Kinetic Mechanism and Metabolic Role of Pyruvate Phosphate Dikinase from *Entamoeba histolytica*

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Marcela Varela-Gómez‡§, Rafael Moreno-Sánchez‡i, Juan Pablo Pardo‡, and Ruy Perez-Montfort‡**

From the ‡Departamento de Bioquímica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70242, 04510 México D. F. México, the †Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México D. F. México, and the ¶Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano No. 1, Col. Sección XVI, 14080 México D. F., México

The kinetic mechanism and the metabolic role of pyruvate phosphate dikinase from *Entamoeba histolytica* were investigated. The initial velocity patterns in double reciprocal plots were parallel for the phosphoenolpyruvate/AMP and phosphoenolpyruvate/pyrophosphate substrate pairs and intersecting for the AMP/pyrophosphate pair. This suggests a kinetic mechanism with two independent reactions. The rate of ATP synthesis at saturating and equimolar concentrations of phosphoenolpyruvate, AMP, and pyrophosphate was inhibited by phosphate, which is consistent with an ordered steady-state mechanism. Enzyme phosphorylation by [32P]pyrophosphate depends on the formation of a ternary complex between AMP, pyrophosphate, and pyruvate phosphate dikinase. In consequence, the reaction that involves the AMP/pyrophosphate pair follows a sequential steady-state mechanism. The product inhibition patterns of ATP and phosphate versus phosphoenolpyruvate were noncompetitive and uncompetitive, respectively, suggesting that these products were released in an ordered process (phosphate before ATP). The ordered release of phosphate and ATP and the noncompetitive inhibition patterns of pyruvate versus AMP and versus pyrophosphate also supported the sequential kinetic mechanism between AMP and pyrophosphate. Taken together, our data provide evidence for a unimolecular bi bi ping pong mechanism for recombinant pyruvate phosphate dikinase from *E. histolytica*. The AG value for the reaction catalyzed by pyruvate phosphate dikinase (+2.7 kcal/mol) determined under near physiological conditions indicates that the synthesis of ATP is not thermodynamically favorable in trophozoites of *E. histolytica*.

Pyruvate phosphate dikinase (PPDK) catalyzes the reversible conversion of AMP, phosphoenolpyruvate (PEP) and pyrophosphate (PPi) to ATP, pyruvate (Pyr) and inorganic phosphate (P). The enzyme is found in bacteria such as *Propionibacterium shermanii* (1) and *Clostridium symbiosum* (2), in the mesophyll cells of C4 plants (3), in leaves and roots of C3 plants (4) and C3-C4 intermediate plants (5), in the protists *Entamoeba histolytica* (6), *Giardia lamblia* (7), trypansomatids, and Euglena (8), and in a thermophilic actinomyces microorganism (9). The functional role of PPDK depends on the organism. For example, it is well established that PPDK is responsible for the synthesis of the primary CO2 acceptor (PEP) in the C4 cycle (10). PPDK also synthesizes PEP in *P. shermanii*, *Acetobacter xylinum*, *Rhodospirillum rubrum*, *Microbipora rosea* (9), and *Rhizobium meliloti* (11). However, in *E. histolytica*, *G. lamblia*, C3 plants, and trypanosomatids the role of PPDK has not been determined.

In the reaction catalyzed by PPDK the transfer of the phosphoryl groups from PEP and PPi to AMP is mediated by a catalytic histidine (His) residue (12). This residue rotates 45 Å from the PEP/Pyr domain to the AMP-ATP/PPi-Pi domain after its phosphorylation by PEP (12) and is also phosphorylated by a phosphoryl group derived from PPi (13). Enzymes that have functional and amino acid sequence similarities to PPDK, such as enzyme I of the bacterial PEP-sugar phosphotransferase system (14) and PEP synthase (12), show the same mechanism of phosphoryl group transfer.

