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Exploring biochemical and functional features of *Leishmania major* phosphoenolpyruvate carboxykinase

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ABSTRACT

This work reports the first functional characterization of leishmanial PEPCK. The recombinant *Leishmania major* enzyme (Lmj_PEPCK) exhibits equivalent k_{cat} values for the phosphoenolpyruvate (PEP) and oxaloacetate (OAA) forming reactions. The apparent K_m towards OAA is 10-fold lower than that for PEP, while the K_m values for ADP and ATP are equivalent. Mutagenesis studies showed that D241, D242 and H205 of Lmj_PEPCK like the homologous residues of all known PEPCKs are implicated in metal ions binding. In contrast, the replacement of R43 for Q nearly abolishes Lmj_PEPCK activity. Moreover, the Y180F variant exhibits unchanged K_m values for PEP, Mn^{2+}, and HCO_3^-, being the k_{cat} for PEP- but not that for OAA-forming reaction more notably decreased. Instead, the Y180A mutant displays an increase in the K_m value towards Mn^{2+}. Therefore in Lmj_PEPCK, Y180 seems to exert different functions to those of the analogous residue in ATP- and GTP-dependant enzymes. Besides, the guanidinium group of R43 appears to play an essential but yet unknown role. These findings promote the need for further structural studies to disclose whether Y180 and R43 participate in the catalytic mechanism or/and in the transitions between the open and the catalytically competent (closed) forms of Lmj_PEPCK

1. Introduction

Leishmania parasites are pathogenic protozoa comprised within the family Trypanosomatidae, which also includes other human pathogens such as *Trypanosoma cruzi* and *Trypanosoma brucei*. Notably, more than 20 species of *Leishmania* are responsible for producing a broad spectrum of clinical manifestations in man (leishmaniasis), ranging from self-healing cutaneous lesions to debilitating mucocutaneous and lethal visceral infections. If untreated, leishmaniasis can lead to severe tissue damage, disfigurement and death. Up to now, no vaccines have been developed and the current clinical treatments are far from being satisfactory [1,2]. In mammals, *Leishmania* parasites are obligate intracellular pathogens, amastigotes proliferate within acidic vacuoles inside macrophages. Therefore, this developmental stage represents the target of anti-parasite therapies.

Phosphoenolpyruvate carboxykinases (PEPCKs) catalyzes oxaloacetate (OAA) decarboxylation and phosphoryl transfer from a nucleoside triphosphate (NTP) to form phosphoenolpyruvate (PEP). Even though this reaction is reversible *in vitro*, PEP formation is normally favored *in vivo*. In man two isoforms are active; one is localized in the cytosol and the other in the mitochondria. Given that the cytosolic PEPCK catalyzes the rate-controlling step in the gluconeogenic pathway, this isozyme has been the most deeply studied. In addition, cytosolic PEPCK also plays an important function in regulating energy homeostasis and flux through the TCA cycle [3].

Based on the specificity of PEPCKs towards the NTP utilized as energy donor, these enzymes are classified in two groups, ATP- and GTP-dependant proteins. Homologues from bacteria, yeast, plant and trypanosomatids use ATP, while PEPCKs from mammals and certain bacteria such as *Mycobacterium tuberculosis* counterpart utilize GTP. Both types of PEPCKs display remarkably low overall sequence relatedness (identity <20%). However, structural studies on PEPCKs from varied sources have shown that the key residues involved in the catalytic mechanism are reasonably conserved among ATP- and GTP-dependant enzymes [for review see [4–6]].

While a wide range of studies have been conducted to disclose the physicochemical properties, 3-D structures and metabolic roles of PEPCKs from diverse organisms, limited information is available for homologues from pathogenic trypanosomatids. Only *T. cruzi* and

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T. brucei PEPCKs have been functionally characterized so far, and notably both enzymes differ in their kinetic parameters [7–9]. The 3-D structure of *T. cruzi* PEPCK has been solved in the absence of substrates or metal ions. Despite this fact, the conformation of the crystallized enzyme resembled that of the closed, ligand-bound form of *Escherichia coli* PEPCK [10].

