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Research Paper

# Molecular and functional characterization of two malic enzymes from *Leishmania* parasites

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ABSTRACT

*Leishmania* parasites cause a broad spectrum of clinical manifestations in humans and the available clinical treatments are far from satisfactory. Since these pathogens require large amounts of NADPH to maintain intracellular redox homeostasis, oxidoreductases that catalyze the production of NADPH are considered as potential drug targets against these diseases. In the sequenced genomes of most *Leishmania* spp. two putative malic enzymes (MEs) with an identity of about 55% have been identified. In this work, the ME from *L. major* (LmjF24.0770, Lmj_ME-70) and its less similar homolog from *L. mexicana* (LmxM.24.0761, Lmex_ME-61) were cloned and functionally characterized. Both MEs specifically catalyzed NADPH production, but only Lmex_ME-61 was activated by L-aspartate. Unlike the allosterically activated human ME, Lmex_ME-61 exhibited typical hyperbolic curves without any sign of cooperativity in the absence of L-aspartate. Moreover, Lmex_ME-61 and Lmj_ME-70 differ from higher eukaryotic homologs in that they display dimeric instead of tetrameric molecular organization. Homology modeling analysis showed that Lmex_ME-61 and Lmj_ME-70 notably differ in their surface charge distribution; this feature encompasses the coenzyme binding pockets as well. However, in both isozymes, the residues directly involved in the coenzyme binding exhibited a good degree of conservation. Besides, only Lmex_ME-61 and its closest homologs were immunodetected in cell-free extracts from *L. mexicana*, *L. amazonensis* and *L. braziliensis* promastigotes. Our findings provide a first glimpse into the biochemical properties of leishmanial MEs and suggest that MEs could be potentially related to the metabolic differences among the species of *Leishmania* parasites.

## 1. Introduction

Malic enzymes (MEs) are widely distributed throughout all kingdoms of life. These enzymes catalyze the reversible oxidative decarboxylation of L-malate to yield pyruvate and $CO_2$ with the reduction of either $NADP^+$ or $NAD^+$, according to the specificity towards the coenzyme. MEs share a relatively high sequence relatedness, which results in highly conserved active sites and structure topology, with most of them exhibiting a tetrameric organization (dimer of dimers). In mammals, MEs have been divided in three groups: the cytosolic $NADP^+$-dependent ME (c-NADP-ME), and the two mitochondrial isoforms, the $NADP^+$-dependent ME (m-NADP-ME) and that $NAD(P)^+$dependent ME (m-NAD(P)-ME) [1,and references therein]. While the cytosolic ME is mainly involved in biosynthetic processes and oxidative stress defense, the mitochondrial isoforms are mostly implicated in energy transduction [2]. In man, the m-NAD(P)-ME differs from the other isoforms in that it is allosterically activated by fumarate [3].

It is well known that among the members of the family Trypanosomatidae, trypanosomes and the about 20 species of the genus *Leishmania* that produce different diseases in man are comprised. These pathogens consume large amounts of NADPH for the detoxification of oxygen and nitrosative species generated during the parasite-host

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interactions. Notably, NADPH represents the only reducing power which guarantees the continuous regeneration of trypanothione. This unique low molecular mass dithiol is essential for the maintenance of intracellular redox homeostasis and survival of trypanosomes and *Leishmania* parasites within their insect vectors and mammalian hosts [for review see 4,5]. Besides, these organisms also depend on NADPH for other key processes such as anabolism [6], synthesis of DNA precursors or the ferrous ion uptake. The latter is particularly critical for the cellular remodeling of leishmanial promastigotes into amastigotes [for review see 7]. Based on the key roles of NADPH in the survival of these pathogenic protozoa, the two dehydrogenases of the pentose phosphate pathway have been proposed as potential drug targets for clinical treatments of the diseases caused in man by trypanosomes and *Leishmania* parasites [8]. The latter route starts from glucose 6-phosphate which is formed by phosphorylation of glucose by hexokinase, in the first reaction-step of the glycolytic pathway. However, leishmanial amastigotes exhibit much reduced rates of glucose uptake, which results in a glucose-sparing metabolism. This switch to more economical metabolic processes occurs regardless of the availability of glucose or other carbon sources in the medium [9]. In this context, MEs might represent the second most important source of NADPH for the intracellular stage of this parasite [for a review see 10]. In line with this hypothesis, the cytosolic ME of *T. brucei* is essential for survival when the expression of glucose-6 phosphate dehydrogenase is repressed [11].

Genome sequencing of *Leishmania* spp. has revealed that, with the exception of *L. major*, parasites such as *L. mexicana*, *L. braziliensis*, *L. donovani* and *L. infantum* exhibit two different gene copies encoding putative MEs (http://www.genedb.org). The two potential isozymes display a sequence identity of about 55%. However, no leishmanial ME has been purified to protein homogeneity or functionally characterized so far.

Keeping in mind that most *Leishmania* spp. are the causative agents of a broad spectrum of clinical manifestations in humans, which, if untreated, lead to severe damaging, morbility and death, the aim of the present study was to evaluate the biochemical properties of the two putative ME isoforms predicted to be operative in *Leishmania* spp. To this end, we selected the putative ME from *L. major* and the less similar homolog from *L. mexicana*. Our results revealed that *Leishmania* parasites exhibit two functional ME isozymes which specifically catalyze NADPH production, but differ in their kinetic properties, structure topology and appear to exhibit a species-specific expression pattern.