Previous studies on PPDK from *P. shermanii* (PsPPDK) and *C. symbiosum* (CsPPDK) described two different kinetic mechanisms. The initial velocity patterns between PEP, AMP, and PPi determined for PsPPDK yielded sets of parallel lines in the double reciprocal plots (15), indicating that each substrate participates in an individual partial reaction according to a non-classical tri uni uni ping pong mechanism (TUU) (Scheme 1). The term non-classical in this mechanism refers to a proposal of three physically and catalytically independent sites (15). On the other hand, the initial velocity patterns for the CsPPDK (16) and the PPDK from C3 plants (10), were intersecting for AMP/PPi and parallel for PEP/AMP and PEP/PPi. This suggests two partial reactions in a non-classical tri uni bi ping pong mechanism (UUBB) (Scheme 2). This mechanism has been labeled as non-classical because the patterns of product inhibition (16) are consistent with a UUBB with two sites that are catalytically and physically independent, instead of only one site.

It is surprising that the same enzyme displays different kinetic mechanisms in different organisms. In this regard, the mechanism that describes the kinetics of EhPPDK (Schemes 1 and 2) has not been established. Therefore the present study was carried out to determine the kinetic mechanism of rEhPPDK. In addition we determined the intracellular concentrations of the substrates and products of PPDK, in...
order to evaluate the metabolic role of PPDK in *E. histolytica*, because there is a lack of information on this relevant subject.

**EXPERIMENTAL PROCEDURES**

**rEhPPDK Purification**

rEhPPDK was overexpressed and purified from the *Escherichia coli* strain BL21DE3pLysS, as described in Ref. 17. A minor modification was the addition of one type of protease inhibitors (Complete Mini EDTA-free tablets from Roche Diagnostics) to 10 ml of bacterial extract at the beginning of the procedure.

**Kinetic Mechanism**

**Initial Velocity Studies**—The mechanism of the reaction catalyzed by PPDK was determined in the direction of pyruvate synthesis by coupling lactate dehydrogenase from rabbit muscle (13 units/ml, Roche Diagnostics) and following the consumption of NADH (0.2 mM) at 340 nm. The reaction was assayed at 25 °C in 50 mM imidazole buffer at pH 6.3 and in the presence of 10 mM MgCl₂ (total) and 4.5 mM NH₄Cl. The initial velocity studies were analyzed by nonlinear regression (Micrococal Origin version 6) by fitting the data to the corresponding equations of steady-state ordered, ping-pong, and rapid equilibrium random bi bi mechanisms (Equations 1, 2, and 1, respectively). In Equations 1 and 2, 

\[
v = \frac{V_{\text{max}} \times [A][B]}{K_A + [A] + K_B + [B] + [A][B]}
\]

(Eq. 1)

\[
v = \frac{V_{\text{max}} \times [A][B]}{[A][B] + K_A + K_B}
\]

(Eq. 2)

v = \text{initial velocity, } V_{\text{max}} = \text{maximal velocity, } [A] \text{ and } [B], K_A \text{ and } K_B = \text{the concentration and Michaelis-Menten constants of substrates A and B, respectively, and } K_{ia} = \text{the dissociation constant for substrate A.}

**Product Inhibition Studies**—Initial velocities of EhPPDK were determined in the absence and in the presence of the inhibitor product Pᵢ (Q), but in the absence of the other two products (ATP and Pyr). The following Equations 8 and 9 are derived from Equations 6 and 7.

\[
v = \frac{V_{\text{max}} [A]^3}{\text{coef}_{ia} + [A]^2 \times \left(\frac{K_{iic} \times K_{iQ}}{K_{ic} \times K_{iQ}} \right)}
\]

(Eq. 8)

\[
v = \frac{V_{f} \times [A]^2}{\text{coef}_{iQ} \times [A] + \text{coef}_{iQ} \times [A]^2 + [A]^3}
\]

(Eq. 9)

At saturating concentrations of the three substrates, Equations 8 and 9 simplify to Equations 10 and 11, respectively.

\[
v_{\text{max}} = \frac{V_f}{1 + \frac{K_{iQ} \times K_{iic}}{K_{ic} \times K_{iQ}}}
\]

(Eq. 10)

\[
v_f = V_{\text{max}}
\]

(Eq. 11)

In Equations 6, 7 and 8, 9, and 10, 11: Vᵢ is the maximal velocity for the synthesis of ATP; Vᵢ is the maximal velocity for the synthesis of FEP; [A], [B], and [C] are the concentrations of substrates PEP, AMP, and Pyr, respectively; [P], [Q], and [R] are the concentrations of prod-

These two equations were determined using the computer program REFERASS (19).

