.065 ± 0.001</td>
<td>0.0032 ± 0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2. Kinetic parameters for the wild type and Thr^{223}Pro variant for PP_i in the direction of acetyl phosphate synthesis.

<table>
<thead>
<tr>
<th>Enzyme/Substrate</th>
<th>PP_i</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (mM)</td>
<td>(k_{cat}) (s(^{-1}))</td>
</tr>
<tr>
<td>wild type acetate</td>
<td>3.6 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>propionate</td>
<td>4.8 ± 0.4</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>butyrate</td>
<td>3.2 ± 0.1</td>
<td>0.029 ± 0.001</td>
</tr>
<tr>
<td>valerate</td>
<td>6.3 ± 0.1</td>
<td>0.051 ± 0.001</td>
</tr>
<tr>
<td>hexanoate</td>
<td>16.3 ± 0.4</td>
<td>0.032 ± 0.001</td>
</tr>
<tr>
<td>Thr^{223} Pro acetate</td>
<td>6.3 ± 0.1</td>
<td>2.0 ± 0.01</td>
</tr>
<tr>
<td>propionate</td>
<td>4.0 ± 0.1</td>
<td>1.1 ± 0.01</td>
</tr>
<tr>
<td>butyrate</td>
<td>4.9 ± 0.1</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>valerate</td>
<td>3.0 ± 0.1</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>hexanoate</td>
<td>1.9 ± 0.1</td>
<td>0.081 ± 0.001</td>
</tr>
</tbody>
</table>
Table 4.3. Kinetic parameters for acetyl phosphate for wild-type and variant ACKs assayed in the direction of acetate synthesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.50 ± 0.006</td>
<td>1938.8 ± 13.7</td>
<td>3845.6 ± 68.9</td>
</tr>
<tr>
<td>Thr$^{223}$Pro</td>
<td>2.00 ± 0.082</td>
<td>8332.9 ± 185.4</td>
<td>4260.5 ± 82.7</td>
</tr>
<tr>
<td>Thr$^{223}$Gly</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ile$^{116}$Ala</td>
<td>1.6 ± 0.094</td>
<td>279.9 ± 2.0</td>
<td>174.9 ± 8.9</td>
</tr>
<tr>
<td>Ile$^{116}$Leu</td>
<td>2.4 ± 0.023</td>
<td>343.81 ± 2.1</td>
<td>143.8 ± 0.73</td>
</tr>
<tr>
<td>Ser$^{230}$Ala</td>
<td>4.5 ± 0.059</td>
<td>2181.0 ± 7.0</td>
<td>485.9 ± 5.1</td>
</tr>
<tr>
<td>Ser$^{230}$Gly</td>
<td>1.2 ± 0.038</td>
<td>1115.6 ± 10.0</td>
<td>911.3 ± 19.9</td>
</tr>
<tr>
<td>Thr$^{133}$Ala</td>
<td>1.1 ± 0.029</td>
<td>988.3 ± 9.5</td>
<td>896.1 ± 15.3</td>
</tr>
<tr>
<td>Thr$^{133}$Lys</td>
<td>1.1 ± 0.044</td>
<td>21.2 ± 0.25</td>
<td>19.0 ± 0.55</td>
</tr>
<tr>
<td>Val$^{87}$Ala</td>
<td>0.53 ± 0.027</td>
<td>120.8 ± 1.6</td>
<td>226.7 ± 10.1</td>
</tr>
<tr>
<td>Val$^{87}$Gly</td>
<td>0.51 ± 0.017</td>
<td>96.9 ± 0.96</td>
<td>188.7 ± 4.4</td>
</tr>
<tr>
<td>Ser$^{271}$Ala</td>
<td>1.0 ± 0.028</td>
<td>953.5 ± 6.6</td>
<td>916.0 ± 18.4</td>
</tr>
<tr>
<td>Ser$^{271}$Asn</td>
<td>0.58 ± 0.013</td>
<td>253.2 ± 0.87</td>
<td>436.5 ± 9.3</td>
</tr>
<tr>
<td>Thr$^{201}$Ala</td>
<td>1.7 ± 0.059</td>
<td>32.2 ± 0.29</td>
<td>18.5 ± 0.50</td>
</tr>
<tr>
<td>Thr$^{201}$Asn</td>
<td>1.2 ± 0.079</td>
<td>5.6 ± 0.06</td>
<td>4.6 ± 0.24</td>
</tr>
<tr>
<td>Asp$^{272}$Asn</td>
<td>0.71 ± 0.03</td>
<td>13.7 ± 0.13</td>
<td>19.3 ± 0.64</td>
</tr>
<tr>
<td>Asp$^{272}$Glu</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Asp$^{272}$Ala</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Arg$^{274}$Ala</td>
<td>0.56 ± 0.019</td>
<td>14.3 ± 0.13</td>
<td>25.9 ± 0.74</td>
</tr>
<tr>
<td>His$^{177}$Ala</td>
<td>8.0 ± 0.072</td>
<td>20.5 ± 0.07</td>
<td>2.6 ± 0.02</td>
</tr>
<tr>
<td>His$^{177}$Ala</td>
<td>0.69 ± 0.074</td>
<td>2.0 ± 0.07</td>
<td>2.8 ± 0.25</td>
</tr>
</tbody>
</table>

* unable to determine kinetic parameters due to low activity
Although the *E. histolytica* ACK Thr\textsuperscript{223}Ala variant was insoluble, an alanine change at the corresponding Pro\textsuperscript{232} residue in *M. thermophila* results in a soluble enzyme with a significantly decreased turnover (26-fold) and catalytic efficiency (126-fold) compared to the wild type [14]. This change in solubility of the enzyme for variant changes indicates a structural variation between these ACKs which is reinforced by these different residues.

In the *M. thermophila* ACK, a leucine is present at position 122 which corresponds to Ile\textsuperscript{116} in the *E. histolytica* ACK. Leu\textsuperscript{122} has been shown to play an important role in acetate binding [14] and is hypothesized to stabilize the methyl group of acetate in the *M. thermophila* ACK [15]. The Ile\textsuperscript{116}Ala variant displayed insignificant changes in kinetics for acetate compared with the wild type kinetic parameters. However, the Ile\textsuperscript{116}Leu variant displayed a 25-fold decrease in the $k_{\text{cat}}$ and 34-fold decrease in the overall enzyme efficiency for acetate compared to the wild type ACK (Table 4.4). Both the Ile\textsuperscript{116}A and Ile\textsuperscript{116}Leu variants were unable to utilize longer acyl substrates. A 26-fold decrease in $k_{\text{cat}}$ and 24-fold decrease in enzyme efficiency for PP\textsubscript{i} assayed with acetate were observed for the Ile\textsuperscript{116}Ala variant (Table 4.5). Kinetic parameters for PP\textsubscript{i} assayed with acetate could not be determined for the Ile\textsuperscript{116}Leu variant due to low activity. Significant decreases for both the $k_{\text{cat}}$ and enzyme efficiency for acetate indicate that Ile\textsuperscript{116} plays an important for catalysis. In the direction of acetate formation, marked decreases of 22-fold and 27-fold in the catalytic efficiency were observed for both Ile\textsuperscript{116}Ala and Ile\textsuperscript{116}Leu respectively (Table 4.3). The decreases in catalytic efficiency for acetyl phosphate as well as the decreases in the turnover and catalytic efficiency in the acetate forming directions for these variants further implicates Ile\textsuperscript{116} as important for
catalysis. Whereas the Leu\textsuperscript{122} in the \textit{M. thermophila} ACK plays a role in acetate binding, Ile\textsuperscript{116} in the \textit{E. histolytica} enzyme has a role in catalysis.

