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Kinetic and functional properties of human mitochondrial phosphoenolpyruvate carboxykinase

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ABSTRACT

The cytosolic form of phosphoenolpyruvate carboxykinase (PCK1) plays a regulatory role in gluconeogenesis and glyceroneogenesis. The role of the mitochondrial isoform (PCK2) remains unclear. We report the partial purification and kinetic and functional characterization of human PCK2. Kinetic properties of the enzyme are very similar to those of the cytosolic enzyme. PCK2 has an absolute requirement for Mn^{2+} ions for activity; Mg^{2+} ions reduce the K_m for Mn^{2+} by about 60 fold. Its specificity constant is 100 fold larger for oxaloacetate than for phosphoenolpyruvate suggesting that oxaloacetate phosphorylation is the favored reaction *in vivo*. The enzyme possesses weak pyruvate kinase-like activity ($k_{cat} = 2.7 s^{-1}$). When overexpressed in HEK293T cells it enhances strongly glucose and lipid production showing that it can play, as the cytosolic isoenzyme, an active role in glyceroneogenesis and gluconeogenesis.

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1. Introduction

Phosphoenolpyruvate carboxykinase (PCK) catalyzes the decarboxylation and subsequent phosphorylation of oxaloacetate to yield phosphoenolpyruvate. GTP is used as phosphoryl donor in this reaction [1]. The reverse reaction is possible; however, it is difficult to occur *in vivo*. The concentration ranges of oxaloacetate and GTP in tissues [2,3] are similar to their K_m values in human PCK1 [4]. Nevertheless, the K_m for PEP and GDP are higher than their physiological concentrations [5]. Two divalent ions are needed for this reaction; one binds directly to the enzyme active site and the second, to the nucleotide substrate [1,6]. PCK plays several metabolic roles in mammals, with its most known regulatory role in gluconeogenesis and glyceroneogenesis [7]. There exist two isoforms of the enzyme, a cytosolic and a mitochondrial one [8]; their relative contribution to total PCK activity varies between

species. Whereas in mice and rat PCK1, the cytosolic isoenzyme, accounts for over 90% of total PCK activity, in both pigs and humans each isoenzyme is responsible for about 50% of total PCK activity [9]. The cytosolic isoform has been extensively studied, and even serves even as a model enzyme to study transcriptional regulation [10], but much less information is available on the precise role of the mitochondrial isoform. However, in the past two years the involvement of the mitochondrial isoenzyme (PCK2) in pathological processes such as cancer and diabetes has been highlighted by well-grounded works in several laboratories [11–14]. Despite these studies, there exist no report on the purification and kinetic characterization of the human PCK2. In this work we present a protocol for the partial purification of recombinant human PCK2, and we determine the kinetic constants for its five substrates. In addition, we present evidence of the gluconeogenic and glyceroneogenic capacity of human PCK2 in cell cultures.

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2. Materials and methods

2.1. Cloning of PCK2

PCK2 gene (Invitrogen, USA) was amplified by PCR using *Pfu* Ultra II HS DNA polymerase (Agilent Technologies, USA) according

to manufacturer's instructions. The following primers were used:

PCK2-BamHI-F:

5'-CTAGGATCGGATCCATGGCCGATTGTACCGCCTG-3'.

PCK2-NotI-R:

5'-CTAGGATCGCGGCCGCTCACATTTTGTGCACACGTCTCTCC-3'.

PCK2-NheI-F:

5'-CTAGGATCGCTAGCATGGCCGATTGTACCGCCTG-3'.

PCK2-myc-NotI-R:

5'-CTAGGATCGCGGCCGCTCACAGGTCTTCTTCAGAGATCAGTTT
CTGTTCATTTTGTGCACACGTCTCTCC-3'

The last primer included a myc tag sequence to detect the protein in cell culture by western blot. PCR products were digested at 37 °C overnight using either BamHI and NotI or NheI and NotI (New England Biolabs, USA) and cloned into either plasmid pET22bSUMO or pCDNA3.1(-) using T4 DNA ligase (Invitrogen, USA) at 16 °C overnight. Constructs were sequenced and transformed into Arctic Express *Escherichia coli* strain.

