Investigating the Structural, Functional, and Biochemical Properties of PP$_i$-dependent PEPCK Paralogs from *Entamoeba histolytica*

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**Abstract**

Phosphoenolpyruvate carboxykinase (PEPCK) is an important metabolic enzyme which functions to interconvert oxaloacetic acid (OAA) and phosphoenolpyruvate (PEP) in the Krebs cycle, a key process of generating cellular energy. There exist three known classes of PEPCK - two of which are nucleotide-dependent, using ATP and GTP. Very little is known about the third, PP$_i$-dependent PEPCK. Comparing classes, nucleotide-dependent PEPCKs are both functionally and structurally similar (~60-70 kDa) whereas PP$_i$-dependent PEPCK bears significant functional and structural differences (~130 kDa). This presented work investigates PP$_i$-dependent PEPCK from a human parasite *Entamoeba histolytica* (EhPEPCK). It is unique from previous work done on another homolog from *Propionibacterium freudenreichii* (PfPEPCK) in that there are three paralogs instead of one. This suggests increased complexity in function and regulation. This work has determined that the interaction between EhPEPCK paralogs gives rise to dimers and heterotrimers, and certain interactions show substrate induced inhibition. Kinetic measurements were completed to determine the metal cofactor of EhPEPCKs, and to determine the kinetic consequences of the aforementioned oligomeric states. The experiments support the conclusion that aggregation causes substrate inhibition, and that dimers are more active than trimers.

**Introduction**

Phosphoenolpyruvate carboxykinase (PEPCK) is an important metabolic enzyme suggested to be the master regulator of TCA cycle flux (Yang et al., 2009). It operates by removing citric acid cycle anions to be used in other metabolic processes, namely glucoseogenesis, glyceroneogenesis, 1-carbon serine synthesis, or works to replenish the TCA cycle (Yang et al., 2009). PEPCK has implications in glucose-stimulated insulin secretion (a process in diabetes), senescence, tuberculosis, and in cancer and has been thought of as a potential therapeutic target (Jeon et al., 2015; Mendez-Lucas et al., 2014; Montal et al., 2015; Park et al., 2014; Marrero et al., 2010; Yuan et al., 2016; Santra et al., 2016; Yang et al., 2009b). The PEPCK family has classically been divided into two nucleotide-dependent classes: those which utilized ATP, and those that utilized GTP as a phosphoryl donor. *In vivo*, PEPCK is thought to primarily catalyze the reaction in which oxaloacetic acid (OAA) is converted into phosphoenolpyruvate (PEP) and carbon dioxide using its nucleotide substrate (McLeod and Holyoak, 2021). However *in vitro*, PEPCK can complete the reverse reaction as well, making them bidirectional. More recently, a third PEPCK which utilizes pyrophosphate (PP$_i$) was discovered, further expanding the family. The two nucleotide-dependent PEPCKs are well characterized in both their structure and function. PEPCK requires two cation metal cofactors for activity, named M1 and M2 (Willard et al., 1969). M1 is an active site cofactor which binds to the enzyme and bridges the substrate and nucleotide binding pockets. M2 binds as a nucleotide-metal complex (McLeod and Holyoak, 2021). In the nucleotide-dependent PEPCKs, the chemical reaction is most activated when the M1 metal is Mn$^{2+}$ and
the M2 metal is Mg$^{2+}$ (Das et al., 2012; Machova et al., 2015; Hebda and Nowak, 1982; Hidalgo et al., 2016; Sokaribo et al., 2020; Escos et al., 2016; Wilkes et al., 1982). The active site of the enzyme contains three important loops (Fig. 1). The P-loop binds the nucleotide and positions it correctly for phosphoryl transfer (Matte et al., 1996). The R-loop binds the substrates (OAA/PEP) and once bound, moves to allow closure of the Ω-loop (Holyoak et al., 2006). Finally, the Ω-loop acts as a gate/lid and protectively encloses the active site while the reaction takes place (Johnson and Holyoak, 2010).

