ELSEVIER

Molecular and Biochemical Parasitology 105 (2000) 203–214

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

www.elsevier.com/locate/parasitology

# Tetrameric and dimeric malate dehydrogenase isoenzymes in *Trypanosoma cruzi* epimastigotes

Giselle Reynoso Hunter [a,1], Ulf Hellman [b], Juan José Cazzulo [c], Cristina Nowicki [a,*]

[a] *IQUIFIB (CONICET — Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires), Junin 956, 1113 Buenos Aires, Argentina*

[b] *Ludwig Institute for Cancer Research, Uppsala Branch, Box 595, S 751 24, Uppsala, Sweden*

[c] *Instituto de Investigaciones Biotecnologicas, Universidad Nacional de General San Martin, Avemida Gral. Paz y Albarellos, INTI, Edificio 24, 1650 San Martin, Buenos Aires, Argentina*

Received 7 May 1999; received in revised form 15 September 1999; accepted 16 September 1999

**Abstract**

Two malate dehydrogenase isoforms, named MDH1 and MDH2, have been purified to homogeneity from *Trypanosoma cruzi* epimastigotes. Both enzymes consist of subunits with a molecular mass close to 33 kDa; native molecular mass determination by gel filtration, however, indicated that MDH1 is a dimer, whereas MDH2 is a tetramer. Both isoforms did not cross-react immunologically. The N-termini of both MDH isoforms and several tryptic peptides of MDH1 (amounting to about one third of the complete molecule) have been sequenced by automated Edman degradation. The tryptic digests of both enzymes have also been analysed by mass spectrometry (MALDI-TOF MS). The apparent $K_m$ values in both directions of the reaction have been determined, as well as the possible inhibition by excess of the substrate oxaloacetate. The sequence data, together with the pI values and the presence or absence of oxaloacetate inhibition indicate that the dimeric MDH1 is the mitochondrial isoenzyme, whereas the tetrameric MDH2 is the glycosomal isoenzyme. No evidence was found for the presence of a cytosolic isoform. 

*Keywords:* *Trypanosoma cruzi*; MDH1; MDH2

*Abbreviations:* CM-Sephadex, carboxy methyl-Sephadex; DTT, dithiothreitol; LDH, lactate dehydrogenase; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MDH, malate dehydrogenase (MDH1 and MDH2 indicate the two *T. cruzi* isoforms; cMDH, mMDH, chMDH, glcMDH, glxMDH and pxMDH indicate the cytosolic, mitochondrial, chloroplastic, glycosomal, glyoxysomal and peroxisomal MDH isoenzymes, respectively); PVDF, polyvinylidene difluoride.

* Corresponding author. Fax: +54-11-4962-5457.

*E-mail address:* cnowicki@criba.edu.ar (C. Nowicki)

[1] Dedicated to Professor Alejandro C. Paladini on his 80th birthday.

0166-6851/00/$ - see front matter 

PII: S0166-6851(99)00176-0

## 1. Introduction

In most eukaryotic cells there are two major isoenzymes of malate dehydrogenase (MDHs), one cytosolic (cMDH) and the other mitochondrial (mMDH). However, in plants and some eukaryotic microorganisms MDH isoenzymes are also present in other organelles such as glyoxisomes (glxMDH), chloroplasts (chMDH), and peroxisomes (pxMDH) [1]. The biological role for the cMDHs and mMDHs has been established. The eukaryotic mMDH is one of the enzymes in the tricarboxylic acid cycle, whereas the cMDH is involved, together with aspartate aminotransferase isoenzymes, in the transfer of reducing equivalents from the cytosol to the mitochondria. The isoenzymes present in microbodies are also supposed to be involved in distributing substrates, as well as reducing equivalents, among different intracellular compartments.

MDHs present extensive structural similarities with lactate dehydrogenases (LDHs), and they are considered to be derived from a common ancestral gene [2], forming the α-hydroxy acid dehydrogenase subfamily within the family of the NAD-dependent dehydrogenases [3,4].

Eukaryotic LDHs are tetrameric [5], whereas bacterial LDHs can form dimers or tetramers, both of which are more active than the monomers [6]. By contrast, all mammalian, plant, and most of the bacterial NAD-linked MDHs reported so far are homodimers with a subunit molecular mass of 32.5–37 kDa. The presence of tetrameric MDHs has been observed in some *Bacillus* spp. [7,8].

Dimeric MDHs isolated from a large number of sources have been thoroughly studied. A number of full amino acid sequences have been obtained (see [9] for review), and detailed 3-D structures of eukaryotic and prokaryotic MDHs are known [10–13].