2.2. Purification of PCK2

PCK2 had an unstable behavior during purification steps, either rendering insoluble or inactive protein. Several *E. coli* strains and purification protocols were used to obtain soluble active protein. The most successful protocol that yielded soluble active PCK2 was the following.

Arctic Express *E. coli* cells were grown in 100 mL of 2xYT medium supplemented with ampicillin (100 µg/mL) at 37 °C overnight and 220 rpm. This bacterial preculture was poured into 2 L of the same medium and incubated at 37 °C and 160 rpm until OD₆₀₀ reached 0.6. Then, cells were induced using IPTG at a final concentration of 1 mM and incubated at 12.5 °C for 72 h and 160 rpm. Cells were harvested by centrifugation at 4 °C, 8000 × g for 15 min and resuspended in buffer A (25 mM HEPES, pH 8, 500 mM NaCl, 10% glycerol, 1 mM TCEP and 10 mM imidazole) with 1 mg lysozyme, 1 µM PMSF, 10 µM benzamide, 0.5 µM leupeptin and 0.1% benzonase (Sigma-Aldrich, USA). Cells were lysed with a Vibra-Cell sonicator (Sonics & Materials, USA) performing 10 cycles, each for 30 s on-30 s off, on ice. The lysate was clarified by centrifugation at 18,000 × g for 15 min and 4 °C. Finally, the pellet was discarded and the supernatant was filtered through a 0.45 µm filter and used in further purification steps.

Protein was loaded into a 5 mL FF crude HisTrap column (GE Healthcare, USA) equilibrated in buffer A. Protein was eluted with buffer B (same as buffer A but with 300 mM imidazole) using a 0–100% gradient. Aliquots were analyzed by SDS-PAGE. Fractions were concentrated using Amicon Ultra Centrifugal Filters 30 kDa (Millipore, Germany). After two washes in buffer C (25 mM HEPES pH 8, 150 mM NaCl, 1 mM TCEP) protein was diluted up to 10 mL in buffer C and quantified using NanoDrop 1000 (Thermo Scientific, USA). PCK2 was incubated with SUMO protease (Thermo Scientific, 1:100 mg protease/mg protein) for 6 h at 4 °C. The protein was then loaded into a HisTrap column equilibrated in buffer C. PCK2 was collected in the flow-through. PCK2 was detected and quantified by SDS-PAGE and using NanoDrop1000. Since PCK2 was partially purified with chaperone 60 (Cpn60), Image J (NIH, USA) was used to quantify the portion of PCK2 in the mixture, comparing the intensity with that of known standards. Total protein (2 mg/mL) aliquots were flash-frozen in liquid nitrogen and stored at –80 °C.

To avoid the potential interference of metals (Mn²⁺ and Mg²⁺) derived from purified PCK2 preparations we performed a control purification including a metal chelating step. Purified PCK2 was mixed with 5% Chelex 100 chelating resin (Sigma-Aldrich, USA) and was gently shaken for 1 h at 4 °C. The sample was then decanted to remove the chelating resin. Total manganese and magnesium contents were analyzed by inductively coupled plasma

atomic emission spectroscopy (ICP-AES) using IRIS Intrepid Radial Thermo-Elemental (Thermo Scientific, USA). The detection limit of the method was 1 µg/L.

2.3. Kinetic properties of PCK2

All kinetic assays were performed at 30 °C using a Unicam UV500 spectrophotometer (Thermo Scientific, USA) in a total volume of 1 mL. Readings were measured at 340 nm. The maximum duration of the assays was 10 min. We report no activity if no change in absorbance above 0.001 units was detected after the maximum assay duration. Three different reactions were performed:

a) Oxaloacetic acid (OAA) decarboxylation. $\text{OAA} + \text{GTP} \rightarrow \text{PEP} + \text{CO}_2 + \text{GDP}$.