At equimolar concentrations of the three substrates of the reaction (PEP, PPᵢ, and AMP) and in presence of the product Pᵢ (Q), but in the absence of the other two products (ATP and Pyr), the following Equations 8 and 9 are derived from Equations 6 and 7.

\[
v = \frac{V_{\text{max}}[A]^3}{\text{coef}_{ia} + [A]^2 \times \left(\frac{K_{iic} \times K_{iQ}}{K_{ic} \times K_{iQ}} \right)}
\]

(Eq. 8)

\[
v = \frac{V_{f} \times [A]^2}{\text{coef}_{iQ} \times [A] + \text{coef}_{iQ} \times [A]^2 + [A]^3}
\]

(Eq. 9)

At saturating concentrations of the three substrates, Equations 8 and 9 simplify to Equations 10 and 11, respectively.

\[
v_{\text{max}} = \frac{V_f}{1 + \frac{K_{iQ} \times K_{iic}}{K_{ic} \times K_{iQ}}}
\]

(Eq. 10)

\[
v_f = V_{\text{max}}
\]

(Eq. 11)
Pyruvate Phosphate Dikinase from Entamoeba histolytica

Determination of Keq and ΔG—The determination of ΔG for the reaction PEP + AMP + PPi ⇌ Pyr + ATP + P was required to determine the concentration of the third substrate. The kinetic patterns and the apparent Michaelis-Menten constants obtained by non-linear regression analysis are summarized in Table I. The patterns for PEP and PPi (concentration of AMP constant) and PEP and AMP (concentration of PPi constant) were parallel, indicating a ping-pong kinetic mechanism for PEP versus PPi, and AMP. The pattern for AMP and PPi (concentration of PEP constant) was intersecting (Fig. 1); the same pattern was obtained when AMP was the variable substrate, suggesting that these substrates have a sequential kinetic mechanism in which AMP or PPi binds first (ordered mechanism), or one in which AMP or PPi binds last (disordered mechanism).

† Ref. 1.

RESULTS AND DISCUSSION

rEhPPDK Purity and Stability—The densitometric analysis of the SDS-electrophoresis gel routinely showed purity greater than 90% for all rEhPPDK used in these experiments. The stability of the enzyme stored in imidazole buffer was increased more than 90% for all rEhPPDK used in these experiments. The densitometric analysis of the SDS-electrophoresis gel routinely showed purity greater than 90% for all rEhPPDK used in these experiments. The stability of the enzyme stored in imidazole buffer was increased more than 90% for all rEhPPDK used in these experiments.

Initial velocities of EhPPDK were determined at different concentrations of P, and constant and saturating concentrations of AMP, PPi, and PEP (1 mM). Pyrophosphorylation of rEhPPDK with 32PPi—The reaction was started by the addition of rEhPPDK (0.4 mg of protein, equivalent to 1.04 nmol) to 500 μl of medium containing 50 mM imidazole (pH 6.3), 10 mM MgCl2, 4.5 mM NH4Cl, 2 mM 32PPi (14 mCi; Perkin Elmer), and the indicated substrates. The reaction was stopped after 0.5–1.5 s of incubation at room temperature by the addition of 10% trichloroacetic acid. Longer times of incubation (30–60 s) did not increase the 32Pi incorporation into PPDK. After centrifugation, the supernatant was removed; the precipitated protein was washed another five times with 10% trichloroacetic acid. It was finally resuspended in 1.5 ml of 10% SDS and mixed with 5.5 ml of water. The covalent incorporation of 32PPi into rEhPPDK was determined in a Beckman LS100C counter by measuring the Cerenkov radiation.