Initial studies on the PP$_i$-dependent class of PEPCK were completed in the 1960s and 1970s (Siu et al., 1961; Lochmuller et al., 1966; Willard and Rose, 1973; O’Brien et al., 1973; Wood et al., 1969; Haberland et al., 1972) In these studies, PP$_i$-dependent PEPCK from Propionibacteriumfreudenreichii (PfPEPCK) was evaluated. These studies were then re-evaluated to determine the differences between the nucleotide and pyrophosphate using classes (McLeod and Holyoak, 2021). First, PP$_i$-dependent PEPCKs are approximately twice the size (~130kDa) of nucleotide-dependent PEPCKs (~70kDa), with a mostly conserved (in relation to nucleotide-dependent PEPCKs) core structure including the active site residues/loops (Fig.1). However, this extra mass has manifested as additional “lobes” around this conserved core. Second, PfPEPCKs preferentially use Fe$^{2+}$ as the M1 cofactor instead of Mn$^{2+}$. Third, although ATP- and GTP-dependent PEPCKs preferentially catalyzed the OAA→PEP reaction, PP$_i$-dependent PEPCK favors the PEP→OAA reaction (Lochmuller et al., 1966). Fourth, PP$_i$-dependent PEPCK exhibits substrate inhibition where high concentrations of PEP lead to inactivation, whereas ATP- and GTP-dependent PEPCKs do not. Finally, in this substrate-induced inactivated state, the enzyme oligomerizes from monomers to homodimers. In addition to substrate (PEP or OAA), malate was shown to bind to an allosteric site which presumably causes this dimerization. In contrast, nucleotide-dependent PEPCKs are typically monomeric and have not been observed to have any regulation by ligand-induced quaternary structure changes (Fukuda et al., 2004).

The PP$_i$-dependent class has been relatively understudied despite PfPEPCK having unique characteristics when compared to nucleotide-dependent PEPCKs. A new isozyme of PP$_i$-dependent PEPCK from Entamoeba histolytica (EhPEPCK) was previously studied (McLeod and Holyoak, 2021). Entamoeba histolytica is a human parasite and studying it may provide a therapeutic avenue to target this organism (Chou and Austin, 2022). Deeper insight into the structure and function may help to discover how to selectively target this enzyme. In the Chiba et al. study, a unique trimer was observed (McLeod and Holyoak, 2021). However, there was no understanding as to what oligomeric states are possible, how these oligomeric states arise (ligand-induce or paralog interactions), or the paralog/oligomer activities. Here, I structurally and functionally characterize these three isoforms in isolation and in combination to understand these structure-function relationships.

Structural characterization was completed using small angle X-ray scattering (SAXS). SAXS uses X-ray scattering to determine low-resolution structural details on macromolecules. Specifically, structural information that can be obtained are: the radius of gyration ($R_g$) (mean distance from the center of mass or central axis to the outer edges), estimated molecular
weight, the \( D_{\text{max}} \) (is the maximum dimension of the molecule), and low-resolution shape information (ie. long rod vs sphere). Further analysis of structural information yields scattering curves and Kratky plots. Kratky plots qualitatively determine the flexibility and degree of unfolding in the sample. Dimensionless Kratky plots normalize scattering profiles by mass and concentration (Hopkins et al., 2017). An unfolded protein displays a plateau with a high q, while globular proteins display a bell shaped curve, and a combination of the two may show characteristics of both. As the oligomerization of \( E\hbox{h} \)PEPCK will lead to large changes in size, SAXS accurately assessed the oligomerization states of the paralogs. SAXS data was collected both with and without substrates and known effectors of \( P\hbox{j} \)PEPCK to determine how the addition of substrates affects oligomerization state. In addition to structural characterization, kinetic experiments were done to map functional consequences to given structures. Prior to full kinetic measurements, the optimal metal cofactor (M1) was determined as this was shown to be variable between classes. The activity of each paralog in isolation, and in combination was measured (work ongoing). These experiments have revealed a general mechanism of substrate regulation of \( E\hbox{h} \)PEPCKs which can then be compared to \( P\hbox{j} \)PEPCK to determine similarities and differences.