The reaction consisted of 100 mM HEPES pH 7.4, 1 mM ADP (Sigma-Aldrich, USA), 10 mM DTT (Sigma-Aldrich, USA), 0.5 mM GTP (Sigma-Aldrich, USA), 0.2 mM MnCl₂ (Panreac, Spain), 2 mM MgCl₂ (Panreac, Spain), 0.2 mM NADH (Sigma-Aldrich, USA), 5 units each of pyruvate kinase and lactate dehydrogenase (Sigma-Aldrich, USA), 1 µg PCK2 and 0.4 mM OAA (Sigma-Aldrich, USA). Reaction was started by adding OAA.

b) Phosphoenolpyruvate (PEP) carboxylation. $\text{PEP} + \text{GDP} + \text{CO}_2 \rightarrow \text{OAA} + \text{GTP}$.

The reaction consisted of 100 mM HEPES pH 7.4, 2 mM PEP (Sigma-Aldrich, USA), 100 mM KHCO₃ (Panreac, Spain), 2 mM GDP (Sigma-Aldrich, USA), 2 mM MgCl₂, 2 mM MnCl₂, 10 mM DTT, 0.2 mM NADH, 2 units of malic dehydrogenase (Sigma-Aldrich, USA) and 1 µg of PCK2.

c) Pyruvate formation. $\text{OAA} + (\text{GTP or GDP}) \rightarrow \text{Pyruvate} + \text{CO}_2 + \text{GDP}$.

These reactions were identical to reaction (a) but without addition of ADP and pyruvate kinase to the mixture.

2.4. Gluconeogenic and glyceroneogenic activity of PCK2 in cell cultures

HEK293T cells (2 × 10⁵ per well) were seeded on 24-well plates in complete medium (DMEM supplemented with 10% FBS, 10 mM L-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin). Poly-L-lysine (Sigma-Aldrich, USA) was used to attach cells to the culture surface in glyceroneogenic assays. Cells were incubated at 37 °C and 5% CO₂ for 24 h. Then, cells were transfected using GeneJuice (Novagen, UK) following manufacturer's instructions. The amount of DNA used per well was 1 µg.

For glucose production assays cells were incubated for 48 h after transfection. Medium was replaced with 1 mL of DMEM without glucose and phenol red and supplemented with 2 mM sodium pyruvate (Sigma-Aldrich, USA) and 20 mM sodium lactate (Sigma-Aldrich, USA). After a 6-hour incubation at 37 °C, half of the medium was collected and a colorimetric assay was performed (GAGO20, Sigma-Aldrich, USA).

For glyceroneogenic assays, 24 h post-transfection DMEM medium was replaced with complete DMEM medium, as described above, supplemented with 250 µM palmitic acid. After 24 h, cells were washed with 1xPBS once and fixed in 1xPBS with 3.7% formaldehyde for 1 h at room temperature. Cells were washed twice with distilled water. Water was discarded and 60% isopropanol was added. They were then incubated for 5 min, dried, and treated with Oil Red-O stain (Sigma-Aldrich, 0.2% in 60% isopropanol) for 30 min. Stain was discarded and cells were washed four times with distilled water. Oil Red-O stain was dissolved in 1 mL of isopropanol for 1 h with shaking and readings were performed at 500 nm.

Data was normalized to the total protein content of whole cell lysates. Units are mg glucose/mg protein and absorbance units/mg protein for gluconeogenic and glyceroneogenic assays, respectively.

2.5. Data analysis

All kinetic data were analyzed using Origin Pro (OriginLab, USA) adjusting kinetic data to the Michaelis-Menten equation using a molecular weight for PCK2 of 70,700 Da. Data normality was checked by Shapiro-Wilk tests. Two-sample *t*-tests were performed to analyze glucose production experiments and one-way ANOVA following a Tukey *post-hoc* test was performed to analyze glyceroneogenic activity data.