In trypanosomatids, PEPCK localizes within glycosomes (peroxisome-like organelles), and like the mammalian homologues, it is also expected to be involved in gluconeogenesis and anaplerotic processes. However in these pathogenic protozoa, the reactions catalyzed by PEPCK take part in uniquely organized pathways [11]. This enzyme plays a major role in maintaining NAD^+/NADH and ATP/ADP balance *via* the glycosomal succinate fermentation pathway (gSF). PEPCK catalyzes the first committed step of the gSF by converting PEP into OAA. The subsequent transformation of OAA into succinate allows the reoxidation of two molecules of NADH into NAD^+, per molecule of PEP. Moreover, the generated C4 dicarboxylic acids (malate and succinate) are further utilized for Krebs cycle anaplerosis. On the other hand, PEPCK also participates in gluconeogenesis by means of the conversion of the OAA derived from amino acid catabolism into PEP. Both roles are supported by genetic and metabolic studies performed in the insect and mammalian stages of *Leishmania mexicana* [12–14]. Besides, recent findings have demonstrated that PEPCK is an essential enzyme for the survival of the mammalian stage of *T. brucei* [15].

Early studies have shown that the specific activity of PEPCK was about 6-fold higher in cell-free extracts of *L. mexicana* amastigotes than in the crude extracts of promastigotes, the insect stage of these parasites [16]. In line with those findings, proteomic approaches have also provided evidence for the presence of PEPCK in amastigotes from different *Leishmania* species [17–19]. However, no leishmanial PEPCK has been purified to protein homogeneity and none has been functionally characterized yet.

Taking advantage of the completely sequenced genomes of various *Leishmania* species, we cloned and functionally characterized *Leishmania major* PEPCK. Moreover, to further explore the functional properties of *L. major* PEPCK, the kinetic consequences of the replacement of seven strictly conserved residues in the ATP- and GTP-dependant homologues were also examined. Our results show that in regards to the kinetic properties, the recombinant *L. major* enzyme more closely resembles *T. brucei* than *T. cruzi* PEPCK. Interestingly, *L. major* PEPCK also differs from *T. cruzi* homologue in that 3-mercaptopicolinic acid (3MPA), a typical inhibitor of PEPCKs acts by means of a mixed and a non-competitive mechanism when PEP or HCO_3^- are tested as substrates, respectively. Moreover, mutagenesis studies suggest that as compared with the ATP- and GTP-dependant enzymes, Y180 and R43 might be differently involved either in the transitions between the open and closed forms or in the catalytic mechanism of *L. major* PEPCK.