On the other hand, very little is known about the oligomeric MDHs. Wynne et al. have proposed, after cloning and overexpressing the tetrameric MDH from a thermophilic *Bacillus*, that some minor changes in this enzyme amino acid sequence may lead to small changes on the surface, making the dimers more likely to aggregate and form a more stable tetramer [14].

*Trypanosoma cruzi*, the causative agent of the American Trypanosomiasis, Chagas disease, has two isoforms of MDH, which, at variance with most other eukaryotes, are placed one in the mitochondrion (mMDH) and the other in a microbody characteristic of the order Kinetoplastida, called glycosome, since it contains most of the glycolytic enzymes (glcMDH) [15]. On the basis of isoelectrofocusing of cell free extracts and subcellular localisation experiments, the MDH isoenzyme with a strongly basic isoelectric point was considered mitochondrial, whereas the acidic isoform was shown to be glycosomal [15]. No evidence for a third isoform, such as a cMDH, has been found, in good agreement with the fact that in *T. cruzi* the L-malate-mediated transport of reducing equivalents from glycolysis to the respiratory chain occurs between the glycosome and the mitochondrion [16].

The complete amino acid sequences of the *T. brucei* mMDH and glcMDH have been recently obtained from the nucleotide sequences of the cloned genes [17,18]. A putative MDH sequence from *T. cruzi*, is present in the GenBank/EMBL/DDBJ databases under the Accession number AF051893. However, none of these enzymes have been purified to homogeneity, and little is known about their biochemical and structural properties.

These facts, together with our recent proposal that the aromatic α-hydroxyacid dehydrogenase [19] present in *T. cruzi* may have evolved from a no longer present cMDH (Cazzulo et al., unpublished results), prompted us to investigate the molecular properties of the MDH isoenzymes from this parasite in greater detail. We report here the purification to homogeneity of two different MDH isoforms, one dimeric and the other tetrameric, as well as their partial structural and kinetic characterisation. Different lines of evidence, including their partial amino acid sequences, suggest that they correspond to the mMDH and glcMDH previously reported in cell-free extracts [15]. So far as we are aware, this is the first report on the presence of a tetrameric MDH in a eukaryote, and of the coexistence of both types of MDH, dimeric and tetrameric, in a single cell.

## 2. Materials and methods

### 2.1. Organism and culture

Epimastigotes of *T. cruzi*, strain Tul 2 were grown and harvested as previously described [20]. The cells were washed twice in a solution containing 25 mM potassium phosphate buffer, pH 7.8, 120 mM KCl, 1mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.5 mM tosyl lysylchloromethylketone, 5 μM leupeptin, 2 μM pepstatin (buffer 1).

### 2.2. Extraction and purification of the MDH isozymes

The cells were suspended (0.5–0.6 g wet weight.ml$^{-1}$) in buffer 1, and disrupted by 6 cycles of freezing and thawing. The homogenate was centrifuged at 27 000 × *g* for 45 min at 4°C, and the supernatant was dialysed at 4°C against 20 mM potassium phosphate buffer, pH 7.8 containing 1 mM DTT, 1 mM EDTA (buffer 2) and kept as cell-free extract for enzyme purification. The pellet was suspended in buffer 1 and disrupted by 3 cycles of sonic disintegration, 15 s each, at 4°C, at maximum power of a Branson Sonifier. The sonicated homogenate was centrifuged as described before, and the supernatant was used to monitor the degree of glycosome disruption, by measuring hexokinase as a glycosomal enzyme marker [21].

The dialysed cell-free extract was applied to the top of a Red 120-Sepharose column (10 × 2 cm) equilibrated with buffer 2. All the MDH activity was adsorbed to the column, and after washing with five column volumes of buffer 2, two different fractions with MDH activity, named MDH1 and MDH2, were eluted by means of two consecutive KCl linear gradients in buffer 2, the first from 0 to 300 mM and the second from 300 mM to 1M. The total volume of each gradient was 100 ml. MDH1 eluted at a lower ionic strength (about 200 mM KCl) than MDH2 (about 500 mM KCl). The fractions with the higher specific activity in each peak were pooled separately and dialyzed overnight against buffer 2 adjusted to pH 7.4 (buffer 3).

For purification of MDH1, the active fractions were applied to a CM-Sephadex column (3 × 2 cm). Most of the proteins did not bind to the column and were obtained in the run-through fractions, but the bulk of the MDH1 activity was adsorbed, and after washing with two column volumes of buffer 3, it was eluted with a linear KCl gradient (0–300 mM) in buffer 3. The fractions with the higher specific activity were pooled and used for the experiments described.