3. Results and discussion

3.1. Cloning of PCK2

The full-length cDNA of human PCK2, including the mitochondrial targeting sequence, was cloned in a pET22bSUMO plasmid. The DNA sequence encoding an N-terminal histidine-tagged *Saccharomyces cerevisiae* SMT3 (SUMO protein) was synthesized and codon-optimized by GenScript to be expressed in *E. coli*. The DNA contained at the 5' end a recognition sequence for NdeI, and at the 3' end a polylinker sequence for BamHI, EcoRI, NcoI, SacI, Sall, HindIII, NotI, and XhoI, and was cloned into the pUC57 vector (GenScript). Following digestion with NdeI and XhoI, the construct was subcloned into the protein expression vector pET22b, resulting in the plasmid pET22bSUMO. The DNA sequence encoding residues 403–621 of ScUlp1 was also synthesized and codon-optimized by GenScript to be expressed in *E. coli*. The DNA, containing at the 5' end a recognition sequence for NdeI and a histidine tag, and at the 3' end a sequence for XhoI and a stop codon, was cloned into the pUC57 vector (GenScript). Following digestion with NdeI and XhoI the construct was subcloned into the protein expression vector pET22b, resulting in the expression plasmid pET22bScUlp1. Sequencing of the cloned cDNA showed no differences with published human PCK2 sequence [15].

3.2. PCK2 purification

After several unsuccessful attempts to purify PCK2 using *E. coli* BL21 DE3 Star or Rosetta-gami strains with several plasmid constructs that yielded insoluble or inactive PCK2 (data not shown), we decided to use Artic Express *E. coli* cells. This strain over-expresses chaperones Cpn10 and Cpn60 from *Oleispira antarctica*, which form one of the most important intracellular folding machines that facilitate protein folding and stability [16]. Fig. 1 shows the partially purified protein preparation obtained after separating the SUMO moiety and the His-tag using SUMO protease. Three bands can be appreciated on the gel: the two most prominent ones correspond to PCK2 and Cpn60. The latter was identified by mass spectrometry (data not shown) to determine that it was not a proteolysis fragment of the former. The third band has a molecular weight of about 10 kDa, corresponding to the SUMO moiety. The specific activity of this PCK2 preparation, calculated based on the content of just PCK2 and excluding therefore Cpn60 and SUMO, was similar to the specific activity of other PCK2 from different species [17,18].

ICP-AES analysis showed that the PCK2 preparation without the chelating resin had manganese levels below the detection limit ($< 1 \mu\text{g/L}$). Magnesium concentration was $21 \pm 2 \mu\text{g/L}$. Therefore, the kinetic assay had only around $0.01 \mu\text{M}$ final concentration of magnesium. Manganese and magnesium levels were below the detection limit when the chelating resin was used. PCK2

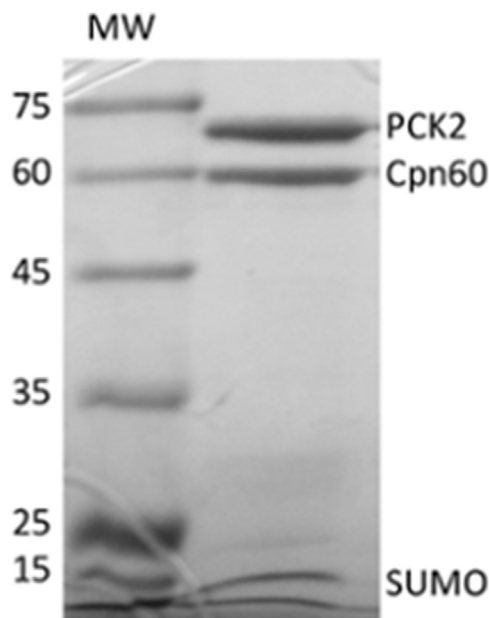


Fig. 1. SDS-PAGE analysis of partially purified PCK2 protein and Cpn60 after separating the SUMO moiety. A Coomassie blue-stained gel is shown. MW: Molecular weight markers (in kDa) run alongside.

preparation without the chelating resin was used in the kinetic assays because PCK2 precipitated in its presence.

