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Kinetic and functional properties of human mitochondrial phosphoenolpyruvate carboxykinase

Miriam Escós [b], Pedro Latorre [a,b], Jorge Hidalgo [a,b], Ramón Hurtado-Guerrero [b,e], José Alberto Carrodeguas [b,c,d,**], Pascual López-Buesa [a,b,*]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, 50013 Zaragoza, Spain
[b] Instituto de Biocomputación y Física de Sistemas Complejos (BIFI), BIFI-IQFR (CSIC) Joint Unit, Universidad de Zaragoza, 50009 Zaragoza, Aragón, Spain
[c] Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, 50009 Zaragoza, Spain
[d] IIS Aragón, 50009 Zaragoza, Spain
[e] Fundación ARAID, Gobierno de Aragón, Zaragoza, Spain

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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.

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.

* Corresponding author at: Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, 50013 Zaragoza, Spain.
** Corresponding author at: Instituto de Biocomputación y Física de Sistemas Complejos (BIFI), BIFI-IQFR (CSIC) Joint Unit, Universidad de Zaragoza, 50009 Zaragoza, Aragón, Spain.
E-mail addresses: carrode@unizar.es (J.A. Carrodeguas), plopezbu@unizar.es (P. López-Buesa).

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

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

PCK2-BamHI-F:
5′-CTAGGATCGGATCCATGGCCGCATTGTACCGCCCTG-3′.
PCK2-NotI-R:
5′-CTAGGATCGCGGCCGCTCACATTTTGTGCACACGTCTCTCC-3′.
PCK2-NheI-F:
5′-CTAGGATCGCTAGCATGGCCGCATTGTACCGCCCTG-3′.
PCK2-myc-NotI-R:
5′-CTAGGATCGCGGCCGCTCACAGGTCTTCTTCAGAGATCAGTTT CTGTTCCATTTTGTGCACACGTCTCTCC-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 pET22b*SUMO* 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 OD_{600} 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 benzamidine, 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 (Mn^{2+} and Mg^{2+}) 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. $OAA + GTP \rightarrow PEP + CO_2 + GDP$.
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 $MnCl_2$ (Panreac, Spain), 2 mM $MgCl_2$ (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. $PEP + GDP + CO_2 \rightarrow OAA + GTP$.
The reaction consisted of 100 mM HEPES pH 7,4, 2 mM PEP (Sigma-Aldrich, USA), 100 mM $KHCO_3$ (Panreac, Spain), 2 mM GDP (Sigma-Aldrich, USA), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM DTT, 0,2 mM NADH, 2 units of malic dehydrogenase (Sigma-Aldrich, USA) and 1 μg of PCK2.
c) Pyruvate formation. $OAA + (GTP \text{ or } GDP) \rightarrow Pyruvate + CO_2 + GDP$.

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 (2×10^5 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% CO_2 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.