For purification of MDH2, the dialysed Red 120-Sepharose pool was applied to the top of a Reactive Blue 4-Agarose column (3 × 2 cm) equilibrated with buffer 3. Elution was achieved by means of a linear KCl gradient (0–700 mM) in buffer 3. The active fractions were pooled, concentrated by ultrafiltration in Centriprep-10 tubes (Amicon) at 3000 × *g*, and applied to the top of a Sephacryl S-400HR column equilibrated with 10 mM Tris–HCl, pH 8.0, 150 mM NaCl. The enzyme was eluted with the same buffer solution; the fractions with the higher specific activity were pooled, concentrated by ultrafiltration in Centriprep-10 tubes (Amicon) at 3000 × *g*, and used for the experiments described.

### 2.3. Determination of enzyme activities

The activity of MDH, in the direction of oxaloacetate reduction, was determined spectrophotometrically at 37°C as the decrease in absorbance at 340 nm, in a reaction mixture containing 100 mM phosphate buffer, pH 7.5, 0.35 mM oxaloacetate, 0.12 mM NADH, and the enzyme, which was added last to start the reaction. The MDH activity in the direction of oxaloacetate formation was measured using citrate synthase as coupled enzyme [15]. The reaction mixture contained 50 mM Tris–acetate buffer, pH 9.0, 1.9 mM NAD, 10 mM L-malate, 0.06 mM acetyl-CoA, and citrate synthase, 0.3 U.ml$^{-1}$, and the enzyme, which was added last to start the reaction. Enzyme units are expressed as μmoles.min$^{-1}$.

The activity of hexokinase was determined spectrophotometrically at 37°C as the increase in absorbance at 340 nm, using glucose 6-phosphate dehydrogenase as coupled enzyme [21]. The reaction mixture contained (in a final volume of 1 ml)

20 mM Tris–HCl buffer, pH 7.6, 15 mM glucose, 20 mM $MgCl_2$, 10 mM EDTA, 0.13 mM NADP, 30 mM ATP, glucose-6-P dehydrogenase 0.2 $U.ml^{-1}$, and enzyme, which was added last to start the reaction.

All apparent kinetic constants were determined with a computer program fitting the data to a hyperbola by applying the Gauss–Newton algorithm [22]

### 2.4. Protein determination

The protein content was determined in cell free extracts using the method of Bradford [23], with BSA as standard.

### 2.5. Gel electrophoresis

Enzyme samples were subjected to SDS-PAGE at room temperature in 10% acrylamide slab gels, under reducing conditions as described by Schägger and von Jagow [24]. The gels were stained for proteins with silver nitrate as described by Blum et al. [25].

MDH activity was detected after electrophoresis in submerged horizontal 1% agarose gels, run under native conditions using 5 mM Tris–38 mM glycine buffer, pH 8.4. The partially purified enzymes were dialysed against the same buffer containing 10% sucrose to add density to the samples and were directly subjected to electrophoresis. In order to visualize the MDH activity bands, the gels were stained as previously described [15], except for the use of Nitro Blue Tetrazolium instead of Blue Tetrazolium as dye.

### 2.6. Determination of the isoelectric point

Isoelectric points were determined in the Phast System (Amersham Pharmacia Biotech, Uppsala, Sweden), with a Phast gel pH 3–9 pre-cast pH gradient gel, using as markers glucose oxidase, soybean trypsin inhibitor, β-lactoglobulin A, carbonic anhydrase B, human carbonic anhydrase, horse myoglobin acidic band, lentil–lectin acidic band, lentil-lectin middle band, lentil–lectin basic band and trypsinogen (pI values of 4.15, 4.55, 5.20, 5.85, 6.55, 6.85, 7.35, 8.15, 8.45, 8.65, and 9.3, respectively). The gels were stained for protein with silver nitrate and for MDH activity as described above.

### 2.7. Determination of the molecular mass by gel filtration

The approximate native molecular mass of the MDH isoforms was estimated by gel filtration in a Sephacryl S-200 HR column ($120 \times 0.8$ cm) equilibrated with 10 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl. The proteins were eluted with the same buffer solution. α-amylase from sweet potato (200 kDa), yeast alcohol dehydrogenase (150 kDa), tyrosine aminotransferase from *T. cruzi* (90 kDa), bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa) were used as molecular mass markers. Their elution volumes in ml were 50, 55, 62, 66,2 and 99, respectively.