3.3. Ion requirements for activity

PCK isoenzymes of diverse origins need divalent cations for activity. Two cations play a role in their catalytic mechanism: one binds directly to amino acids of the active site and the other binds and stabilizes the negative charged phosphates of the nucleotide substrate [1]. Whereas there exist an almost absolute requirement for Mn^{2+} to fulfill the first role, nucleotide stabilization can be achieved by either Mg^{2+} or Mn^{2+} , although both ions are not equally efficient in this task. Human PCK2 is no exception to this behavior (see Fig. 2), since no activity can be observed if only Mg^{2+} is present in the assay medium. However if only Mn^{2+} is added (the assay contains also $0.01 \mu\text{M}$ Mg^{2+} due to the ion content of the PCK2 preparation) the activity reaches values of k_{cat} up to 21.3 s^{-1} (Table 1). The addition of 2 mM Mg^{2+} to Mn^{2+} -containing assays has two effects: it enhances the maximal activity up to k_{cat} values of 31.3 s^{-1} and it reduces drastically K_{m} values from 200 to $3.5 \mu\text{M}$. These changes result in an increase in the specificity constant of almost 200-fold (see Table 1).

3.4. Kinetic parameters of PCK2

We calculated K_{m} , k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ values for the five PCK substrates. The divalent cation concentration in the assays was optimized to reach maximal activity. Assays in the direction of OAA decarboxylation were performed at 0.2 mM Mn^{2+} because higher Mn^{2+} concentrations resulted in high rates of spontaneous OAA decarboxylation (data not shown) whereas assays performed in the direction of OAA synthesis were performed at 2 mM Mn^{2+} concentration. Mg^{2+} was set at 2 mM in all the assays. PCK2 followed Michaelis-Menten kinetics for the five substrates (see Fig. 3A–E). The values of K_{m} , k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ are presented in Table 2. The specificity constant ($k_{\text{cat}}/K_{\text{m}}$) is a useful parameter for comparing alternative and competing substrates for the same enzyme [19]. Specificity constants are similar for nucleotides in both reaction directions but about 100-fold larger for OAA than for

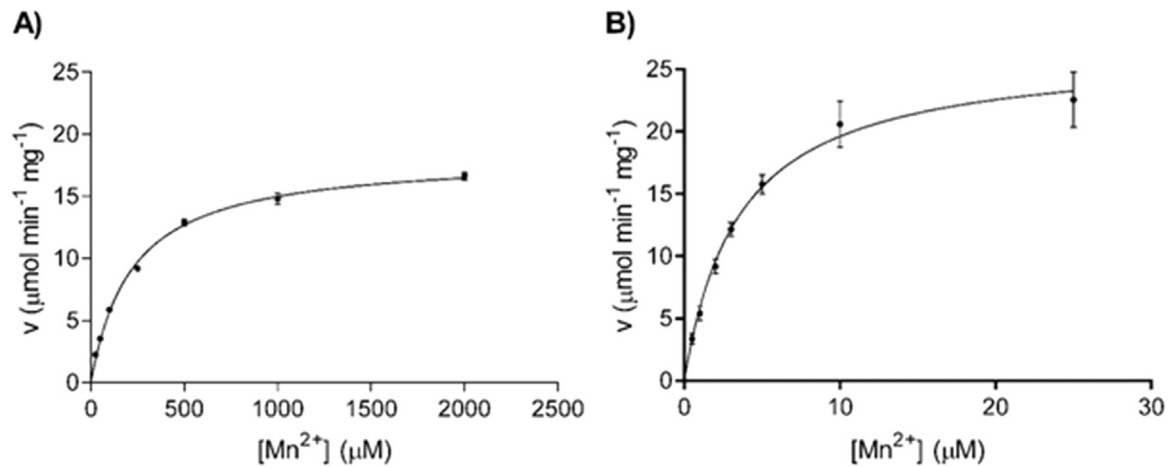


Fig. 2. Effect of concentration of divalent cations on PCK2 activity. (A) Effect of variable Mn^{2+} (25–2000 μM) and 0.01 μM of Mg^{2+} . (B) Effect of variable Mn^{2+} (0.5–25 μM) in the presence of fixed Mg^{2+} concentration (2 mM). All reactions were performed in the PEP carboxylation direction as described in Materials and Methods. Mean \pm SD were adjusted to the Michaelis-Menten equation.

Table 1
PCK2 kinetic parameters for divalent cations.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	Figure
Mg^{2+}	Not detected ^a			
Mn^{2+}	200 ± 11	21.3 ± 0.3	1.1×10^5	2A
$\text{Mn}^{2+} + \text{Mg}^{2+}$	3.5 ± 0.4	31.3 ± 1.2	8.9×10^6	2B

^a See Section 2.