Val\textsuperscript{87} in the \textit{E. histolytica} ACK corresponds to Val\textsuperscript{93} in the \textit{M. thermophila} ACK, which has been shown to be important for acetate binding [14] by providing stabilization to the methyl group of acetate [15]. The Val\textsuperscript{87}Ala and Val\textsuperscript{87}Gly variants were not active in the acetyl phosphate-forming directions and therefore kinetic parameters were unable to be determined. Loss of the more prevalent hydrophobic interaction indicates that this residue plays an important role in the active site of the \textit{E. histolytica} ACK. Significant decreases in turnover and catalytic efficiency were observed for Val\textsuperscript{87}Ala and Val\textsuperscript{87}Gly in the direction of acetate synthesis (Table 4.3). 91-fold and 16-fold decreases in turnover and 202-fold and 16-fold decreases in catalytic efficiency were observed for Val\textsuperscript{87}Ala and Val\textsuperscript{87}Gly respectively. These significant decreases in the direction of acetate synthesis indicate that Val\textsuperscript{87} plays an important role in catalysis. Val\textsuperscript{87} is located in a hydrophobic pocket of the putative active site with another hydrophobic residue (Phe\textsuperscript{117}) at a distance of only 4.3 Å. These residues may also participate in hydrophobic interactions with the methyl group of acetate and acetyl phosphate in the \textit{E. histolytica} ACK.
Table 4.4. Kinetic parameters for acetate for wild-type and variant ACKs assayed in the direction of acetyl phosphate synthesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>106.8 ± 1</td>
<td>1.8 ± 0.01</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>Ile$^{116}$Ala</td>
<td>219.6 ± 9.9</td>
<td>0.88 ± 0.01</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>Ile$^{116}$Leu</td>
<td>146.2 ± 5.3</td>
<td>0.069 ± 0.001</td>
<td>0.00047 ± 0.00001</td>
</tr>
<tr>
<td>Ser$^{230}$Ala</td>
<td>142.6 ± 9.8</td>
<td>0.84 ± 0.013</td>
<td>0.0059 ± 0.0003</td>
</tr>
<tr>
<td>Ser$^{230}$Gly</td>
<td>145.9 ± 1.7</td>
<td>0.57 ± 0.001</td>
<td>0.0039 ± 0.0001</td>
</tr>
<tr>
<td>Thr$^{15}$Lys</td>
<td>59.1 ± 6.6</td>
<td>0.27 ± 0.001</td>
<td>0.0045 ± 0.0001</td>
</tr>
<tr>
<td>Thr$^{15}$Ala</td>
<td>114.0 ± 0.85</td>
<td>0.61 ± 0.001</td>
<td>0.0054 ± 0.0001</td>
</tr>
<tr>
<td>Ser$^{271}$Asn</td>
<td>140.3 ± 2.8</td>
<td>0.16 ± 0.001</td>
<td>0.0012 ± 0.0001</td>
</tr>
<tr>
<td>Arg$^{274}$Ala</td>
<td>186.4 ± 21.7</td>
<td>0.11 ± 0.003</td>
<td>0.00056 ± 0.00005</td>
</tr>
<tr>
<td>Ser$^{271}$Ala</td>
<td>173.7 ± 0.92</td>
<td>0.93 ± 0.01</td>
<td>0.0054 ± 0.0001</td>
</tr>
</tbody>
</table>
Table 4.5. Kinetic parameters for PP$_i$ for wild-type and variant ACKs assayed in the direction of acetyl phosphate synthesis in the presence of acetate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>3.9 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>1.1 ± 0.02</td>
</tr>
<tr>
<td>Ile$^{116}$Ala</td>
<td>0.2 ± 0.01</td>
<td>3.3 ± 0.1</td>
<td>0.043 ± 0.001</td>
</tr>
<tr>
<td>Ser$^{230}$Ala</td>
<td>1.1 ± 0.01</td>
<td>1.4 ± 0.1</td>
<td>0.78 ± 0.01</td>
</tr>
<tr>
<td>Ser$^{230}$Gly</td>
<td>1.2 ± 0.01</td>
<td>5.2 ± 0.1</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Thr$^{15}$Ala</td>
<td>0.73 ± 0.01</td>
<td>3.3 ± 0.1</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Ser$^{271}$Asn</td>
<td>0.22 ± 0.01</td>
<td>1.1 ± 0.1</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Arg$^{274}$Ala</td>
<td>0.23 ± 0.01</td>
<td>2.8 ± 0.1</td>
<td>0.085 ± 0.001</td>
</tr>
<tr>
<td>Ser$^{271}$Ala</td>
<td>1.3 ± 0.01</td>
<td>5.7 ± 0.1</td>
<td>0.22 ± 0.01</td>
</tr>
</tbody>
</table>
Characterization of other catalytically important residues.

A lysine at position 14 in the *M. thermophila* ACK has been shown to be catalytically important in the active site, acting as a site for hydrogen bonding during substrate interaction [14]. Lys$^{14}$ corresponds to Thr$^{15}$ in the *E. histolytica* enzyme. With acetate as the acyl substrate, alteration of Thr$^{15}$ to alanine resulted in a 12-fold increase in $k_{cat}$, but did not significantly alter other kinetic parameters (Table 4.4). Kinetic parameters for propionate were not significantly altered (Table 4.6), but information on longer acyl chain substrates could not be garnered due to loss of activity. No significant changes in the kinetic parameters for acetyl phosphate were observed for the Thr$^{15}$Ala variant in the acetate forming direction. However, 91-fold and 202-fold decreases in turnover and catalytic efficiency of acetyl phosphate were observed for the Thr$^{15}$Lys variant in the direction of acetate synthesis (Table 4.3).
Table 4.6. Kinetic parameters for propionate for wild-type and variant ACKs assayed in the direction of acetyl phosphate synthesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>79.8 ± 1.94</td>
<td>1.2 ± 0.005</td>
<td>0.015 ± 0.0003</td>
</tr>
<tr>
<td>Thr$^{223}$Pro</td>
<td>98.7 ± 0.18</td>
<td>0.61 ± 0.001</td>
<td>0.0062 ± 0.00001</td>
</tr>
<tr>
<td>Ser$^{230}$Ala</td>
<td>104.4 ± 4.19</td>
<td>0.36 ± 0.003</td>
<td>0.0034 ± 0.0001</td>
</tr>
<tr>
<td>Ser$^{230}$Gly</td>
<td>128.4 ± 0.05</td>
<td>0.36 ± 0.001</td>
<td>0.0028 ± 0.00001</td>
</tr>
<tr>
<td>Thr$^{15}$Ala</td>
<td>108.8 ± 0.98</td>
<td>0.26 ± 0.001</td>
<td>0.0024 ± 0.00002</td>
</tr>
<tr>
<td>Ser$^{271}$Asn</td>
<td>64.4 ± 0.82</td>
<td>0.058 ± 0.001</td>
<td>0.00091 ± 0.00001</td>
</tr>
<tr>
<td>Ser$^{271}$Ala</td>
<td>101.5 ± 0.60</td>
<td>0.33 ± 0.0002</td>
<td>0.0032 ± 0.00002</td>
</tr>
</tbody>
</table>
The change in observed kinetic parameters as compared to those of the corresponding Lys\textsuperscript{14} residue in the \textit{M. thermophila} enzyme, imply that Thr\textsuperscript{15} in the \textit{E. histolytica} ACK may have a different role from its archaeal Lys\textsuperscript{14} counterpart. Theoretically, the Thr\textsuperscript{15}Lys variant would result in lysine protruding into the core of the putative active site, likely interfering with catalysis (Figure 4.2). The Thr\textsuperscript{15} residue is located on the periphery of the putative active site and likely plays a role in catalysis upon close of the active site.