### 2.8. Tryptic digestion

The purified MDH1 was reduced with DTT and pyridylethylated, and afterwards desalted by HPLC in a $250 \times 4.6$ mm $C_4$ column, using a linear gradient of 0–80% (v/v) acetonitrile in 0.1% (w/v) trifluoroacetic acid. After taking the sample to dryness in a Speed Vac rotatory dessicator, approximately 80 μg of purified 4VP-MDH1 was dissolved in 8 M urea and tryptic digestion was performed in presence of 2 M urea and 0.1 M ammonium bicarbonate at a Trypsin: MDH1 ratio of 1:100, at 37°C, for 18 h. The digest was acidified with trifluoroacetic acid and centrifuged for 10 min at 12 000 rpm. The supernatant was submitted to HPLC in a $250 \times 2.1$ mm Vydac $C_{18}$ column. The peptides were eluted using a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 0.2 $ml.min^{-1}$.

In order to obtain some amino acid sequence information about the MDH2 isozyme, in-gel tryptic digestion was performed [26], using 3 μg of purified enzyme run in SDS-PAGE. A similar amount of MDH1 was simultaneously submitted to in-gel digestion, in order to compare the tryptic peptide maps of both enzymes. Both digests were separately submitted to reversed-phase HPLC in a $150 \times 1$ mm Kromasil $C_{18}$ column and to mass

spectrometric analyses in a Bruker Biflex III MALDI-TOF-MS, using α-cyano-4-hydroxycinnamic acid as matrix. Samples were prepared by the 'dried-droplet' technique and the instrument was externally calibrated using a known peptide mixture.

### 2.9. Peptide sequencing

The intact MDH isoforms, blotted onto a polyvinylidene difluoride membrane (PVDF, Pro-Blott), as well as selected tryptic peptides from MDH1 and MDH2, were sequenced in an Applied Biosystems Model 477A or 494 A Automatic Sequencer (Applied Biosystems, Foster City, CA, USA), run according to the manufacturer's instructions.

### 2.10. Immunological cross reactivity

The proteins run in SDS-PAGE as described before were electroblotted onto nitrocellulose membranes, and whole rabbit sera raised against purified *T. cruzi* MDH1 (1:15 000) and MDH2 (1:3000) were used as primary antibodies. The Western blots were developed using anti-rabbit goat antibodies coupled to alkaline phosphatase.

For dot-blot assays the same amounts of *T. cruzi* MDH1 and MDH2 (10 and 100 ng), as well as similar amounts of other α-hydroxy acid dehydrogenases, were spotted onto a nitrocellulose membrane, and developed with the rabbit polyclonal antisera as described above.

### 2.11. Chemicals

Sephacryl S-200HR, Sephacryl S-400HR, CM-Sephadex, Red 120-Sepharose, Reactive Blue 4-Agarose, molecular mass markers, and all enzymes were purchased from Sigma Chemical Co., St Louis, MO, USA. Reagents for peptide sequencing were obtained from Applied Biosystems, Foster City, CA, USA. All other chemicals were analytical grade reagents.

## 3. Results

### 3.1. Purification of two MDH isozymes from T. cruzi epimastigotes

Hexokinase was selected as an enzyme marker for glycosomal integrity during parasite cell disruption. About 80% of the extractable hexokinase activity was present in the soluble fraction obtained after freezing and thawing. We assumed, therefore, that this treatment led to a substantial disruption of glycosomes, and the cell-free extract thus obtained, containing 75–80% of the total MDH activity and far less contaminating proteins than the sonicate (not shown), was used as source of the isoenzymes for the purification procedure.

Two different peaks with MDH activity could be separated when this cell-free extract was chromatographed in the Red 120-Sepharose column (Fig. 1). The fractions with MDH activity eluted as a sharp peak at about 200 mM KCl were named MDH1, and those eluted as a broader peak at about 500 mM KCl were named MDH2. The latter broad peak may be due to a strong interaction of the enzyme with the chromatographic matrix.

Table 1 shows the results of a typical purification of both isoenzymes. MDH1 was purified nearly 160-fold with a yield of 40%, whereas MDH2 was purified nearly 200-fold, with a much lower yield.

When both activity peaks were submitted to electrofocusing in pre-cast pH gradient gels MDH1 and MDH2 presented quite different pI values. MDH1 presented two very close activity bands with a pI value close to 9.5, whereas one band with a pI value close to 5 was obtained for MDH2 (Fig. 2a). Both enzymes also had different electrophoretic mobility when analysed by native agarose gel electrophoresis (Fig. 2b). These results indicate that the two activity peaks in the Red-120 Sepharose chromatography indeed correspond to two different MDH isoenzymes.