PEP. All k_{cat} values are slightly lower than those of human PCK1. Most K_m values are also of the same order of magnitude than those of human PCK1. Only the K_m for GDP is an order of magnitude lower in PCK2 than in PCK1 [4]. OAA and GTP K_m values are in the same range of OAA and GTP physiological concentrations [2,3], however the PEP K_m value is higher than the PEP physiological concentration [5]. The difference in K_m values and specificity

constants shows that the enzyme favors the reaction in the direction of PEP synthesis (or OAA decarboxylation) as it happens with other PCK isoenzymes [4,18,20,21].

Some PCK isoenzymes show pyruvate kinase-like activity [21,22]. This activity derives from the instability of the enolate reaction intermediate and its tendency to yield pyruvate. To avoid pyruvate formation, the active site of PCK is closed during catalysis by an Ω -loop that helps to position the substrates correctly in the active site and that stabilizes the enolate reaction intermediate protecting it against protonation [1]. To check if human PCK2 was able to accelerate the transformation of OAA into pyruvate we excluded the auxiliary enzyme pyruvate kinase and its substrate ADP from the assays. No pyruvate formation was detected if GTP was present, but in the presence of GDP pyruvate was readily formed (Fig. 3F). The K_m and k_{cat} values for OAA in this reaction were 17 μM and 2.7 s^{-1} , respectively. The resulting k_{cat}/K_m value is $1.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). These results are similar to those of rat