In the \textit{M. thermophila} ACK, His\textsuperscript{180} plays an important role in catalysis [16], and is hypothesized to interact with the \(\alpha\)-phosphate of ATP during acetyl phosphate synthesis by stabilizing the transition state [15]. When the corresponding residue in \textit{E. histolytica} (His\textsuperscript{172}) was altered to alanine activity in the acetyl phosphate-forming direction could not be detected. In the direction of acetate formation a 995-fold and 1354-fold decrease in respective turnover and catalytic efficiency for acetyl phosphate was observed for His\textsuperscript{172}Ala (Table 4.3). This significant decrease in turnover and enzyme efficiency indicates that this residue is critical for catalysis. The His\textsuperscript{172} residue lies directly in the core of the active site, providing one or more hydrogen atoms from the imidazole ring of histidine as lone pair acceptors. Alteration of this residue to alanine would theoretically remove this residue from the core of the putative active site, providing only limited, if any, hydrophobic interaction with the substrates.
Figure 4.2. The putative active site of the *E. histolytica* ACK. Residues important for acetate binding and catalysis (turquoise). Residues that may interact with substrates upon closure of the active site (red). The putative active site is modeled from data received from the laboratory of Dr. Tina Iverson, Vanderbilt University [6].
His\textsuperscript{123} is located near the active site in the \textit{M. thermophila} ACK and has been reported to be important for acetyl phosphate binding [16]. When the corresponding His\textsuperscript{117} in the \textit{E. histolytica} enzyme was changed to alanine, acetyl phosphate synthesis could not be detected. In the direction of acetate synthesis the His\textsuperscript{117}Ala variant displayed a 16-fold increase in the $K_m$ for acetyl phosphate, indicating that this residue may be important for acetyl phosphate binding (Table 4.3). Furthermore, a 95-fold and 1496-fold decrease in respective turnover and catalytic efficiency for acetyl phosphate was observed for His\textsuperscript{117}Ala. This significant decrease implicates this residue's importance in catalysis. This residue lies near the core of the putative active site (Figure 4.2), and like His\textsuperscript{172}, may provide hydrogen atoms for electrons from an oxygen located one or more of the phosphate groups of P\textsubscript{i} or PP\textsubscript{i}. The His\textsuperscript{117}Ala variant would result in removal of these lone pair acceptors and the resulting methyl group side chain would likely be so far removed from the core of the putative active site that interaction with ACK substrates would no longer be possible.

**Characterization of other possible active site residues.**

The \textit{E. histolytica} ACK was crystallized in the presence of PP\textsubscript{i} and an electron density found to be present within the structure was originally modeled as PP\textsubscript{i} (Figure 4.3). However, further refinement suggests that PP\textsubscript{i} is not present in the structure. Residues Ser\textsuperscript{230}, Ser\textsuperscript{271}, Asp\textsuperscript{272}, Arg\textsuperscript{274}, and Thr\textsuperscript{201} were hypothesized to participate in PP\textsubscript{i} binding and/or catalysis in the initial structure. Protein sequence alignments comparing the corresponding residues in the \textit{M. thermophila}, \textit{E. coli}, and fungal ACK sequences indicate a number of these changes are unique to the \textit{Entamoeba} enzymes (Figure 4.4).
Figure 4.3. An early model of the active site of the \textit{E. histolytica} ACK. Residues Ser$^{230}$, Ser$^{271}$, Asp$^{272}$, Arg$^{274}$, and Thr$^{201}$ are hypothesized to participate in PP$_i$ binding and/or catalysis. Structure image produced by Tarjani Thaker and Dr. Tina Iverson, Vanderbilt University.
To investigate the roles of these residues in the *E. histolytica* ACK, each of the above residues was changed to either alanine or the corresponding amino acid found in the *M. thermophila* ACK.

Ser$_{230}^{\text{Ala}}$ and Ser$_{230}^{\text{Gly}}$ variants did not display significant changes in kinetic parameters for acetate (Table 4.4), or PP$_i$ (Table 4.5), or propionate (Table 4.6). However, parameters for longer acyl chain substrates could not be determined due to loss of activity. No significant changes in the kinetic parameters for acetyl phosphate were observed for either variant.

Another serine residue (Ser$_{271}^{\text{Ala}}$) is located adjacent to Ser$_{230}^{\text{Ala}}$ in the putative active site (Figure 4.4). Variants Ser$_{271}^{\text{Ala}}$ and Ser$_{271}^{\text{Asn}}$ had insignificant changes in their kinetic parameters for acetate with exception to Ser$_{271}^{\text{Asn}}$ which displayed a 10-fold decrease in enzyme efficiency in acetyl phosphate synthesis (Table 4.4). A 17-fold decrease in the $k_{\text{cat}}$ for PP$_i$ was observed for acetyl phosphate synthesis (Table 4.5). Both Ser$_{271}^{\text{Ala}}$ and Ser$_{271}^{\text{Asn}}$ were unable to utilize acyl substrates longer than propionate. A 30-fold decrease in the $k_{\text{cat}}$ and 17-fold decrease in enzyme efficiency was observed for Ser$_{271}^{\text{Asn}}$ for propionyl phosphate synthesis (Table 4.6). No significant changes in kinetic parameters were observed for Ser$_{271}^{\text{Ala}}$ and Ser$_{271}^{\text{Asn}}$ in the direction of acetate synthesis.

In the refined structure, Ser$_{230}^{\text{Ala}}$ and Ser$_{271}^{\text{Asn}}$ are located on the margins of the putative active site and would appear to have little to no interaction with acetyl phosphate (Figure 4.2). However, changes to these residues had an effect on propionate and longer acyl substrates during acetate synthesis. The absence of activity of substrates longer than
propionate can be attributed to the use of high concentrations of protein in the assay to
detect ACK activity in the direction of acetate synthesis. The use of such high
concentrations of protein is necessary for the detection of ACK activity in this direction
and is likely the reason that Reeves and Guthrie [5] were unable to detect acetyl
phosphate forming ACK activity more than 35 years earlier.
Figure 4.4. Alignment of sequences identified from early analysis of the *E. histolytica* structure along with corresponding residues from other *Eukarya*, *Bacteria*, and *Archaea*. Residues proposed to play a role in either ATP binding and catalysis in the *M. thermophila* ACK are highlighted in green. Active site residue differences in the *Entamoeba* ACKs (blue) and fungal ACKs (red) are noted. Residue numbers above the alignments are of the *E. histolytica* ACK. Residue numbers below the alignments are of the *M. thermophila* ACK. *Entamoeba histolytica*, *Ehistolytica*; *Entamoeba invadens*, *Einvadens*; *Phytophthora ramorum*, *Pramorum*; *Phaeosphaeria nodorum*, *Pnodorum*; *Cryptococcus neoformans*, *Cneoformans*; *Escherichia coli*, *Ecoli*; *Methanosarcina thermophila*, *Mthermophila*. 
Thr^{201} in the *E. histolytica* ACK was altered to alanine and asparagine. Both the Thr^{201}Ala and Thr^{201}Asn variants were not active in the acetyl phosphate-forming direction indicating that these residues may play an important role in the active site. In the direction of acetate synthesis, significant decreases in both the $k_{cat}$ and catalytic efficiency for acetyl phosphate were observed for both variants. A 60-fold and 343-fold decrease in the $k_{cat}$ were observed for Thr^{201}Ala and Thr^{201}Asn respectively (Table 4.3). Decreases of 207-fold and 837-fold in catalytic efficiency were respectively observed for Thr^{201}Ala and Thr^{201}Asn. These significant decreases in both enzyme turnover and catalytic efficiency for acetyl phosphate indicate that this residue plays an important role in catalysis, most especially in the direction of acetate formation. Thr^{201} projects the methyl group of the side chain into the core of the putative active site; which leads to the hypothesis that this residue may provide some hydrophobic interaction during catalysis (Figure 4.2). The Thr^{201}Ala variant contains a truncated side chain in comparison to the wild type enzyme, reducing the hydrophobic interaction in this region of the putative active site, and would theoretically point in a direction perpendicular to the direction of the methyl group of threonine at this position. Thr^{201}Asn variant alters the hydrophobicity, and likely ejects a polar side chain into the putative active site. The significant decreases in both catalysis and catalytic efficiency noted above for the Thr^{201}Asn variant, but less significant for the Thr^{201}Ala variant support the presence of a hydrophobic side chain interacting with one or more of the substrates utilized by the enzyme. Thr^{201} could also participate in hydrogen bonding with one or more of the ACK substrates.
Asp\textsuperscript{272} was altered to asparagine, glycine, and alanine. The variants were not active in the acetyl phosphate-forming direction indicating that this residue may be important for acetate and/or PP\textsubscript{i} binding or catalysis. Furthermore, the Asp\textsuperscript{272}Asn variant displayed significant decreases of 142-fold and 200-fold turnover and catalytic efficiency for acetyl phosphate in the direction of acetate formation (Table 4.3). Aspartate variants Asp\textsuperscript{272}Asn and Asp\textsuperscript{272}Gly displayed specific activities too low to determine kinetics. The significant decreases in turnover and catalytic efficiency for the Asp\textsuperscript{272}Asn variant and extremely low specific activity for Asp\textsuperscript{272}Ala and Asp\textsuperscript{272}Gly in the direction of acetate synthesis implies that this residue plays an important role in catalysis. This residue may be important in enzyme folding or in structural integrity of the putative active site. The aspartate residue faces away from the internal portion of the putative active site, and therefore, would not be hypothesized to participate in substrate interaction (Figure 4.2).