Table I

| Substrate | Varied | Variable fixed | Fixed | K_{app}^{a} | Observed pattern |
|-----------|--------|----------------|-------|-------------|-----------------|
| PEP       | AMP    | PEP            | parallel | 0.024 ± 0.012 | parallel         |
| PEP       | AMP    | PEP            | parallel | 0.032 ± 0.010 | parallel         |
| AMP       | PEP    | AMP            | parallel | 0.004 ± 0.001 | parallel         |
| AMP       | PEP    | PEP            | intersecting | 0.023 ± 0.011 | parallel         |
| PEP       | AMP    | PEP            | parallel | 0.062 ± 0.023 | parallel         |
| PEP       | AMP    | AMP            | intersecting | 0.126 ± 0.031 | parallel         |

a Concentrations were PEP, 0.6 mM; PPi, 1 mM; AMP, 0.6 mM.

Pyrophosphorylation of rEhPPDK with 32PPi—The reaction was started by the addition of rEhPPDK (0.4 mg of protein, equivalent to 1.04 nmol) to 500 μl of medium containing 50 mM imidazole (pH 6.3), 10 mM MgCl2, 4.5 mM NH4Cl, 2 mM 32PPi (14 mCi; Perkin Elmer), and the indicated substrates. The reaction was stopped after 0.5–1.5 s of incubation at room temperature by the addition of 10% trichloroacetic acid. Longer times of incubation (30–60 s) did not increase the 32Pi incorporation into PPDK. After centrifugation, the supernatant was removed; the precipitated protein was washed another five times with 10% trichloroacetic acid. It was finally resuspended in 1.5 ml of 10% SDS and mixed with 5.5 ml of water. The covalent incorporation of 32PPi into rEhPPDK was determined in a Beckman LS100C counter by measuring the Cerenkov radiation.
either substrate binds first (random mechanism). This intersecting pattern (Fig. 1) suggested that the reaction catalyzed by EhPPDK involves a partial reaction in which a ternary AMP-EP-PP_i complex is formed.

These patterns (Table I) corresponded to those predicted for a UUBB ping-pong kinetic mechanism. Identical double-reciprocal patterns were reported for the PPDK from C. symbiosum under similar conditions (16), whereas the AMP/PP_i patterns were parallel for the enzyme from P. shermanii (15). This is the main difference between these two kinetic mechanisms (shown in Schemes 1 and 2).

**Product Inhibition Studies**—The intersecting pattern of the AMP/PP_i pair (Fig. 1) may result from three different sequential kinetic mechanisms: a random rapid-equilibrium, a steady-state ordered or a steady-state random mechanisms (22). In an attempt to discern between the three of them, initial velocity data determined with different concentrations of P_i were analyzed using Equations 10 and 11. Equation 10 predicts that in a UUBB ping-pong mechanism, with a steady-state ordered addition of AMP and PP_i, the second product (P_i) will inhibit the enzyme activity. In contrast, Equation 11 predicts that in a UUBB ping-pong mechanism with a random rapid equilibrium for the reaction between AMP/PP_i, the effect of P_i on the initial rate is negligible in the presence of saturating and equimolar concentrations of the three substrates. The equation considering a steady-state random mechanism between these substrates was not derived because its analysis is exceedingly complex. Thus, based on Equations 10 and 11, the hyperbolic decay in the plot of apparent maximal velocity versus P_i concentration (Fig. 2) suggested that the interaction of the enzyme with AMP and PP_i could not be explained by a random rapid equilibrium mechanism.

To distinguish between steady-state random and ordered mechanisms other product inhibition patterns were examined. These were determined by measuring the initial velocity catalyzed by PPDK as a function of a variable substrate, in the direction of ATP synthesis. The results from these experiments are summarized in Table II. The product inhibition patterns for P_i versus AMP and PP_i, and ATP versus PP_i and AMP, were noncompetitive (Table II), indicating that P_i and ATP, and PP_i and AMP bind to different enzyme forms and indicating that substrates and products were reversibly interconnected. This may occur in the pyrophosphorylated form of the enzyme (EPP), because the AMP/PP_i and ATP/P_i pairs participate in the same reversible partial reaction. The noncompetitive inhibition exerted by ATP against AMP (Table II) also supported a reversible conversion between these two ligands.