Materials and Methods

\( \text{PP}_i, \text{PEP}, \) and \( \text{NADH} \) were purchased from ChemImpex. \( \text{BME} \) was purchased from Sigma Aldrich. \( \text{MDH} \) was purchased from Calzyme Laboratories. \( \text{HEPES} \) was obtained from Gold Biotechnology. Sodium Bicarbonate was purchased from Mallinckrodt Pharmaceuticals. The enzyme was purified previously at Cornell University after recombinant expression. All other chemicals were purchased from the highest grade available.

Small Angle X-ray Scattering of \( E\hbox{h} \)PEPCK paralogs (SAXS)

SAXS was used to determine the oligomeric states of \( E\hbox{h} \)PEPCKs in isolation, in combination and with different ligands. Screening for optimal conditions was performed using a BioXolver (Xenocs) home-source instrument at Cornell’s Laboratory of Atomic and Solid-State Physics. The data was recollected at Sector 7A1 (BioSAXS) at the Cornell High Energy Synchrotron Source (CHESS) to extend signal/noise and resolution limits (Table 1). Final samples were in 50 mM TRIS pH 8.0 and 2 mM TCEP at a final volume of 20 uL and the total concentration of \( E\hbox{h} \)PEPCK was 1.25 mg/mL.

<table>
<thead>
<tr>
<th>Wavelength (Å)</th>
<th>1.103</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector distance (cm)</td>
<td>1735</td>
</tr>
<tr>
<td>Exposure number/sample</td>
<td>10</td>
</tr>
<tr>
<td>Exposure length (s)</td>
<td>1</td>
</tr>
<tr>
<td>Q range (Å⁻¹)</td>
<td>0.000123 - 0.577</td>
</tr>
</tbody>
</table>

Table 1. CHESS SAXS collection parameter details for \( E\hbox{h} \)PEPCK structural studies.

**Paralog activity measurements using Michaelis-Menten Kinetics**

\[
P\hbox{EP} + \text{P}_i + \text{CO}_2 \rightarrow \text{OAA} + \text{PP}_i
\]

The kinetic constants were determined for the paralog \( \text{Eh1} \), and the combinations \( \text{Eh1}+2 \) and \( \text{Eh1}+3 \) of \( \text{Entamoeba histolytica} \), with experimentation in progress for every paralog and combination possible for the three paralogs. The assays were done in duplicate at room temperature at a final volume of 1 mL. A Varian 50 Bio UV-Visible Spectrophotometer was used to monitor enzyme activity. The conversion of PEP to OAA in each assay was monitored at 340 nm via a coupled reaction using malate dehydrogenases by observing the change of NADH to \( \text{NAD}^+ \). An R script from McLeod and Holyoak (2021) was used to determine Michaelis-Menten parameters by fitting data to the substrate inhibition model (Eq. 1)

\[
V_{\text{max}} = \frac{\text{subrate concentration}}{(K_{\text{m}} + \text{substrate concentration}) \cdot (1 + (\text{substrate concentration} / K_{\text{i}}))}
\]

**Equation 1**

The standard assay mix for PEP carboxylation was composed of 100 mM HEPES pH 7.5, 20 mM BME, 300 μM NADH, 10 mM KH\(_2\)PO\(_4\).
5 mM MgCl$_2$, 250 μM MnCl$_2$ (or other M1 metal substitute), 40 mM KHCO$_3$ (bubbled with dry ice), PEP varying from 2.5 uM to 10,000 uM, 5U of malate dehydrogenase (MDH) and 5 μg of PP$_i$-dependent PEPCK (Eh1, Eh2, or Eh3). Reactions were initiated with the addition of PP$_i$-dependent PEPCK. To determine what metal is used most effectively as a cofactor by EhPEPCK, kinetic assays with Eh1 were performed with each one using 250 µM of the following metals: iron (FeCl$_3$), manganese (MnCl$_2$), magnesium (MgCl$_2$), calcium (CaCl$_2$), zinc (ZnCl$_2$), copper (CuCl$_2$), and cobalt (CoCl$_2$). As zinc and copper did significantly react in the initial round of testing, they both were not included in final testing, and therefore are not included in the discussion.