2. Material and methods

2.1. PCR and cloning

Total DNA from *L. major* promastigotes was isolated [20]. *L. major PEPCK* (*LmjF.27.1810*, Lmj_*PEPCK*) was amplified by PCR using genomic DNA as template and *Pfu*-Turbo DNA-polymerase (Stratagene). In order to perform the PCR, specific primers were designed on the basis of the predicted ORF in the genome project database (http://www.genedb.org): *Lmj_pepck-fw-NdeI*: 5′-CATATGGCCCCGATCATCCAC-3′ and *Lmj_pepck-rev-EcoRI*: 5′-GAATTCCTACAGATGAGCCGTCTCC -3′. The PCR reaction settings were as follows: 5 min at 95 °C and 25 cycles under the next conditions: (i) denaturation at 95 °C for 45 s, (ii) annealing at 58 °C during 45 s, (iii) extension at 72 °C for 1 min 40 s, in addition a final extension step was performed for 10 min. The resulting DNA fragments were cloned into pGEM-T Easy vector and fully sequenced to confirm the predicted ORF. The DNA fragment encoding Lmj_*PEPCK* was excised by digestion with NdeI and EcoRI and ligated into pET28 vector. The generated pET28-Lmj_*PEPCK* plasmid allowed the expression of the recombinant PEPCK with a 6xHis extension at its N-terminus. Besides, in order to produce an untagged recombinant enzyme, Lmj_*PEPCK* was cloned into pET24 vector. Subsequently, the constructed plasmids were used to transform *E. coli* Rosetta (DE3) pLysS. A selected bacteria colony was grown at 37 °C in LB medium supplemented with 30 μg/ml kanamycin and 34 μg/ml chloramphenicol. When an OD_{600nm} of 0.6 was reached, protein expression was induced by adding isopropyl-D-thiogalactopyranoside at final concentration of 0.1 mM. Then, cultures were further grown overnight at 20 °C. The recombinant His-tagged Lmj_PEPCK was purified by affinity chromatography on a Ni^{2+}-nitrilotriacetic (Ni-NTA) column (Qiagen, Germany) following standard procedures. Instead, the untagged enzyme was purified by cation exchange and gel filtration chromatography. The bacteria cell-free extract was applied onto a cation exchange matrix equilibrated in 75 mM triethanolamine buffer, pH 7.4. The weakly interacting proteins were washed with the same buffer while bound proteins were eluted with a linear gradient from 0 to 500 mM KCl in the same buffer. Lmj_PEPCK eluted at about 300 mM of KCl. Subsequently, the fractions with the highest specific activities were pooled and subjected to gel filtration chromatography on a Sephacryl-S200 HR column equilibrated with 50 mM HCl-Tris buffer, pH 8, supplemented with 150 mM NaCl. Protein homogeneity of the recombinant Lmj_PEPCK was analyzed by SDS-PAGE [21], and protein concentration was determined using the method of Bradford and bovine serum albumin as standard [22]. The N-terminal sequence of the recombinant untagged protein was determined by Edman degradation in an Automatic Sequencer (Applied Biosystems, Foster City, CA, USA), run according to the manufacturer's instructions at Lanais-Pro (UBA-CONICET).

2.2. PEPCK activity assays

PEPCK activity was measured spectrophotometrically by monitoring the absorbance decrease at 340 nm resulting from NADH oxidation in the carboxylation and decarboxylation assays. The reactions in both directions were conducted in 75 mM triethanolamine (TEA) buffer, pH 7.4 at 37 °C. PEP-carboxylation was followed by coupling the production of oxaloacetate to NADH oxidation in the presence of malate dehydrogenase. A typical assay mixture contained 2 mM ADP, 6 mM PEP, 100 mM $NaHCO_3^-$, 3.5 mM $MgCl_2$, and 0.15 mM $MnCl_2$, 0.28 mM NADH, 8 units of malate dehydrogenase. The PEP-carboxylation reaction was started by adding Lmj_PEPCK. On the other hand, the standard assay mixture of the OAA-decarboxylation reaction contained 1 mM ATP, 1 mM OAA, 3.5 mM $MgCl_2$, 0.15 mM $MnCl_2$, 0.28 mM NADH, and 20 units of both lactate dehydrogenase and pyruvate kinase. The OAA-decarboxylation reaction was started by adding OAA. For each reaction, blanks were run to measure the unspecific OAA decarboxylation. The obtained values were substracted from those measured in the presence of Lmj_PEPCK. One unit of enzyme activity was defined as the amount of enzyme that produces either 1 μmol of OAA or PEP per min. The optimal pH of the recombinant Lmj_PEPCK reaction was determined using a wide range of buffers: 75 mM sodium acetate/acetic acid (pH 3.6–5.6), 75 mM Bis-Tris (pH 5.8–7.2), 75 mM TEA-HCl (pH 7.3–8.3), and 75 mM glycine-sodium hydroxide, (pH 8.6–10.6). Initial velocity studies were performed by varying the concentration of one of the substrates around its K_m value while the concentrations of the other substrates were maintained constant at saturating levels. The kinetic parameters