In the \textit{M. thermophila} ACK, Arg\textsuperscript{285} is involved in binding acetate [17] and the corresponding residue Arg\textsuperscript{274} is also near putative acetate pocket in the \textit{E. histolytica} active site. An Arg\textsuperscript{274}Ala variant displayed a 16-fold and 17-fold decrease in $k_{\text{cat}}$ for acetate (Table 4.4) and PP\textsubscript{i}, respectively (Table 4.5). The enzyme efficiencies for acetate and PP\textsubscript{i} were also decreased by 28-fold and 12-fold, respectively. The Arg\textsuperscript{274}Ala variant was unable to use longer acyl substrates. In the direction of acetate synthesis a 135-fold and 148-fold decrease in respective turnover and catalytic efficiency were observed for acetyl phosphate (Table 4.3), implying that this residue may be important for catalysis. The decreases observed in the acetyl phosphate forming direction for PP\textsubscript{i} combined with the decreases observed in the kinetic parameters for acetyl phosphate in acetate synthesis
highlights that this residue may interact with one or more of the phosphates of PP\textsubscript{i} and/or acetyl phosphate. One, if not both of the amine groups of Arg\textsubscript{274} face into the active site, allowing for one or more of the hydrogen atoms a part of the amine to act as a lone pair acceptor (Figure 4.2). The truncated side chain in the Arg\textsubscript{274} Ala variant would be hypothesized to no longer interact with substrates in the putative active site due to distance from the site core. While, Arg\textsubscript{285} in the \textit{M. thermophila} ACK plays a role in acetate-binding, the corresponding Arg\textsubscript{274} residue in the \textit{E. histolytica} enzyme is involved in catalysis.

\textit{Conclusions.}

Kinetic analyses of ACKs variants identified from sequence alignments have resulted in the identification of residues important for acetate and acetyl phosphate binding, and catalysis. Furthermore, a number of residues that may be important for PP\textsubscript{i}-binding have also been identified.

Multiple attempts were made to generate variants of Glu\textsubscript{323}, but site specific changes could not be generated at the DNA level possibly due to primer interactions or temperature annealing problems.

While a number of important residues have been identified, additional residues that have been identified in both sequence alignments (Figure 4.1 and Figure 4.3) and in recent refined structures [6] should be further investigated. Ser\textsuperscript{11}, Ser\textsuperscript{12}, Ser\textsuperscript{13}, and Asn\textsuperscript{8} are located in the margins of the putative active site and may provide sites for interaction with phosphate oxygen atoms. His\textsuperscript{198} and Arg\textsuperscript{232} protrude into the core of the putative active site, and their close proximity to other residues that have been shown to be
important in catalysis implicates them as candidates for site specific changes. The results presented here have not only broadened our understanding of the ACK enzyme family but have provided a foundation for future investigations.
IV. REFERENCES


CHAPTER V
RNA INTERFERENCE AND THE PHYSIOLOGICAL ROLE OF THE ACETATE KINASE IN *ENTAMOEBA HISTOLYTICA*

Matthew L. Fowler, Madison A. Foster, Ann M. Guggisberg, Cheryl J. Ingram-Smith, and Kerry S. Smith

ABSTRACT

*Entamoeba histolytica* is a protozoan parasite and the third leading cause of parasitic morbidity and mortality worldwide. The enzyme acetate kinase (ACK), a prokaryotic metabolic enzyme, has recently been discovered in several eukaryotic organisms, including *E. histolytica*. In prokaryotes, ACK catalyzes the reversible phosphorylation of acetate to acetyl phosphate utilizing ATP. The ACK identified in *E. histolytica* differs from the prokaryotic ACK in that the enzyme uses pyrophosphate (PP_i) in place of ATP as the phosphoryl donor. Common partners for ACK, phosphotransacetylase (PTA) and xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP) are also absent in the current *E. histolytica* genome assembly. Here, the dependency of the PP_i-dependent ACK from *E. histolytica* is investigated. Short hairpin RNA interference constructs were generated in order to post-transcriptionally knock down the expression of the *E. histolytica* PP_i-ACK. Specific activities from purified *E. histolytica* extracts indicate that the enzyme functions in the direction of acetyl phosphate formation, suggesting a physiological means of generating PP_i. The
introduction of plasmid DNA into *E. histolytica* was unsuccessful in these experiments. However, the results here will be important for future attempts at gene knockdown and investigations into parasite dependency on this enzyme. Further knowledge of the dependency of metabolic enzymes in *E. histolytica* may lead to new drug targets and treatments of amoebic dysentery.
I. INTRODUCTION

*Entamoeba histolytica* is a protozoan parasite and the causative agent of amoebic dysentery. Affecting nearly 50 million people worldwide, *E. histolytica* is the third leading cause of death world-wide due to parasitic disease resulting in more than 100,000 deaths annually [1]. Infection of the host is accomplished through the fecal-oral route. Contaminated food or water is ingested which contain quadra-nuclear cysts. Once these cysts reach the lumen of the small intestine, the cysts undergo excystation, resulting in the production of the amoebic trophozoites. These mobile parasites can then disseminate to other organs such as the liver, lungs, or brain and generate abscesses which result in a high rate of mortality [2]. Trophozoites can undergo encystation in the large intestine where they are passed into the feces (Figure 5.1) [3].

Initially thought to be present only in prokaryotes, ACK has been identified in the genomes of several eukaryotes, including *E. histolytica* [4]. ACK functions in the interconversion of acetate to acetyl phosphate with ATP as the phosphoryl donor and is an important enzyme in bacterial and archaeal metabolism for the synthesis of acetyl-CoA [4-6]. In many prokaryotes, and more recently discovered in eukaryotes such as *Phytophthora* spp. and *Chlamydomonas reinhardtii*, a partner enzyme phosphotransacetylase (PTA) is responsible for the interconversion of acetyl phosphate to acetyl-CoA[4]. In fungi, another bacterial enzyme xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP), has been proposed to partner with ACK to convert xylulose 5-phosphate or fructose 6-phosphate to acetate for the generation of ATP [4].
Figure 5.1. Life cycle of *E. histolytica*. The mature cyst is ingested by the human host. Excystation of the cyst occurs in the small intestine where the active trophozoite is produced. The trophozoite can then replicate by binary fission to form additional trophozoites, or can undergo encystation to produce a number of cysts. Cysts are passed into the host’s feces where water-borne transmission allows for continuation of the parasite’s life cycle. Taken from [3].
Previous research on *E. histolytica* has led to the discovery of an ACK in the parasite [7]. The enzyme is novel among other known ACKs in that the enzyme utilizes PPi as the phosphoryl donor in the place of ATP [8]. Furthermore, this ACK is unique in that it is capable of utilizing acyl substrates longer than butyrate. [8]. The enzyme displays 3,500-fold difference in catalytic efficiency in the direction of acetate formation indicating preferential directionality for acetate and PPi formation. Acetyl phosphate could be generated from either XFP or PTA, partners for ACK in other eukaryotes. However, genes encoding either enzyme are absent in the most recent genome assembly.