## 2. Materials and methods

### 2.1. Organisms and culture

*L. mexicana* and *L. major* promastigotes were grown as previously described [12], while *L. braziliensis* (strain MHOM/BR/1975/M2903) and *L. amazonensis* (strain MHOM/BR/1973/M2269) promastigotes were cultured in 25-ml flasks at 26 °C in Schneider's Insect Medium, pH 7.2, (Sigma, St Louis, MO, USA) supplemented with 10% inactivated fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. Cultures were routinely maintained by weekly passages.

### 2.2. Cloning and expression of putative ME isozymes from L. major and L. mexicana

Total DNA was isolated from *L. major* and *L. mexicana* promastigotes as previously described [13]. The coding sequences of the putative *MEs* from *L. major* (*LmjF24.0770*, *Lmj_ME-70*) and *L. mexicana* (*LmxM.24.0761*, *Lmex_ME-61*) were amplified by PCR using genomic DNA as template and Phusion DNA polymerase (New England Biolabs). Specific primers were designed on the basis of the predicted open reading frames (ORFs) in the genome project database (http://www.genedb.org): *Lmj_ME70*-fw 5′- catatgtttgccaagtcgctg-3′; *Lmj_ME70*-rev 5′- gaattcttagcgaatcaactccttctcc-3′; *Lmex_ME61*-fw 5′- catatgtcccctcggaa acgatctgc-3′; *Lmex_ME61*-rev 5′gaattctcacaggaggctggcgtcc-3′. The forward primers contained an *Nde*I restriction site (underlined), whereas the reverse primers contained an *EcoRI* restriction site (underlined). PCR settings were 5 min at 95 °C and 25 cycles involving denaturation at 95 °C for 45 s, annealing at 60 °C and 58 °C for 45 s for *Lmex_ME*-61, and *Lmj_ME*-70 respectively, and extension at 72 °C for 90 s. Besides, a final extension step at 72 °C for 10 min was performed. In each of the two reactions, a single fragment (≅ 1.8 kb) was amplified. Upon agarose gel purification, the PCR products were cloned into the pGEM-T easy vector and the ligated DNA fragments were fully sequenced. The nucleotide sequences coding for both *ME*s were subcloned into the pET28a$^+$ expression vector (Novagen, Darmstadt, Germany), thus generating pET28-*Lmex_ME*-61 and pET28-*Lmj_ME*-70. The 5′-ends of the *L. major* and *L. mexicana* genes were similarly extended with a nucleotide sequence encoding a 6xHis-tag and a thrombin cleavage site. pET28-*Lmex_ME*-61 and an expression plasmid containing the coding sequence for pGro7 were used to transform *Escherichia coli* BL21-CodonPlus (DE3) RIL, whereas pET28-*Lmj_ME*-70 was introduced into *E. coli* Rosetta (DE3) pLysS. Subsequently, both strains of transformed bacteria were grown in LB medium supplemented with 19.9 µg ml$^{-1}$ and 34 µg ml$^{-1}$ of chloramphenicol, respectively, as well as with 30 µg ml$^{-1}$ kanamycin at 37 °C, until an $OD_{600nm}$ of 0.6 was reached. In the case of Lmj_ME-70, the expression was initiated by adding isopropyl-α-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, and the cultures were further grown at 20 °C, for 4 h. Instead, the production of Lmex_ME-61 was induced by adding IPTG to a final concentration of 0.1 mM and arabinose 0.49 mg ml$^{-1}$. The cultures were further grown at 20 °C, for approximately 16 h.

Bacteria were harvested by centrifugation and the pellets suspended in 20 mM HCl-Tris buffer, pH 7.9, containing 5 mM imidazole and 500 mM NaCl. Upon sonication, the recombinant proteins were purified by affinity chromatography on a $Ni^{2+}$-nitrilotriacetic column (Qiagen, Germany), and the fractions with enzymatic activity were pooled and immediately desalted in a PD-10 column (GE-Healthcare) equilibrated with 75 mM Triethanolamine (TEA) buffer, pH 7.4. The protein homogeneity of both recombinant enzymes was analyzed by SDS-PAGE [14]. The method of Bradford was used to determine protein concentration; bovine serum albumin was used as standard [15]. Moreover, to evaluate the molecular organization of the two ME isozymes in native conditions, Lmj_ME-70 and Lmex_ME-61 were subjected to gel-filtration chromatography on a Sephacryl-S200 h column equilibrated with 75 mM TEA buffer, pH 7.4. The elution was monitored at 280 nm, and the molecular-mass markers used to calibrate the column were: cystathionine β synthase (156.8 kDa), phosphoenolpyruvate carboxykinase (103 kDa), tyrosine aminotransferase (90 kDa), hemoglobin (68 kDa); and ovoalbumin (45 kDa).

### 2.3. Kinetic characterization of the ME isozymes from L. major and L. mexicana

The activities of the recombinant ME isozymes were assayed spectrophotometrically at 37 °C, in 75 mM TEA buffer, pH 7.4. The oxidative decarboxylation of malate was examined in the presence of 0.5 mM $MnCl_2$, 0.5 mM $NADP^+$ and 5 mM L-malate. The ability to catalyze pyruvate carboxylation was tested in 100 mM acetate buffer, pH 5.4, containing 0.5 mM $MnCl_2$, 0.5 mM NADPH, 5 mM $NaHCO_3$ and 50 mM pyruvate. The reactions were initiated by the addition of the corresponding recombinant enzyme and the activities were determined by following the absorbance increase (due to NADPH production) or decrease (due to NADPH consumption) at 340 nm. The kinetic parameters were determined by varying the concentration of the tested substrate while the concentrations of the other substrates were kept at saturating levels. One unit of ME activity was defined as the amount of enzyme that catalyzes the production of 1 µmol of NADPH per minute. The kinetic constants are the means of at least four determinations and were