Taking into account the strongly basic pI observed for MDH1 (Fig. 2a), CM-Sephadex chomatography was used as a second purification step (Table 1). This procedure allowed a further fivefold purification and the enzyme preparation

obtained, when run in SDS-PAGE, presented a single band with an apparent molecular mass of 33 kDa (Fig. 3). The molecular mass of the native MDH1, determined by gel filtration on Sephacryl S-200 was about 70 kDa, as indicated by an elution volume of 65.7 ml, suggesting that, like

Fig. 1. Separation of the two MDH isoenzymes (MDH1 and MDH2) present in the cell-free extracts of *T. cruzi* epimastigotes. The parasite soluble extract, applied onto the Red 120-Sepharose column, was eluted with KCl linear gradients as described in Section 2. The eluted fractions were assayed for enzymatic activity using oxalacetate and NADH as substrates.

Table 1
Purification of the MDHs isoenzymes from *T. cruzi* epimastigotes[a]

| Step | Protein (mg) | Enzyme activity | | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| | | Total (U) | Specific ($U.mg^{-1}$) | | |
| Cell-free extract | 780 | 9494 | 12 | 100 | 1 |
| MDH1 | | | | | |
| Red 120 Sepharose | 16 | 6857 | 416 | 73 | 35 |
| CM Sephadex | 2 | 3752 | 1914 | 40 | 159 |
| MDH2 | | | | | |
| Red 120 Sepharose | 7.8 | 1256 | 161 | 13 | 13.5 |
| Reactive Blue Agarose 0.4 | 0.4 | 768 | 1920 | 8 | 159 |
| Sephacryl S-400 | 0.05 | 127 | 2490 | 1.3 | 207 |

[a] The enzymes were purified from 35 g (wet weight) of epimastigotes and the fractions were assayed for protein and for enzymatic activity in the direction of oxalacetate reduction as described in Section 2.

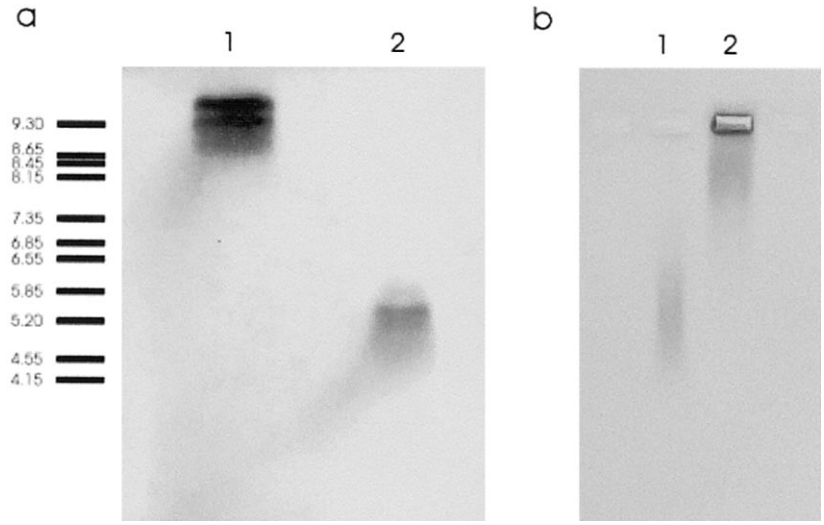


Fig. 2. (a) Isoelectrofocusing pH gradient gel of the MDHs isoenzymes eluted from Red 120-Sepharose column, MDH1 (lane 1) and MDH2 (lane 2). The pIs values of the protein markers are indicated on the left side. (b) Native agarose submerged gel electrophoresis of both MDHs isoenzymes: MDH1 (lane 1) and MDH2 (lane 2). Both gels were stained for MDH activity as indicated in Section 2 using Nitro Blue Tetrazolium as dye.

most MDHs, the native enzyme is a dimer made up of two similar subunits.

The purified MDH2, obtained after Blue 4-agarose chromatography and gel filtration on Sephacryl S-400HR, presented a single band with apparent molecular mass of 33 kDa (Fig. 3) when run under denaturing conditions in SDS-PAGE. When the molecular mass of the native MDH2 was determined by gel filtration on Sephacryl S-200 the elution volume was 51.4 ml, corresponding to a value of about 160 kDa. This suggests that the native MDH2 is a tetrameric enzyme made up of four similar subunits.