We are currently investigating the role of the PPi-dependent ACK in *E. histolytica*. An acetate-forming PPi-ACK activity was detected in cellular extracts, but an ATP-ACK activity was not. Activities for PTA and XFP, the proposed partners for ACK in other eukaryotes, were also not detected in cellular extracts of the parasite. Here we describe an attempt at short hairpin RNA (shRNA) interference in *E. histolytica* in order to down-regulate expression of the enzyme. The introduction of shRNAs into the parasite is expected to target and inhibit expression of the ACK. While we were unable to successfully perform this technique as previously described [9, 10], the results of RNAi experiments are important for the future development of this system and determination of the importance of this enzyme in the parasite’s metabolic processes. In addition, the human host lacks an ACK, implicating this gene as a possible target for the development of new treatments for infection by *E. histolytica*. 
II. MATERIAL AND METHODS

*Cultivation of E. histolytica*

Trophozoites of *E. histolytica* strain HM1:IMSS were routinely cultured under axenic conditions in TYI-S-33 medium [11].

*Cell extract preparation*

To prepare *E. histolytica* cell extracts, 4 x 10^6 trophozoites were resuspended in 25 mM Tris, 150 mM NaCl (pH 7.4) and vortexed with acid-washed glass beads for one minute followed by one minute on ice, for three cycles. The extract was centrifuged at 5000 x g for 15 minutes and the supernatant isolated.

*Hydroxamate assay for ACK activity*

The hydroxamate assay [5, 12, 13] was used to determine kinetic parameters in the acyl phosphate-forming direction. The reactions (300 µL) contained 100 mM MES, 5 mM MgCl₂, 600 mM hydroxylamine hydrochloride (pH 7.5), 2 M acetate and 10 mM sodium pyrophosphate. ATP and PP₃ were tested as substrates at a final concentration of 10 mM. The reactions were pre-incubated at 45°C for 1 minute and initiated by the addition of enzyme. Reactions were terminated by the addition of two volumes of 1.25% FeCl₃, 1 N HCl, 5% trichloroacetic acid and the absorbance at 540 nm was measured spectrophotometrically. All assays were performed in triplicate. Kinetic calculations and progress curves were generated using Kaleidagraph (Synergy Software, Reading, PA).

Kinetic parameters in the acetate-forming direction of the reaction were determined using a modified reverse hydroxamate assay [14]. The reactions (300 µL) contained 100 mM Tris (pH 7.0), 10 mM MgCl₂, 200 mM sodium phosphate, and 2 mM
acetyl phosphate. Reactions were pre-incubated for 1 minute at 37°C, initiated by the addition of purified enzyme or native *E. histolytica* extract and terminated by the addition of 50 µL 2 M hydroxylamine hydrochloride (pH 7.0). Following incubation at 60°C for 5 minutes, 100 µL 250 mM FeCl₃, 1.5 N HCl, 15 % trichloroacetic acid was added for color development and the absorbance at 540 nm was measured spectrophotometrically. All assays were performed in triplicate. A standard curve of acetyl phosphate concentrations was used to determine the amount of acetyl phosphate depleted in the reaction.

**Phosphotransacetylase (PTA) assay**

Activity was measured in both the acetyl phosphate-forming and the acetyl-CoA-forming directions by monitoring the decrease or increase in absorbance at 233 nm for 20 minutes in triplicate, indicative of the formation or breakage of the thioester bond of acetyl-CoA, respectively. The reaction mix for the acetyl phosphate-forming assay [6] consisted of 100 mM Tris (pH 7.0), 2 mM DTT, 10 mM sodium phosphate, and 120 µg of native *E. histolytica* extract in a total volume of 200 µL. The reaction was initiated by the addition of acetyl-CoA (0.5 mM final concentration). The reaction mix for the acetyl-CoA-forming assay 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 (2 mM final concentration).

**Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP) assay**

XFP activity in *E. histolytica* cell extracts was analyzed using the hydroxamate assay to detect the formation of acetyl phosphate [5, 12, 13]. 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 \textit{E. histolytica} extract and terminated after 30 minutes by the addition of 2 volumes of 1.25% FeCl$_3$, 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. All assays were performed in triplicate.

\textbf{Short hairpin RNA interference constructs}

In order to knock down the expression of the ACK gene in \textit{E. histolytica} via RNA interference (RNAi), constructs were made using a two-step PCR procedure (Figure 5.2A) [10]. Constructs form short hairpin RNAs (shRNAs), each composed of a 29 base sense strand, a 9 base loop, and a 29 base antisense strand (Figure 5.2C). To generate shRNAs against the \textit{EhACK} gene, four sequences were selected from the \textit{E. histolytica} ACK gene sequence using the siRNA target finder at http://www.ambion.com/techlib/misc/siRNA_finder.html. Each sequence was denoted according to its starting nucleotide position (441, 462, 600, and 842) within the \textit{EhACK} gene sequence. Chosen sequences contained 21 nucleotides, beginning with AA and possessing GC content between 30 and 50 percent. A 29 nucleotide sequence was generated by the addition of 8 nucleotides from the \textit{EhACK} gene sequence by addition to each 21 nucleotide sequence. A scrambled negative control sequence was generated by inputting the experimental construct 441 sequence into a word scrambler (http://www.lerfjhax.com/scrambler) to jumble the sequences. The scrambled control
along with the other 29 nucleotide shDNA sequences were blasted against the *E. histolytica* genome to ensure that there were no possible off-target effects.

The reverse primers for each round of PCR were designed using the reverse complements of the desired constructs (Table 5.1). The reverse primer for the first round of PCR contained the reverse complement of the 3’ end of the *E. histolytica* U6 promoter (GGATAAAAAGAAAAATAAAAATT), the *Apa*I site (GGGCC), the sense strand of the 29-mer shRNA sequence chosen with the siRNA target finder, and the 9 base loop (TTCAAGAGA). The reverse primer for the second round of PCR contained the reverse complement of the 9 base loop, the antisense strand of the shRNA sequence, the T6 terminator (TTTTTT), and the *Not*I site (GCGGCCGC). Because the shRNA sequence ends in TT, only TTTT was inserted as the T6 terminator. Additionally, the reverse complement of GATCGA was added to the end of the sequence to allow for optimal *Not*I digestion.

The first round of PCR was conducted using KOD Hot Start DNA polymerase (EMD Chemicals), along with the U6 *HindIII* forward primer (CTACTGAAGCTTTTATGAAAAAGTGTATTTGC, 10 µM) and the first round reverse primers (10 µM) (Table 5.1). pGIR310:U6 + PATscram (45µg/ml) plasmid (kindly provided by Dr. William Petri [10]), containing the RNA polymerase III U6 promoter originally cloned from *E. histolytica* genomic DNA was used as the template. Two 50 µl reactions were performed for each shRNA construct using 2 µl and 5 µl of plasmid template, respectively, and the published PCR conditions [10]. Round 1 PCR products were confirmed by gel electrophoresis.
The second round of PCR was conducted using KOD Hot Start DNA polymerase, along with the U6 *Hind*III forward primer (10 µM) and the second round reverse primers (10 µM) (Table 5.1). Two 50 µl reactions were performed for each shRNA construct using 2 µl of the respective Round 1 PCR products as the template and the published PCR conditions [10]. Final PCR products from Round 2 were confirmed by gel electrophoresis.