### Results and Discussion

#### Paralog oligomerization determined by SAXS

Small-angle x-ray scattering (SAXS) uses X-rays to determine low resolution structural details on macromolecules. SAXS was used here to determine the quaternary state of the paralogs in isolation (Eh1, Eh2, and Eh3) and in combination (Eh1+2, Eh1+3, Eh2+3, Eh1+2+3), both with and without substrate (both OAA and PEP) as well as a known allosteric effector of PfPEPCK (malate) (Table 2). It was observed that in isolation, all three paralogs have approximately the same D$_{\text{max}}$ values of 140, 139, and 142 Å (respectively) and R$_g$ values of 47.1, 44.2, and 48.4 Å (Table 2). They also have the near identical scattering curves, Kratky plots, and P(r) functions (Fig.2). Eh2+3 is approximately the same as the paralogs in isolation, with an R$_g$ of 45.6 Å and D$_{\text{max}}$ of 139 Å. The dimer structure from crystallized PfPEPCK was found to have an R$_g$ of 46.4 Å and D$_{\text{max}}$ of 139 Å (Manalastas-Cantos et al., 2021). Hence, Eh1, 2, 3, and Eh2+3’s R$_g$ and D$_{\text{max}}$ values are, without the addition of substrate or ligand, suggestive of a dimer complex. It is unknown if Eh2 and Eh3 combine to form a heterodimer, or if they are only present as homodimers.

<table>
<thead>
<tr>
<th>EhPEPCK</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1+2</th>
<th>1+3</th>
<th>2+3</th>
<th>1+2+3</th>
</tr>
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<tbody>
<tr>
<td>D$_{\text{max}}$ (Å)</td>
<td>140</td>
<td>139</td>
<td>142</td>
<td>192</td>
<td>192</td>
<td>139</td>
<td>195</td>
</tr>
<tr>
<td>R$_g$ (Å)</td>
<td>47.1 ± 0.277</td>
<td>44.2 ± 0.379</td>
<td>48.4 ± 0.358</td>
<td>66.1 ± 0.618</td>
<td>62.4 ± 0.568</td>
<td>45.6 ± 0.381</td>
<td>61.4 ± 0.187</td>
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<table>
<thead>
<tr>
<th>10 mM PEP</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1+2</th>
<th>1+3</th>
<th>2+3</th>
<th>1+2+3</th>
</tr>
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<tbody>
<tr>
<td>D$_{\text{max}}$ (Å)</td>
<td>Agg.*</td>
<td>160</td>
<td>165</td>
<td>Agg.*</td>
<td>Agg.*</td>
<td>150</td>
<td>480</td>
</tr>
<tr>
<td>R$_g$ (Å)</td>
<td>53.5 ± 0.511</td>
<td>53 ± 0.673</td>
<td>Agg.*</td>
<td>Agg.*</td>
<td>56.2 ± 0.607</td>
<td>120.6 ± 1.18</td>
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<table>
<thead>
<tr>
<th>10 mM OAA</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1+2</th>
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<tbody>
<tr>
<td>D$_{\text{max}}$ (Å)</td>
<td>Agg.*</td>
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<td>155</td>
<td>Agg.*</td>
<td>Agg.*</td>
<td>150</td>
<td>235</td>
</tr>
<tr>
<td>R$_g$ (Å)</td>
<td>57.3 ± 0.694</td>
<td>51.7 ± 0.378</td>
<td>Agg.*</td>
<td>Agg.*</td>
<td>49.1 ± 0.374</td>
<td>72.2 ± 0.257</td>
<td></td>
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</table>

<table>
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<tr>
<th>40 mM Malate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1+2</th>
<th>1+3</th>
<th>2+3</th>
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<tr>
<td>D$_{\text{max}}$ (Å)</td>
<td>139</td>
<td>144</td>
<td>145</td>
<td>255</td>
<td>250</td>
<td>145</td>
<td>250</td>
</tr>
<tr>
<td>R$_g$ (Å)</td>
<td>53.3 ± 0.503</td>
<td>46.5 ± 0.357</td>
<td>48.2 ± 0.373</td>
<td>65.6 ± 0.882</td>
<td>68.6 ± 1.65</td>
<td>45.7 ± 0.304</td>
<td>72.1 ± 0.274</td>
</tr>
</tbody>
</table>