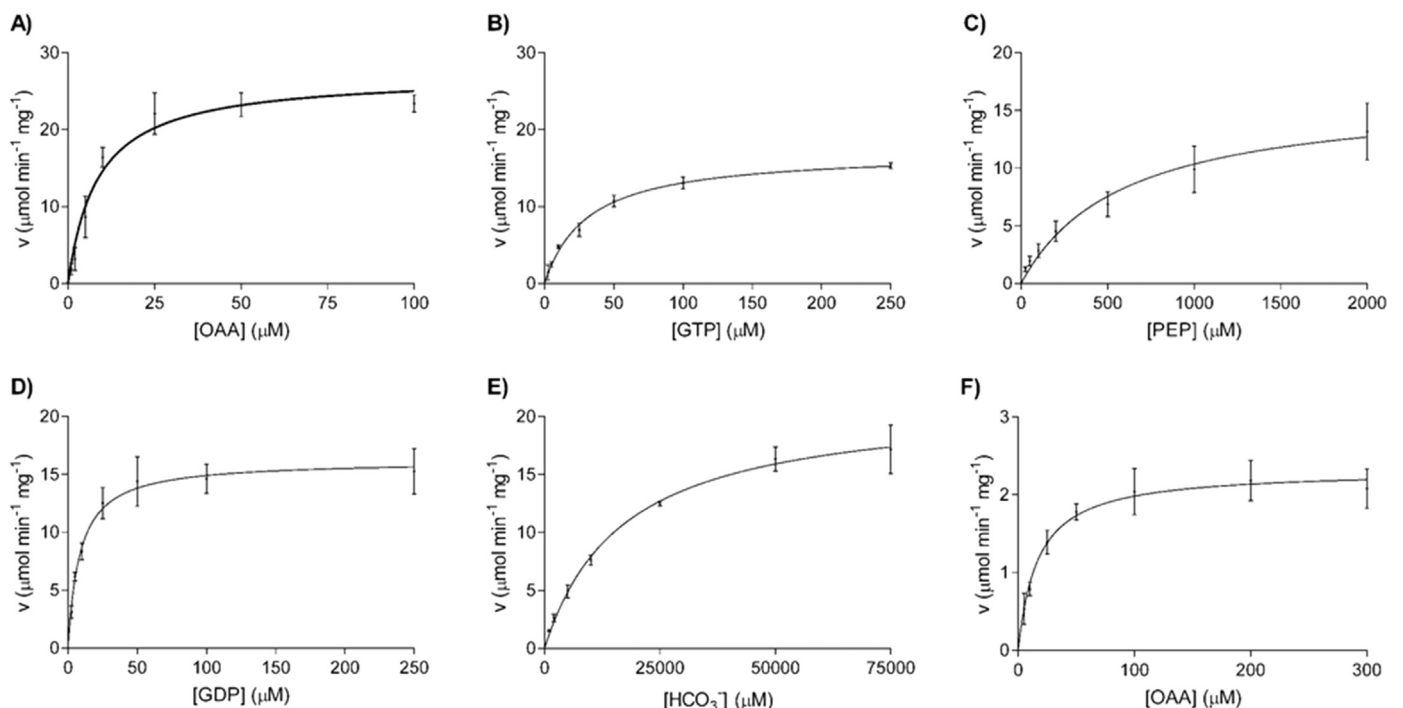


Fig. 3. Kinetic characterization of human PCK2. Effect of OAA (A), GTP (B), PEP (C), GDP (D) and HCO_3^- (E) concentrations on PCK2 activity. (F) Effect of OAA concentration on pyruvate kinase-like activity of PCK2. Mean \pm SD were adjusted to the Michaelis-Menten equation.

Table 2
Kinetic parameters of human PCK2.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	Figure
OAA decarboxylation. $\text{OAA} + \text{GTP} \rightarrow \text{PEP} + \text{CO}_2 + \text{GDP}$				
OAA	8.7 ± 2.5	32.0 ± 2.5	3.7×10^6	3A
GTP	29 ± 7	19.8 ± 1.5	6.9×10^5	3B
PEP carboxylation. $\text{PEP} + \text{GDP} + \text{CO}_2 \rightarrow \text{OAA} + \text{GTP}$				
PEP	585 ± 120	18.8 ± 1.7	3.2×10^4	3C
GDP	8.6 ± 1.0	19.1 ± 0.5	2.2×10^5	3D
HCO_3^-	$17,454 \pm 2529$	25.3 ± 1.3	1.4×10^3	3E
Pyruvate formation. $\text{OAA} + \text{GTP} \rightarrow \text{Pyruvate} + \text{CO}_2 + \text{GDP}$ Not detected ^a				
Pyruvate formation. $\text{OAA} + \text{GDP} \rightarrow \text{Pyruvate} + \text{CO}_2 + \text{GDP}$				
OAA	17 ± 2.7	2.7 ± 0.1	1.6×10^5	3F

^a See Section 2.

[21] and human [22] PCK1 in terms of k_{cat} . However, there are differences in K_m values; rat and human values were 10-fold higher and 40% lower than the PCK2 value, respectively. The pyruvate kinase-like activity of human PCK2 in the presence of GDP, together with its larger affinity for GDP than PCK1, could, at least theoretically, enhance mitochondrial pyruvate formation, and its subsequent transformation into oxaloacetate, when energy levels in the mitochondria (GTP/GDP ratio) are low, contributing in this way to the Krebs cycle. This is an unlikely scenario and perhaps only possible under extreme exercise or long fasting conditions. However, to the best of our knowledge, there is no interpretation in the literature of the biological role, if any, of the PCK pyruvate kinase-like activity.

3.5. Gluconeogenic and glyceroneogenic activity of PCK2 in cell cultures

PCK2 was transiently overexpressed in HEK293T cells in a glucose-free medium. Cells overexpressing PCK2 yielded 3.3-fold more glucose (48.1 ± 6.9 mg glucose/mg protein) than cells that did not express PCK2 (14.7 ± 0.5 mg glucose/mg protein). To analyze its effects on lipid production, PCK2 was transiently overexpressed in HEK293T cells in complete DMEM supplemented

with palmitic acid to enhance lipid synthesis. Cells overexpressing PCK2 produced 2.3-fold more lipids (3.25 ± 0.2 absorbance units/mg protein) in the presence of palmitate than cells transfected with an empty pCDNA3.1 plasmid (1.42 ± 0.1 absorbance units/mg protein). In both experiments, differences between PCK2-overexpressing cells and controls were statistically significant (Fig. 4).