Final PCR products were purified used the Wizard SV PCR Clean-Up System (Promega) and digested separately in two consecutive reactions with *Hind*III and *Not*I, along with expression vector pGIR310: EV+PL (also known as pGIR310 2.1.2) an empty vector pGIR310 modified with a *Hind*III-*Sall*-NotI-NotI-NotI polylinker. After gel-purification of the digested products, the RNAi construct inserts were directly cloned into pGIR310: EV+PL between the *Hind*III and *Not*I sites of the polylinker using Novagen 2x clonables (EMD Chemicals) (Figure 5.2B).

Recombinant plasmids were transformed into NovaBlue competent *E. coli* cells (Novagen, Inc.) and plated on LB-ampicillin plates. Proper ligation was confirmed by PCR colony screen using the conditions described in the creation of shDNA sequences. Transformed colonies were grown up into LB-ampicillin medium, and the recombinant plasmids purified using the Zyppy Plasmid Miniprep Kit (Zymo Research Corporation). Purified plasmids were digested with *Hind*III and *Not*I and run on an agarose gel for further verification of proper RNAi construct insertion into pGIR310. Confirmed clones were sequenced using the Sp6 forward primer and the hgl reverse primer by the Clemson University Genomics Institute. Recombinant plasmids verified by sequencing to contain
the correct shRNAi construct were produced and purified on a large-scale using Zyppy Plasmid Maxiprep Kit (Zymo Research Corporation) or the Plasmids Mega Prep Kit (Qiagen, Inc.).

**Control Transfection Plasmids**

The pGIR308 and pGIR209 plasmids were obtained from the laboratory of Dr. Lesly Temesvari at Clemson University. These plasmids are used as part of a tetracycline inducible system often used to express or over express modified proteins in *E. histolytica*. The plasmid were obtained and transformed into NovaBlue (Novagen, Inc.) *E. coli* cells. Plasmid was purified using a Plasmid Mega Prep Kit (Qiagen, Inc.).

**Transfection of E. histolytica**

Two separate methods of transfecting *E. histolytica* cells were utilized. For electroporation cells were cultivated as described above. Cells grown to confluence were washed twice using ice-cold phosphate buffered saline, and washed once with ice-cold Cytomix (120 mM potassium chloride, 0.15 mM calcium chloride, 10 mM potassium phosphate, 25 mM HEPES, 2 mM EGTA, 5 mM magnesium chloride, pH 7.6) [15]. The cells were resuspended to a concentration of $1 \times 10^6$ cells/800 µL in freshly generated Cytomix-AG (Cytomix containing 4 mM ATP and 10 mM glutathione). $1 \times 10^6$ cells were placed in a 4 mm electroporation cuvette along with 200 µL of sterile water for controls, or 60-80 µg of prepped plasmid RNAi constructs and pulsed twice at 1200 volts + 25 µFD using a Gene Pulser Xcell Total System, Model No. 165-2660 (BioRad, Inc.) Time constants of 0.3-0.6 msec were routinely observed. The cells were immediately returned to 50 mL sterile cell culture flasks containing pre-warmed media. The cells were
incubated at 37°C in a water-jacketed incubator. Hygromycin or G418 were added at concentration of 5-15 µg/mL and 3-6 µg/mL respectively where indicated after 24-72 hours.

For the alternative method of transformation, cells were cultivated as described above. Cells grown to confluence were washed twice with phosphate buffered saline and resuspended to a concentration of 1 x 10^6 cells/mL. 15 µL of Superfect® (Qiagen, Inc.) reagent was added to 20-40 µg of plasmid and incubated at ambient temperature for 5-10 minutes to allow transformation complexes to form. The Superfect®-plasmid complex added to either microcentrifuge tubes or 5 mL sterile glass vials along with 1 x 10^6 cells and incubated horizontally at 37°C for 3-4 hours. Following the incubation, cells were returned to 50 mL sterile flasks containing pre-warmed media and incubated at 37°C. Antibiotics were added at concentration described above after 24-72 hours.

**Generation of a hygromycin killing curve**

1 x 10^6 cells were inoculated into 50 mL sterile flasks containing pre-warmed media. Hygromycin was added at concentrations of 0, 2.5, 5, 10, 15, and 20 µg/mL in equivalent volumes and incubated at 37°C. The cells were counted by light microscopy using a hemocytometer every 24 hours over a period of 4 days. The data representing cells counted after 48 hours was transformed to percent cells surviving and a smooth, second order regression curve was generated using Kaleidagraph (Synergy Software). Manual interpolation was used to determine the LD_{50}.

**Chemicals**
Chemicals were obtained from Sigma-Aldrich, ThermoFisher Scientific, or VWR Scientific Products.
Table 5.1. Reverse primer sequences to create shRNAi constructs.

<table>
<thead>
<tr>
<th>EhACK sequence</th>
<th>Round 1 of PCR</th>
<th>Round 2 of PCR</th>
</tr>
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<td>TCGATCGCGGGCGCAAAAAAACCAG</td>
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</tr>
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<tr>
<td></td>
<td>TTTATTTTTCTTTTTTATCC</td>
<td>ATCTCTGGAA</td>
</tr>
</tbody>
</table>
A

1st PCR: Template is Genomic DNA

HindIII
5' U6 forward

5' U6 forward

HindIII

U6 Promoter

Apal

3' (Sense) Reverse 1

2nd PCR: Template is 1st PCR product

HindIII

5' U6 forward

HindIII

U6 Promoter

Sense

Loop

Apal

3' (Antisense) Reverse 2

Final PCR Product

HindIII

Apal

U6 Promoter

Sense

Loop

Antisense

TTTTTT

B

BamHI
XbaI
EcoRI
HindIII
BamHI
EcoRI

3' fdx

5' fdx

5' hgl

hyg

(Sall/Xhol)

pGIR310

pGEM-luc

HindIII

XbaI

EcoRI

NcoI

SaiI

Modified pGIR310

HindIII

BamHI

EcoRI

3' hgl

hyg

(Sall/Xhol)

C

Sense strand (29 nt)

Antisense strand (29 nt)
Figure 5.2. shRNA system for *Entamoeba histolytica*. (A) Diagram of the two-step PCR process for generating short hairpins shRNA constructs. Genomic DNA (or subsequently, the cloned U6 promoter) was used as a template to amplify the *E. histolytica* U6 promoter and to add the hairpins. The primers in the first PCR round were the forward primer, containing a *HindIII* site and 5' end of the U6 promoter, and a first reverse primer, containing the U6 promoter 3' end, the shRNA sense strand sequence, and the 9-nucleotide loop. To yield the final product, in the second PCR round, the same forward primer was used, with a second reverse primer containing the loop sequence, the antisense strand sequence, the termination sequence, and a *NotI* recognition site, using the first round product as a template. The primers used to generate the PCR products are listed in Table 5.1. (B) PCR products were cloned into the *HindIII* and *NotI* sites. pGIR310 confers hygromycin resistance in amoebae and ampicillin resistance in *E. coli* bacteria. (C) Expected structure of 29-basepair shRNA before processing by Dicer. The 29-basepair stem and 9-nucleotide loop are shown. Taken from [9].
III. RESULTS

*Acetate kinase operates preferentially in the direction of acetate synthesis*

Native extracts of *E. histolytica* cultured cells only demonstrated PP\(_i\)-forming ACK activity in the acetate-forming (0.594 ± 0.005 µmol min\(^{-1}\) mg\(^{-1}\)) direction. The absence of observable activity in the acetyl phosphate-forming direction is likely due to the low concentrations of enzyme isolated from the cells, a problem encountered upon initial discovery of the enzyme more than 35 years ago [7]. This finding supports previous work [8] on the recombinant *E. histolytica* ACK that the enzyme functions physiologically in the direction of acetate/PP\(_i\) formation. We were unable to detect an ATP-dependent ACK activity when extracts were prepared axenically. An ATP-dependent ACK activity was reported nearly 50 years prior, however cellular extracts prepared were from xenically cultured *E. histolytica* cells [16].