*Aggregation
To determine the molecular origins of the previously reported trimer (McLeod and Holyoak, 2021), each combination was tested. In combination \( Eh_{1+2}, 1+3, \) and \( 1+2+3 \) have approximately the same \( D_{\text{max}} \) (192, 192, and 195 Å) and \( R_g \) (66.1, 62.4, and 61.4 Å) values, which are higher than the aforementioned paralogs in isolation (or \( Eh_{2+3} \)) (Table 2). This suggests that this complex is a heterotrimer as opposed to \( Eh_1 \) forming a homotrimer with itself as \( Eh_2 \) or 3 is also required to form this structure. Comparing \( Eh_1 \) (dimer) with \( Eh_{1+2} \) (trimer), there is a clear change in the structure as observed by the scattering curve (Fig. 3) which is the same structure as \( Eh_{1+2+3} \) (Fig. 4). At a sequence level, both \( Eh_2 \) and \( Eh_3 \) share 98% sequence similarity with one another, whereas they only share 90% sequence similarity with \( Eh_1 \). It is therefore not surprising that \( Eh_1 \), being most unique of the three, is the required component for the heterotrimer species.

**Figure 2.** \( Eh_1, Eh_2, \) and \( Eh_3 \) in isolation form dimeric structure. SAXS scattering profiles indicate that each paralog in isolation is the same dimeric structure (when compared to theoretical \( R_g/D_{\text{max}} \) of known dimeric structure). A) Scattering Curve of \( Eh_1 \) (blue), \( Eh_2 \) (orange) and \( Eh_3 \) (green). B) Normalized Kratky Plot. C) Normalized \( P(r) \).

**Figure 3.** \( Eh_1 \) in isolation forms a dimeric structure, while \( Eh_{1+2} \) forms a trimeric structure. \( Eh_1 \) SAXS curve shows the characteristic dimeric scattering curve whereas \( Eh_{1+2} \) shows different scatter of the proposed trimeric state. A) SAXS scattering curve of \( Eh_1 \) in isolation (blue) and with \( Eh_{1+2} \) (orange). B) Normalized Kratky plot. C) Normalized \( P(r) \).
Effector molecules induced changes in structure
When substrates or effector molecules bind to their partners, they can often induce structural changes. *PfPEPCK* for instance, formed a homodimer upon binding of PEP, OAA, and malate (unpublished). Similar experiments were completed here to determine the effect of substrates and malate on *EhPEPCK* structure. It was found that the effect of OAA and PEP were identical. First, when PEP is present with *Eh2, Eh3* or both *Eh2+3*, an increase in both $D_{max}$ (160, 165, and 150 Å) and $R_g$ (53.5, 53, and 56.2 Å) is observed (Table 2). This suggests that the addition of PEP causes a larger structure to form. However, it is unclear what this structure is. The aforementioned values are greater than the known dimer dimensions, but less than a trimer’s, so it could perhaps be an expanded dimer. Second, the addition of 10 mM PEP to *Eh1*, as well as *Eh1+2* and *Eh1+3* caused aggregation (the accumulation and/or clumping together of structures) such that the $R_g$ and $D_{max}$ values could not reliably be determined. This suggests that substrates specifically interact with *Eh1* causes it to destabilize and aggregate, whereas *Eh2* and *Eh3* are unaffected. Finally, with *Eh1+2+3* and 10 mM PEP (only) there is a substantial change compared to the unbound trimer (Fig. 5). This conformational change is evident by all diagnostic plots, and the combination with substrate almost doubles the $R_g$ from 61.4 to 120.6 Å and increases the $D_{max}$ considerably from 195 to 480 Å, which points to the probable formation of a much larger structure, whose specifics are unknown but may be a trimer-of-trimers.