PCK2 has arisen as an important regulatory protein in cell energy metabolism that is also linked to pathological processes such as cancer [23]. In particular, PCK2 is a pro-survivor protein because it enables tumor cells to adapt to low-glucose environments [12]. Although the capacity of PCK1 to produce glucose has been previously reported [24,25], this is the first time that PCK2 is overexpressed in cell culture and tested for glucose production. Our results indicate that PCK2 behaves as a gluconeogenic enzyme, similar to PCK1. PCK2 is the only isoform that provides PCK activity in pancreas due to the involvement of PCK2 in glucose-stimulated insulin secretion, linking the production of mitochondrial GTP by succinyl-CoA synthetase to anaplerotic PEP cycling [26] and it is responsible for half of PCK activity in human liver. However, to date only PCK1 has been a drug target for diabetes. The gluconeogenic activity of PCK2 suggests that this protein could play an important role in diabetes [14], and this is further supported by the results obtained when testing the ability of PCK2 to increase lipid synthesis. In the case of PCK1, its overexpression is responsible for lipid deposition in diabetic liver and its silencing improves dyslipidemia in mice [27]. These results in PCK1 agree with our findings showing that PCK2 overexpression can lead to lipid deposition in cells and support the potential role of PCK2 in diabetes.

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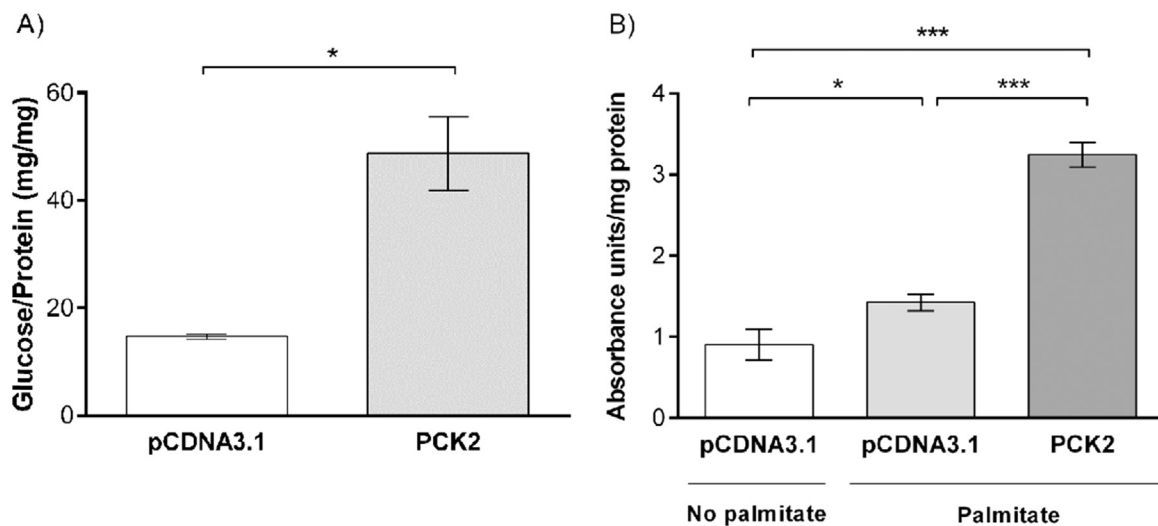


Fig. 4. Effect of PCK2 overexpression on glucose and lipid production. (A) Glucose production in HEK293T cells using an empty pCDNA3.1 plasmid as control (14.7 ± 0.5 mg glucose/mg protein) or overexpressing PCK2 (48.1 ± 6.9 mg glucose/mg protein). Shapiro-Wilk test was performed to test normality and a two-sample *t*-test, to analyze glucose production ($*p < 0.05$). (B) Lipid production in HEK293T cells using an empty pCDNA3.1 plasmid as control in absence (0.9 ± 0.2 absorbance units/mg protein) or presence (1.42 ± 0.1 absorbance units/mg protein) of palmitate or overexpressing PCK2 in presence of palmitate (3.25 ± 0.2 absorbance units/mg protein). Shapiro-Wilk test was performed to test normality. One-way ANOVA following a Tukey *post-hoc* test was performed to analyze lipid production ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.06.007>.

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