### 3.2. Peptide sequences of the T. cruzi MDH isoenzymes

When both purified MDH isoenzymes were blotted onto PVDF membranes and submitted to sequence analysis, the following N-terminal amino acid sequences were obtained:

MDH1: SKVAVLGAAGGIGQPSSLSD

MDH2: VNVAVIGAAGGIGQSLSLLL.

These sequences presented 70% identity and 75% similarity when compared to each other. A Blast [27] search, performed at the NCBI sequence data base, revealed that they had strong similarity with MDHs of different sources, the most similar being the *T. brucei* and some plant MDHs. As it may be expected for two different isoenzymes, when the tryptic digests of MDH1 and MDH2 were submitted to reversed-phase HPLC their peptide maps were quite different

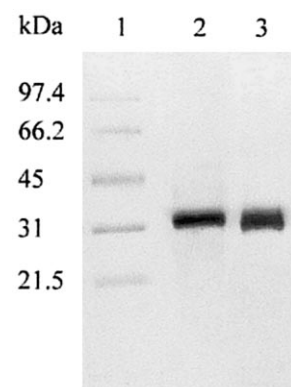


Fig. 3. SDS/PAGE of the purified MDH isoenzymes purified from *T. cruzi* epimastigotes. Electrophoresis was carried out in a 10% polyacrylamide gel, under reducing conditions, as described in Section 2. Molecular weight mass markers (lane 1), MDH1 (lane2) and MDH2 (lane 3). The proteins were visualised by silver staining.

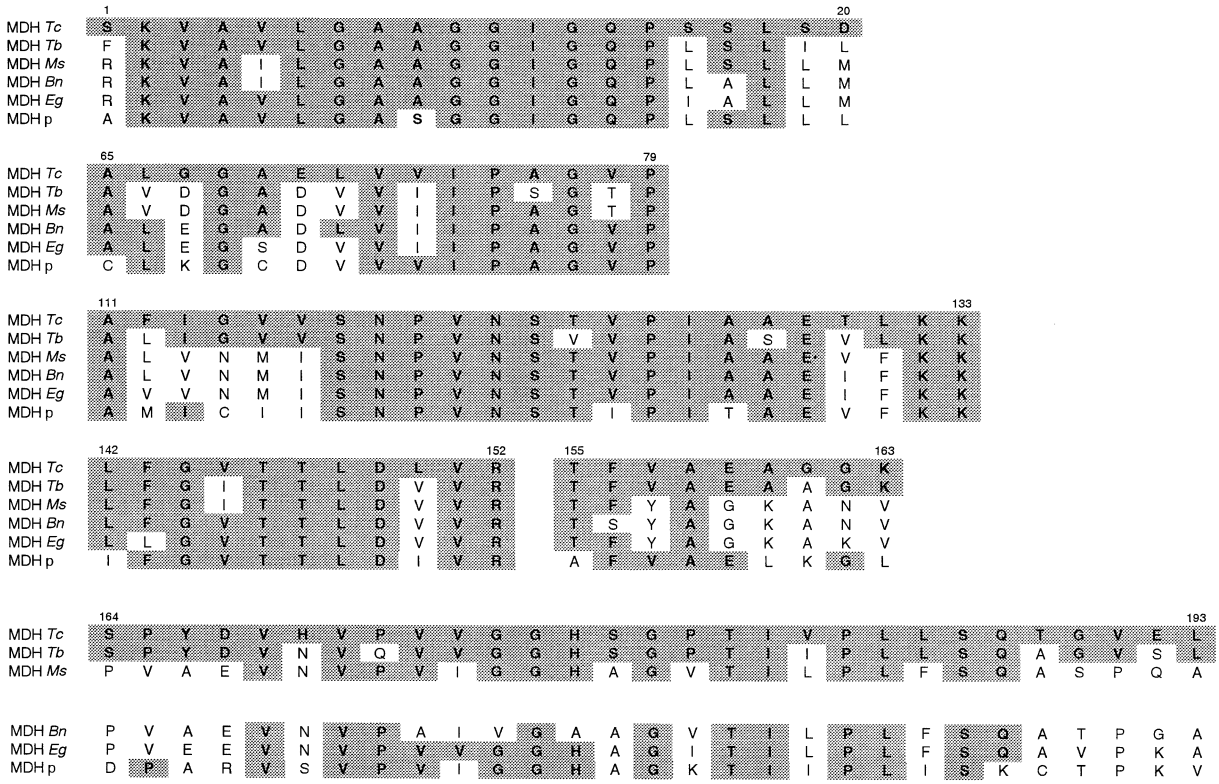


Fig. 4. Comparison of the amino acid sequences of isolated peptides from *T. cruzi* MDH1 isoenzyme. MDH1 tryptic peptides were aligned with different mitochondrial MDH sequences obtained from the data banks: *T. brucei* (MDH*Tb*), *Medicago sativa* (MDH*Ms*), *Eucalyptus gunii* (MDH*Eg*), *Brassica napus* (MDH*Bn*) and *Sus scrofa* (MDHp). Identical amino acid residues are shaded.