*PTA and XFP activities are absent in *E. histolytica* extracts.*

Other eukaryotes that have ACK also have an ORF encoding one of two bacterial partner enzymes. In the green algae *Chlamydomonas* and the oomycete *Phytophthora*, the presence of a gene encoding PTA provides a pathway for the interconversion of acetate to acetyl-CoA. A gene encoding XFP has been identified in all fungi that have an 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. The absence of ORFs encoding PTA or XFP in the most recent *E. histolytica* genome assembly indicates the possibility that either an XFP or PTA is in a region of the genome that has not been assembled or a new class of XFP or PTA structurally different from the
known bacterial enzymes is present. To rule out the presence of a 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 using fructose-6 phosphate as the substrate.

**Problems with the RNA interference protocol in *E. histolytica***

Initial antibiotic selection for pGIR310 was performed using 15 μg/mL of hygromycin. After unsuccessful transfection attempts using this antibiotic, a hygromycin killing curve was generated (Figure 5.3). Manual interpolation of the curve indicated an LD$_{50}$ of approximately 1 μg/mL. Using 10X the LD$_{50}$, a new concentration of 10 μg/mL hygromycin was utilized for future experiments. Where half the antibiotic dose was to be used, 5 μg/mL of hygromycin was used. This antibiotic selection was also utilized in the last transformation for cells transfected with pGIR308. Utilizing half of the antibiotic dose did not improve the experimental outcomes.
Figure 5.3. Hygromycin killing curve generated using *E. histolytica* cultures. A LD$_{50}$ of approximately 1 µg/mL was extrapolated manually.
We were unable to produce RNA interference with short hairpin RNAs using two separate protocols due to problems with transfection. The first protocol involves the use of electroporation, thereby damaging the cell membranes and encouraging the uptake of plasmids. The method was attempted three times with similar results. On each occasion, with one exception, cells that were electroporated were returned to media and began to divide normally until the addition of antibiotics. During one trial all cells that were electroporated failed to survive the 24 hour recovery period. Cells that survived electroporation were allowed to recover for 24, 48, or 72 hours. For cells transfected with pGIR310 or pGIR308 plasmids (60 µg, 80 µg, or 100 µg), 10µg/mL or 15 µg/mL of hygromycin was added to the cells. In each case after 24 hours all cells containing 15 µg/mL of hygromycin did not survive. When 10 µg/mL of hygromycin was employed the transfected cells survived 24-48 hours. Cells transfected with pGIR209 were selected for using 3 µg/mL or 6 µg/mL of G418. Cells selected with 6 µg/mL of G418 did not survive more than 24 hours. Cells selected with 3 µg/mL of G418 survived only 48-72 hours.

A second protocol involving the use of the commercial transfection reagent Superfect® was employed after the electroporation attempts. Superfect® reagent allows for the formation of vesicle transformation complexes which have been used previously [9, 10] to successfully transfect *E. histolytica* cells. In this procedure, cells are incubated with pre-formed transformation complexes and allowed to naturally endocytose the plasmid. This process is thought to be advantageous over the electroporation procedure in that recipient cell competency is not lost due to directed damage to the cell membranes. Researchers utilizing this protocol for *E. histolytica* transfection have reported a higher
success rate than transfection by electroporation (personal communication, Dr. Upinder Singh and Dr. Richard Pearson, Stanford University).

The Superfect® transformation protocol was attempted on four separate occasions without success. In each case, 20 µg of plasmid DNA was transfected with a contact incubation time of three or four hours. Antibiotics employed for selection were added after 48 or 72 hours at concentrations of 10 µg/mL or 5 µg/mL of hygromycin for pGIR310 series or pGIR308 plasmids and 6 µg/mL or 3 µg/mL of G418 for cells transfected with the pGIR209 plasmid. Concentrations of 5 µg/mL of hygromycin and 3µg/mL G418 or half the antibiotic dosing concentrations were utilized in the last transformation attempt to encourage cell growth during selection. Cells receiving full doses of hygromycin (10 µg/mL) or G418 (6 µg/mL) did not survive more than 48 hours.

During the last transformation where half concentrations of antibiotics were utilized (5 µg/mL hygromycin and 3 µg/mL G418) the cells transformed with plasmids survived up to 96 hours, but were inconsistent with the constructs transformed. The cells that survived transformation were very small in quantity (25-50 living, motile cells) after 48 hours. While some of these cells continued to survive, the cells doubled only once in another 48 hours resulting in very small cells numbers that did not survive more than 96 hours. One exception was the flask containing cells that were incubated with Superfect® and no plasmid, but did contain 5 µg/mL of hygromycin. Despite the addition of antibiotics this culture continued to divide and increase the rate of doubling after 72 hours. By 96 hours 10 colonies containing more than 200 cells were observed.
IV. DISCUSSION

The PP$_i$-dependent ACK found in *E. histolytica* offers a unique opportunity to investigate the physiological roles of this novel enzyme. All of the other known ACKs found in all other eukaryal, bacterial, and archaeal organisms utilize ATP as a phosphoryl donor [4, 17, 18]. Phylogenies constructed using eukaryotic ACK sequences also support the novelty of this enzyme, as *Entamoeba* ACKs form a clade separate from the other eukaryotic ACKs [4].

As previously mentioned, *E. histolytica* lacks ORFs [8] for PTA and XFP, the partners for ACK found in other eukaryotes that have an ACK. Furthermore, *E. histolytica* lacks the genes for other potential partner enzymes which are found in other organisms (Figure 5.4). However, the absence of these enzymes does not preclude the possibility that an evolutionarily distinct PTA or XFP is present in the parasite. We were unable to detect PTA and XFP activities in *E. histolytica* cellular extracts. The absence of bacterial ACK partner enzymes in *Entamoeba* suggests that a novel acetyl phosphate generating enzyme is the partner for the PP$_i$-ACK in this protist. While the identification of this partner enzyme remains unknown, *E. histolytica* possesses a complete pentose phosphate pathway, but lacks a mitochondrion, and therefore TCA cycle. A novel partner enzyme could generate acetyl phosphate from an intermediate of the pentose phosphate pathway or the end product of a yet to be identified catabolic pathway.
Figure 5.4. Network of pathways involved in the metabolism of acetyl-CoA. Pathways in green have been identified in *Entamoeba histolytica*, while pathways in gray have been observed in species other than *E. histolytica*. Acetate kinase (ACK) functions in the interconversion of acetate and acetyl phosphate. *E. histolytica* specifically utilizes a PP_i-dependent ACK, which differs from the ATP-dependent ACK found in prokaryotes and archaea. To date, there are no known partner enzymes for PP_i-dependent ACK in *E. histolytica*. 
Preferentially operating in the direction of acetate formation, the *E. histolytica* ACK could provide PP$_i$ which can be utilized by phosphofructokinase (PPi-PFK) and PP$_i$-dependent phosphoenolpyruvate dikinase (PPDK) in glycolysis during substrate-level phosphorylation; the primary source of energy for the parasite (Figure 5.5). Further evidence supporting this physiological role includes previous observations of acetate fermentation by the parasite [19]. Microarray data also indicates the presence of acetate kinase transcripts in both cyst and trophozoite life stages of the parasite [20]. However, an unknown enzyme partner must exist in order to synthesize acetyl phosphate from an intermediate in pentose phosphate pathway or another metabolic process (Figure 5.6).
Figure 5.5. Proposed metabolic pathway(s) for acetate synthesis and utilization in *E. histolytica*. An extended glycolytic pathway is present in *E. histolytica* in which pyruvate is converted to acetyl-CoA by the PFOR. Acetyl-CoA can then be converted to acetate by and ADP-ACS or by a ACK/PTA pathway. HK, hexokinase; PPi-dependent phosphofructokinase, PPi-PFK; 3-PGK, 3-phosphoglycerate kinase; PPDK, phosphoenolpyruvate dikinase; HYD, hydrogenase; PFOR, pyruvate ferrodoxin oxidoreductase; ADP-ACS, ADP-forming acetyl-CoA synthetase; phosphotransacetylase, PTA; acetate kinase, ACK.
Figure 5.6. Proposed metabolic pathway(s) for PP$_i$ synthesis and utilization in *E. histolytica*. An extended glycolytic pathway is present in *E. histolytica* in which glucose is converted to acetyl-CoA utilizing PP$_i$-dependent enzymes. Acetyl-CoA can then be converted to acetate by an ADP-ACS. Glucose 6-phosphate can enter the pentose phosphate pathway in order to generate acetyl phosphate from an unknown intermediate and unknown enzyme partner. Other unknown enzyme partners could be utilized to generate acetyl phosphate from unknown sources. PP$_i$-dependent phosphofructokinase, PP$_i$-PFK; 3-PGK, 3-phosphoglycerate kinase; PPDK, phosphoenolpyruvate dikinase; ADP-ACS, ADP-forming acetyl-CoA synthetase; acetate kinase; PP$_i$-ACK.
Our attempts at transfections to perform RNA interference in *E. histolytica* were unsuccessful. In all experimental trials, cells transformed with plasmids containing the scrambled sequences did not survive any longer than cells transfected with plasmids capable of coding for shRNAs. Should ACK be essential for the survival of *E. histolytica*, we would expect that the parasites would not survive selection. In this case, an alternative inducible RNA interference system could be constructed. A tetracycline inducible system has already been employed in *E. histolytica* [21, 22] and could be modified to serve this purpose.