The release order for ATP and P_i was determined from their product inhibition patterns against PEP. The kinetic prediction for an ordered mechanism between these two products is that the last product released should act as a competitive inhibitor against PEP, whereas the first released product is an uncompetitive inhibitor. For a random mechanism, the prediction is noncompetitive inhibition by both products. The inhibition patterns (Table II) showed that ATP and P_i were released in an ordered sequence, although in a non-classical manner, since the observed inhibition patterns did not strictly correspond to those expected. The uncompetitive inhibition of P_i against PEP indicated that these ligands bind to different enzyme forms and that P_i was released before ATP. However the noncompetitive inhibition of ATP versus PEP did not fit the predicted pattern for the last released product. Alternative explanations for this noncompetitive inhibition are the formation of dead-end complexes, the existence of an iso mechanism (23), or the existence of a non-classical UUBB ping-pong mechanism (15, 16).

Evidence for the presence of dead-end complexes with ATP came from noncompetitive inhibition by GMP with respect to AMP (data not shown). The data suggested that GMP and AMP bind to the same enzyme form (EP), but that GMP interacts with another form, possibly the free enzyme, leading to a dead-end complex. This finding agrees with the noncompetitive pattern observed for ATP versus PEP and with the existence of dead-end complexes.

Another explanation for the noncompetitive inhibition of ATP versus PEP is an iso mechanism; this involves a rate-limiting step associated with isomerization of the free enzyme in the catalytic cycle. In this mechanism, the free enzyme that binds the first substrate is conformationally different from the free enzyme form that binds the last product released. Thus, the inhibition pattern between the first substrate and the last product is noncompetitive, instead of competitive (23). PPDK exhibits two-site catalysis with two independent active sites separated by 45 Å. To complete a catalytic cycle the enzyme undergoes a transition between two different conformations,
which have been identified by x-ray crystallography in the protein from C. symbiosum (12, 24). A full catalytic turnover involves a transition of conformer 1 to conformer 2 that brings the dissociation of the products AMP/PPi from their active site, preparing the enzyme for catalysis at the PEP/Pyr active site (25). The partial reaction involving PEP/Pyr in CsPPDK is slower than the partial reaction involving ATP/Pi. It has been proposed that the movement of the central domain between the C-terminal and N-terminal domains could be the rate-limiting step (26). The high level of sequence identity among PPDKs (50–60%) suggests a conserved structure and the same catalytic conformers for rEhPPDK. The putative presence of two different conformers for the free enzyme could explain the noncompetitive inhibition by ATP with respect to PEP. However, a simpler explanation for the noncompetitive inhibition of ATP versus PEP, which is supported by our data, is that EhPPDK catalysis occurs through a non-classical kinetic mechanism (two independent sites), like the one mentioned previously for CsPPDK. The binding order between AMP and PPi was determined from the Pyr inhibition patterns. Competitive inhibition by Pyr with respect to the first substrate and noncompetitive inhibition with respect to the second substrate is predicted for a classical UUBB kinetic mechanism. The noncompetitive inhibition by Pyr with respect to PPi suggested that this substrate stands after AMP. However, the inhibition pattern of Pyr with respect to AMP was also noncompetitive, suggesting that the kinetic mechanism between AMP and PPi could be random (because the Pyr inhibition with both substrates was of the same type). Alternatively, these inhibition patterns may correspond to and support the non-classical mechanism mentioned above. Thus, Pyr may bind to the binary complex EP AMP, and AMP to the Pyr EP complex since both substrates bind to different sites (Scheme 3). There are experimental data of Pyr inhibition against PPi at saturating concentrations of AMP, and of the inhibition patterns predicted from the equation derived from Scheme 3 that support the formation of the Pyr EP AMP dead end complex (data not shown). The patterns of Pyr inhibition versus AMP and PPi were not reported for CsPPDK, but they were determined for PsPPDK (15). This last enzyme exhibited noncompetitive inhibition for Pyr versus both AMP and PPi.