The addition of 40 mM malate does not seem to have much effect on the enzyme. In *Eh1* on, *Eh2*, and *Eh3*, no change was seen in the $D_{max}$ values, which stayed at approximately 140 Å each, which is in contrast to the addition of OAA and PEP, which caused aggregation in *Eh1*. However, an increase in the $R_g$ value of *Eh1* was seen from 47.1 to 53.3 Å, while *Eh2* and *Eh3* saw little to no change. Malate caused a slight increase in size in *Eh1+2*, *Eh1+3*, and *Eh1+2+3*, as the $D_{max}$ values increased from 192, 192, and 195 Å respectively, to 255, 250, and 250 Å. The $R_g$ values for *Eh1+2*, *Eh1+3*, and *Eh1+2+3* stayed approximately the same, as they changed from 66.1, 62.4, and 61.4 Å to 65.6, 68.6, and 72.1 Å respectively.
The SAXS data has revealed the structures of these paralogs both in isolation, and in combination with one another and with the addition of substrate and ligand. However, the activity of these structures is unknown. In order to understand the functionality of each structure, kinetic activity experiments were performed. Before these experiments could be carried out, the optimal conditions for activity had to be determined. Therefore, different metals were tested to see which one functioned as the most activating M1 cofactor. The first experiment done testing metal-dependency to determine if Fe$^{2+}$ is the most activating cation for \( E_{h}PEPCK \) as it was in \( P_fPEPCK \). Various metals were tested: iron (Fe$^{2+}$), manganese (Mn$^{2+}$), magnesium (Mg$^{2+}$), calcium (Ca$^{2+}$), and cobalt (Co$^{2+}$). It was found after testing was complete that manganese was the best M1 Metal cofactor (Table 3).

### Activity of \( E_{h}PEPCK \) Paralogs

With the optimal metal determined, Michaelis-Menten plots while varying PEP were obtained. \( E_{h}1 \) was tested first, and there was an increase in activity at small concentrations of PEP, with the activity gradually decreasing as substrate concentration increases, showing classical substrate inhibition (Fig. 6). The SAXS analysis suggests that \( E_{h}1 \) is a dimer in isolation with no evidence of trimer formation but with the addition of PEP, \( E_{h}1 \) aggregates suggesting this may be the structural cause of the strong substrate inhibition. Next, \( E_{h}1+2 \) and \( E_{h}1+3 \) start as a trimer and have stronger substrate inhibition than \( E_{h}1 \). Like \( E_{h}1 \), aggregation was shown by SAXS and is also likely the origin of the substrate inhibition. Looking at the kinetic constants collected \( E_{h}1 \) and \( E_{h}1+2/1+3 \) have approximately the same Michaelis-Menten constant (\( K_M \)), however, the maximal specific activity of \( E_{h}1 \) is over three times greater than that of \( E_{h}1+2/1+3 \) (Table 4). While \( E_{h}1 \) undergoes substrate inhibition, the determined \( K_i \) values suggest that the inhibition is stronger for \( E_{h}1+2/1+3 \). Thus, \( E_{h}1 \) started as a dimer with higher activity than the trimer, and while both dimer and trimers experienced aggregation with PEP, the trimers appear to be more sensitive.

**Figure 5.** \( E_{h}1+2+3 \) forms a proposed trimeric structure, while \( E_{h}1+2+3 \) with 10 mM PEP forms an unknown larger structure. A) Scattering Curve \( E_{h}1+2+3 \) in isolation (blue) and with 10 mM PEP substrate (orange). B) Normalized Kratky plot. C) Normalized P(r).

**Metal Dependency**

The SAXS data has revealed the structures of these paralogs both in isolation, and in combination with one another and with the addition of substrate and ligand. However, the activity of these structures is unknown. In order to understand the functionality of each
Table 3. Metal dependency of \textit{Eh1} PEPCK.