Alternatively to an essential role in parasite metabolism, ACK may play an important role in other cellular processes whose disruption could impede growth and replication. Quantitative reverse transcriptase PCR (qRT-PCR) can be utilized to examine RNA levels of the scrambled control against the experimental constructs to determine ACK gene expression is actually being knocked down by RNA interference. ACK assays can also be employed to detect enzyme activity.

Treatment for infection by *E. histolytica* involves the administration of two drugs. Metronidazole (Flagyl), a nitromidazole which interacts with protozoal ferrodoxin (PFOR reaction in Figure 5.3) to produce toxic byproducts is used to treat invasive trophozoites, and paromomycin (Humatin) an aminoglycoside, is used as a luminal amoebicide [23]. *In vitro* amoebic resistance to metronidazole and *in vivo* cytotoxic and nephrotoxic effects from paromomycin indicates a need for the identification of novel drug targets in this parasite. Should the PPi-dependent ACK be essential to the organisms survival, further
research regarding this novel enzyme as a possible drug target for *E. histolytica* infection should be pursued.
V. ACKNOWLEDGEMENTS

We would like to thank Dr. William Petri and Alicia Linford at the University of Virginia for providing the RNAi construct plasmids (pGIR310 series). We would also like to thank Dr. Lesly Temesvari for providing the pGIR308 and pGIR209 plasmids.
VI. REFERENCES


CHAPTER VI
CONCLUDING REMARKS AND FUTURE PROSPECTS

*Entamoeba histolytica* possesses a novel PP\textsubscript{i}-dependent acetate kinase (ACK).

This is the first PP\textsubscript{i}-dependent ACK to be biochemically and kinetically characterized and is the only known member of the ASKHA superfamily that can utilize PP\textsubscript{i}. The enzyme operates preferentially in the direction of acetate/PP\textsubscript{i} synthesis and is capable of utilizing acyl substrates as long as hexanoate in the direction of acetyl phosphate synthesis. The kinetic parameters for substrate affinity indicate that the enzyme functions as both an acetate and propionate kinase in the acetyl phosphate-forming direction; however, the low $k_{\text{cat}}$ raises question whether this direction of the reaction is physiological. Studies of the mechanism of this enzyme indicates that the PP\textsubscript{i}-ACK follows a sequential mechanism, supporting a direct in-line phosphoryl transfer mechanism previously reported in the most well characterized ATP-dependent ACK found in *Methanosarcina thermophila*.

A modified hydroxamate assay was developed for measuring the synthesis of acetyl phosphate to overcome problems associated with using multiple coupled enzymes to detect ACK activity. The assay can be used to detect ACK activity of both ATP-dependent and PP\textsubscript{i}-dependent acetate kinases in the direction of acetyl phosphate synthesis. Modifications of this assay may be used for other enzymes that utilize acetyl phosphate or acetyl-CoA as a substrate, such as ACS, ADP-ACS, or PTA.

Although a number of conserved catalytically important residues are present in the *E. histolytica* PP\textsubscript{i}-ACK, this investigation revealed a number of significant active site
changes in comparison to the well characterized *M. thermophila* ATP-dependent ACK. The residues His\textsuperscript{117}, His\textsuperscript{172}, Val\textsuperscript{87}, Thr\textsuperscript{201}, Thr\textsuperscript{15}, Arg\textsuperscript{274}, and Asp\textsuperscript{272} appear to be essential for catalysis in the direction of acetate synthesis. Furthermore, His\textsuperscript{117} appears to be critically important for binding acetyl phosphate and therefore important in acetate/PP\textsubscript{i} synthesis reactions. While Thr\textsuperscript{15} of the *E. histolytica* ACK appears to lie outside of the active site, the residue was implicated in catalysis. For this process to occur, the residue would need to be brought into the active site for substrate interaction. Therefore a logical proposition is that the *E. histolytica* ACK active site undergoes a claw-like closure; a process also observed in the *M. thermophila* ACK. Closure of the active site would also bring Thr\textsuperscript{15} into close proximity with Arg\textsuperscript{274} which is also implicated in catalysis. The active site of the *E. histolytica* ACK also lacks the adenosine pocket present in the ATP-dependent, *M. thermophila* ACK.

Based on the results presented in this dissertation, the data supports the hypothesis that the *E. histolytica* ACK operates physiologically in the direction of PP\textsubscript{i} synthesis. PP\textsubscript{i} generated from this reaction may be utilized by the glycolytic enzymes PP\textsubscript{i}-dependent phosphofructokinase (PP\textsubscript{i}-PFK) and PP\textsubscript{i}-dependent phosphoenolpyruvate dikinase (PPDK) in substrate-level phosphorylation for energy generation by the parasite.

RNA interference of *E. histolytica* ACK was unable to be carried out due to problems with transfection of plasmid DNA into the parasite. However, data garnered from cellular extracts indicates the presence of the PP\textsubscript{i}-dependent ACK, and absence of an ATP-dependent ACK activity. Utilization of PP\textsubscript{i} by PP\textsubscript{i}-dependent glycolytic enzymes produced by the parasite’s ACK suggests an important role for this enzyme in
Entamoeba’s metabolism. Prokaryotic ACK partner enzyme activities of phosphotransacetylase (PTA) and xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (XFP) were absent in parasite cellular extracts. The absence of bacterial ACK partner enzymes in Entamoeba suggests that a novel acetyl phosphate generating enzyme is the partner for the PP\textsubscript{i}-ACK in this protist. A novel partner enzyme could generate acetyl phosphate from an intermediate of the pentose phosphate pathway or the end product of a yet to be identified catabolic pathway. Identification and characterization of the Entamoeba PP\textsubscript{i}-ACK partner enzyme will provide unique insights into the metabolism of this important protist.

This study has provided significant knowledge on a novel PP\textsubscript{i}-dependent ACK, and helps gain a better understanding of ACKs and the ASKHA enzyme superfamily. Future experiments investigating additional active site residues will assist in the understanding of the overall mechanism of this novel ACK. Continued research on the ack RNA knockdown may lead to a better understanding of the essentiality of this enzyme in E. histolytica physiology, and the possibility of identifying new drug targets for the treatment of amoebiasis.