Pyrophosphorylation of rEhPPDK with $^{32}$PPi.—It was found that $21 \pm 3\%$ of rEhPPDK bound $^{32}$PPi covalently in the presence of $^{32}$PPi + PEP + AMP in the reaction mixture and 5.7 $\pm 0.7\%$ with either $^{32}$PPi, $^{32}$PPi + AMP or $^{32}$PPi + PEP (Table III). The higher incorporation of $^{32}$PPi into the enzyme in the presence of $^{32}$PPi + PEP + AMP, compared with $^{32}$PPi + PEP (Table III), suggests that the phosphorylated enzyme produced in the first partial reaction (PEP → pyruvate) does not catalyze the second phosphorylation from $^{32}$PPi in the absence of AMP. This finding suggested that the ternary complex EP-AMP-PPi was essential for the pyrophosphorylation of rEhPPDK. The pyrophosphorylated PPDK form was originally identified by following the time course of catalysis of CsPPDK with [γ-$^{32}$P]ATP, in which the steady-state E-PP concentration diminished by increasing the Pi concentration, which is the first phosphate acceptor in the kinetic mechanism for the PEP synthesis (13).

Although these results did not establish the binding order between AMP and PPi, they indicated that the second reaction was sequential and corresponded to a UUBB ping-pong mechanism (supporting the data reported in Table I and Fig. 1) instead of a TUU mechanism. The incorporation of $^{32}$P into the enzyme in the absence of PEP was unexpected (Table III), because, in all mechanisms, PEP is the first substrate that binds to PPDK producing the phosphorylated form (EP), which in turn reacts with either AMP or PPi. However, this incorporation could be caused by the high concentration of $^{32}$PPi used (2 mM). This finding complicates the kinetic mechanism proposed for rEhPPDK because it suggests that the free enzyme may also bind PPi as the first phosphate group donor. It has been reported that with 1 mM of each of the three substrates, 10 mM MgCl2 and after 70 min of reaction in presence of EhPPDK at 25 °C, ADP is generated in addition to $^{32}$PPi in the presence of $^{32}$PPi + PEP + ATP at a concentration 10 times lower than that of ATP (6). ADP could be synthesized by phosphorylation of EhPPDK in an incomplete catalytic cycle, requiring only one phosphate donor, perhaps PPi, in addition to AMP. This observation supports the notion that PPi binds to the free enzyme, as shown here. However, in order to simplify the kinetic scheme of EhPPDK (Scheme 3), the PPi-enzyme complex and other dead end complexes were disregarded.

**Metabolic Role: Substrate and Product Concentrations**—The concentrations of metabolites involved in the PPDK reaction were determined in extracts of *E. histolytica* (Table IV). Using these values we estimated the mass action constant ($K_{MA}$) of 12.7 in Equation 12.
The total concentration of Mg\(^{2+}\) was 9.6 ± 1.6 mM (125 ± 9 nmol/10\(^7\) cells). This concentration is high enough to assume safely that Mg-PEP, Mg-PP\(_i\), and Mg-ATP (the true substrates) are formed. Our data of the concentrations of PEP, Pyr, and PP\(_i\) were in the same order of magnitude of those reported by Reeves et al. (0.21 ± 0.09 mM and 0.38 ± 0.11 mM, 0.35 ± 0.09 mM, respectively, Ref. 27). Also, the concentration of ATP was in the same order of magnitude, but it was approximately two times higher than the value reported before, 1.05 ± 0.41 mM (Table IV) and 0.40 ± 0.08 mM (27). These differences may result from using different E. histolytica strains and/or different growth and incubation conditions.

PPDK catalyzes PEP synthesis in C. plants (10). It is localized in chloroplasts of mesophyll cells, which have a pH of 8 in the stroma, and where the reaction is coupled with pyrophosphatases and adenylate kinases (10). It has been estimated that, under physiological conditions, the reaction catalyzed by CsPPDK goes toward the synthesis of ATP (\(\Delta G^\circ = -1\) kcal/mol, Ref. 28). However, in that work the authors used the concentrations of the metabolites reported for E. coli and not those of C. symbiosum (28–30).