(not shown). The sequences obtained for five MDH1 tryptic peptides were aligned as shown in Fig. 4. Highest similarity was found between MDH1 and the *T. brucei* mitochondrial isoenzyme; considering all the MDH1 peptides sequenced, the identity was 80%. A lower identity (about 63%) was observed with plant and mammalian mMDHs.

In the case of MDH2, the sequence ELPFGSTLSLYDVAGAPG was obtained for a selected tryptic peptide. This sequence, in addition to that obtained from the MDH2 N-terminal region, matched perfectly those predicted for the putative MDH from *T. cruzi* (GenBank, Accession Number AF051893) for the first 40 amino acid residues at the N-terminus. Moreover, the mass spectrum of the MDH2 tryptic digest obtained by MALDI-TOF (not shown) gave nine perfect matches with those predicted for the putative *T. cruzi* MDH, allowing positive identification, with a probability of 1 (Fig. 5). The monoisotopic molecular masses of these peptides differed from the theoretical values at most by 0.4Da. These results suggest that the gene reported with GenBank, Accession Number AF051893 is likely to code for the MDH2 isoenzyme that we have purified.

### 3.3. Immunological cross reactivity

No significant immunological cross reactivity could be detected when both, MDH1 and MDH2, were dotted onto nitrocellulose membranes which were developed with the polyclonal antisera raised against each of the purified enzymes. These results may suggest little surface similarity between

MDH1 and MDH2, probably reflecting their differences in quaternary structure. Fig. 6 also shows lack of cross reactivity when MDHs of different sources were assayed under the same experimental conditions. Due to internal sequence similarities some cross reactivity was observed against the other isoenzyme when the same polyclonal antisera were assayed in Western blots, after SDS-PAGE in denaturing conditions (Fig. 6). The MDH2 sample presented two faint bands of lower apparent molecular mass, which were not detected by silver staining of the same enzyme preparation (Fig. 3). These bands might be minor products of limited proteolysis, or be due to some unspecificity of this rabbit antiserum.

### 3.4. Kinetic characterisation of *T. cruzi* MDH isoenzymes

Although both enzymes are active in a wide

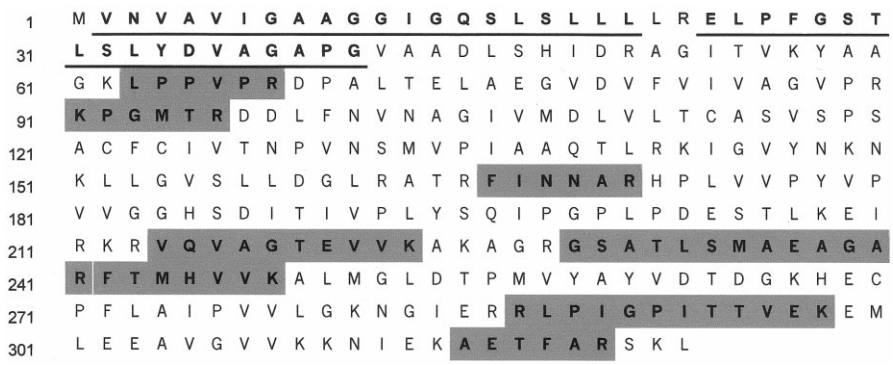


Fig. 5. Amino acid sequence of a putative malate dehydrogenase from *T. cruzi* (GenBank Accession Number AF051893). The underlined sequences were determined by Edman degradation of the N-terminus, and of an internal tryptic peptide, from MDH2; the boxed sequences were identified by MALDI-TOF MS analysis of the same tryptic digest. The initial methionine residue is processed away since it was not found at the N-terminus of the mature MDH2.