<table>
<thead>
<tr>
<th></th>
<th>(\text{Fe}^{2+})</th>
<th>(\text{Mn}^{2+})</th>
<th>(\text{Mg}^{2+})</th>
<th>(\text{Co}^{2+})</th>
<th>(\text{Ca}^{2+})</th>
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</thead>
<tbody>
<tr>
<td>Average rate ((\mu\text{mol/min/mg}))</td>
<td>1.59 ± 0.10</td>
<td>2.82 ± 0.28</td>
<td>2.44 ± 0.25</td>
<td>0.050 ± 0.03</td>
<td>1.97 ± 0.25</td>
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</tbody>
</table>

Figure 6. Substrate Inhibition of paralog \textit{Eh1}. \textit{Eh1}'s activity was measured with PEP concentration of 2 – 10,000 mM PEP. It undergoes substrate inhibition - Its activity decreases as it undergoes oligomerization.

Table 4. \textit{EhPEPCK}s and Respective \(V_{\text{max}}\), \(K_{m}\), and \(K_{i}\) values.

<table>
<thead>
<tr>
<th>\textit{EhPEPCK}(s)</th>
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<th>1+2</th>
<th>1+3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}}) ((\mu\text{mol/min/mg}))</td>
<td>2.3 ± 0.11</td>
<td>0.9 ± 0.14</td>
<td>0.70 ± 0.12</td>
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<tr>
<td>(K_{m}) ((\mu\text{M}))</td>
<td>39.6 ± 4.8</td>
<td>26.9 ± 8.91</td>
<td>39.5 ± 13.3</td>
</tr>
<tr>
<td>(K_{i}) ((\mu\text{M}))</td>
<td>2960 ± 470</td>
<td>511 ± 166</td>
<td>476 ± 154</td>
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</table>

**Conclusion and Future Directions**

Structural analysis showed the oligomerization states of each paralog in isolation and in combination where \textit{Eh1}, \textit{Eh2} and \textit{Eh3} are dimers, while \textit{Eh1+2}, \textit{Eh1+3}, and \textit{Eh1+2+3} exist as heterotrimers. With the addition of substrate, aggregation in \textit{Eh1}, \textit{Eh1+2}, and \textit{Eh1+3} was observed but not seen in the dimeric complexes and with the trimeric \textit{Eh1+2+3}. Instead, \textit{Eh1+2+3} formed large structure with over twice the \(R_g\) value and 2.5x increase in \(D_{\text{max}}\) value. Malate did not affect \textit{Eh2}, \textit{Eh3}, or \textit{Eh2+3}. It caused a \(D_{\text{max}}\) value increase in \textit{Eh1} and an overall increase in \textit{Eh1+2}, \textit{Eh1+3}, and \textit{Eh1+2+3}, which suggests that it interacts especially with \textit{Eh1} and its combinations, causing a different, slightly larger trimer to form. These observations are different from what was observed previously in \textit{PfPEPCK}, which underwent dimerization from the addition of malate, OAA, and PEP, with PEP inducing the least change. SAXS is a useful method, but is low resolution, and prevents the observations of monomers as the high concentrations required force \textit{EhPEPCK} to dimeric (or trimeric) states. Therefore, the monomer-dimer/trimer transition may also be affected by substrates (or malate). In order to further understand conformational changes, it would be useful to repeat these experiments with crystallography, to observe the exact structures under different conditions.

Kinetically, it was discovered that \(\text{Mn}^{2+}\) was the most activating M1 cofactor, which is similar to the nucleotide-dependent classes, but
different from \( \text{PfPEPCK} \) (\( \text{Fe}^{2+} \)). Of the paralogs tested, \( \text{Eh1} \) had the highest activity out of \( \text{Eh1}, \text{Eh1+2}, \) and \( \text{Eh1+3} \), with over twice the \( V_{\text{max}} \) of the other two. All three showed activity at low concentrations of PEP but \( \text{Eh1+2}/\text{Eh1+3} \) are most sensitive. From SAXS, this suggests that aggregation is causing substrate inhibition. In order to further understand these paralogs activity, it should be considered to test the kinetic activity of either \( \text{Eh2} \) or \( \text{Eh3} \) in isolation. These are both dimers, but do not experience aggregation, and should not suffer substrate inhibition.

**Acknowledgements**

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**References**