A PEP synthesizing role has been proposed for PPDK in other organisms such as P. shermanii and M. rosea from the measurement of its activity in different growth media (1, 9). Other authors have suggested that PPDK catalyzes synthesis of ATP in parasite protists such as E. histolytica and Giardia intestinalis because of the lack of pyruvate kinase in these organisms. However, it is noted that recent works showed in G. intestinalis (31) and E. histolytica trophozoites (32) have significant pyruvate kinase activity. Thus, it was judged relevant to evaluate the metabolic role of PPDK in E. histolytica under near physiological conditions. At pH 6.3, 37 °C and the initial concentrations of substrates and Mg\(^{2+}\) (which were in excess over those of the metabolites) described under “Experimental Procedures,” the reaction catalyzed by PPDK reached equilibrium after 40 min (data not shown). The equilibrium concentrations were 0.04 mM for P\(_i\), and 0.015 mM for ATP and pyruvate. The equilibrium concentrations of AMP (0.035 mM), PEP (0.035 mM), and PP\(_i\) (0.01 mM) were calculated by subtracting the concentration of ATP at equilibrium from the initial substrate concentrations. With these values, the equilibrium constant for the reaction was calculated (\(K_{eq} = 0.73; \Delta G^\circ = +0.19\) kcal/mol). This value differed from that reported for the reaction catalyzed by CsPPDK (333 for the same reaction at 25 °C, 5 mM MgCl\(_2\) and pH 7, Ref. 28). In both cases, the \(K_{eq}\) was determined without considering the concentration of protons. This parameter could be important because it has been reported that the reaction catalyzed by EhPPDK requires 2 \(\mu\)mol of H\(^+\), which was in full agreement with that reported by Reeves (6). However, proton consumption was not considered explicitly in the calculation of \(\Delta G\), since \(K_{MA}\) and \(K_{eq}\) were determined at pH 6.3, and the concentration of protons cancels in the equation for the determination of \(\Delta G\) (+2.7 kcal/mol). Therefore, it seems that in the assumed physiological conditions for amoeba trophozoites the PPDK reaction is not close to its thermodynamic equilibrium. In consequence, net ATP synthesis catalyzed by PPDK should be expected when substrates accumulate and products diminish under particular metabolic conditions of the trophozoites. The apparent lack of pyrophosphatases (34) together with an active vacuolar proton-transporting ATPase, which acidifies the intravacuolar milieu and that is involved in the phagocytosis of bacteria and erythrocytes (33, 35), may fulfill the required conditions for driving the synthesis of ATP by EhPPDK.

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**TABLE III**

*Incorporation of \(^{32}\)P (from PEP) into rEhPPDK*

| Assay components | nmoles \(^{32}\)P/nmole | Percentage of \(^{32}\)P incorporated into rEhPPDK |
|------------------|------------------------|-----------------------------------------------|
| \(^{32}\)PP (2 mM) | 0.077 ± 0.020 | 4.2 ± 0.1 (3) |
| \(^{32}\)PP (2 mM) + AMP (0.6 mM) | 0.048 ± 0.015 | 2.5 ± 0.1 (3) |
| \(^{32}\)PP (2 mM) + PEP (0.6 mM) | 0.067 ± 0.012 | 2.0 ± 0.5 (3) |
| \(^{32}\)PP (2 mM) + PEP (0.6 mM) + AMP (0.6 mM) | 0.211 ± 0.032 | 21.0 ± 3.1 (11) |

*a The number of experiments is shown in parentheses.

**TABLE IV**

*Concentrations of metabolites related to the reaction catalyzed by PPDK*

| Substrate | concentration (nmol/10\(^7\) cells) | mass (nmol) |
|-----------|---------------------------------|-------------|
| PEP       | 10.4 ± 4.0 (n = 3)              | 0.8 ± 0.2   |
| AMP       | 12.3 ± 5.2 (n = 6)              | 0.95 ± 0.31 |
| PP\(_i\)   | 5.5 ± 1.4 (n = 6)               | 0.41 ± 0.12 |
| Pyr       | 5.8 ± 2.5 (n = 5)               | 0.45 ± 0.11 |
| ATP       | 1.37 ± 0.60 (n = 7)             | 1.05 ± 0.41 |
| P\(_i\)   | 108.7 ± 50.4 (n = 4)            | 8.36 ± 3.22 |

**Equation (12)**

\[ K_{MA} = \frac{[\text{Pyr}][\text{P}][\text{ATP}]}{[\text{PEP}][\text{PP}_i][\text{AMP}]} \]
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