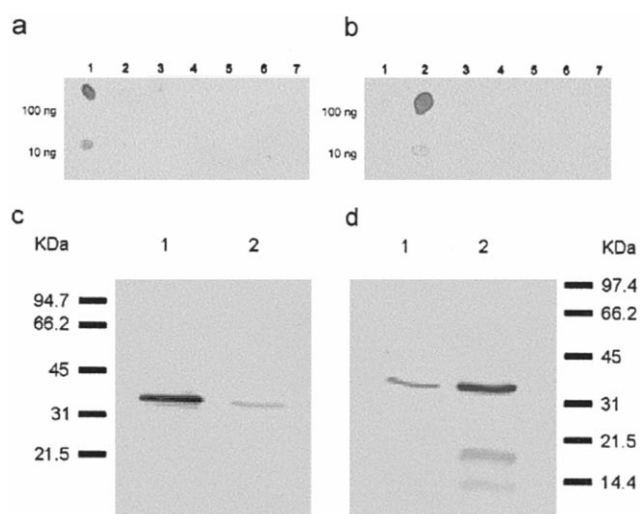


Fig. 6. Immunological cross reactivity of the MDH isoenzymes from *T. cruzi* epimastigotes. (a) and (b) Dot blots developed, as described in Section 2, with polyclonal rabbit antisera raised against purified MDH1 (a) and MDH2 (b), respectively. The samples were applied onto the membrane in native conditions. 1, MDH1; 2, MDH2; 3, aromatic L-α-hydroxy acid dehydrogenase from *T. cruzi*; 4, pig heart mMDH; 5, pig heart cMDH; 6, *Thermus flavus* MDH, 7, rabbit muscle LDH. (c) and (d) Western blots of purified MDH isoenzymes developed with polyclonal rabbit antisera raised against purified MDH1 (c) and MDH2 (d), respectively. Lane 1, MDH1, lane 2, MDH2 Molecular mass markers (in kDa) are shown on the right side of the panels.

Table 2
Apparent $K_m$ and $V_{max}$ values for the MDH isoenzymes from *T. cruzi* epimastigotes[a]

| Substrate | Co-substrate (mM) | App. $K_m$ (mM) | $V_{max}$ (U.mg$^{-1}$) | $V_{max}/K_m$ |
|---|---|---|---|---|
| MDH1 | | | | |
| L-malate | NAD (1.9) | $0.56 \pm 0.008$ | $200 \pm 8$ | 357 |
| NAD | L-malate (10) | $0.32 \pm 0.03$ | $210 \pm 7$ | 656 |
| Oxaloacetate | NADH (0.15) | $0.069 \pm 0.003$ | $1428 \pm 85$ | 20695 |
| NADH | Oxaloacetate (0.3) | $0.054 \pm 0.007$ | $1660 \pm 72$ | 30740 |
| MDH2 | | | | |
| L-malate | NAD (3.8) | $2.9 \pm 0.32$ | $490 \pm 19$ | 169 |
| NAD | L-malate (10) | $0.97 \pm 0.01$ | $481 \pm 16$ | 496 |
| Oxaloacetate | NADH (0.15) | $0.044 \pm 0.006$ | $4200 \pm 189$ | 95454 |
| NADH | Oxaloacetate (0.3) | $0.042 \pm 0.003$ | $3980 \pm 200$ | 94762 |

[a] The values, which are the means of 4–5 determinations ± SE, were obtained as described in Section 2, at the co-substrate concentration stated in brackets.

range of pH, optimum pH values of about 8.5 for MDH1 and 7.5 for MDH2 were determined in the direction of oxaloacetate reduction (not shown).

Table 2 shows the apparent $K_m$ values obtained for the *T. cruzi* MDH isoenzymes in both directions of the reaction, oxaloacetate reduction and malate oxidation. The apparent $K_m$ and $V_{max}$ values obtained for oxaloacetate and NADH for both, MDH1 and MDH2, are similar to those reported for the mammalian enzymes [28]. The MDH2 isoenzyme did not deviate from Michaelis Menten kinetics when the activity was determined in the presence of oxaloacetate concentrations up to 1 mM; on the other hand, the MDH1 isoenzyme presented non-Michaelian kinetics, being inhibited 35% by 1 mM oxaloacetate. The isoenzymes did not present any substrate inhibition or activation by L-malate, up to 20 mM. Both isoenzymes were inhibited about 65% by 10 mM citrate, when assayed in the presence of 0.35 mM oxalacetate 0.12 mM NADH.

## 4. Discussion

We have purified from *T. cruzi* epimastigotes two different MDH isoenzymes to electrophoretic homogeneity. MDH1 and MDH2 possessed similar specific activities, 1914 and 2490 U.mg$^{-1}$, respectively. The results of Red 120-Sepharose chromatography suggest that MDH1 accounts for at least 75% of the total MDH activity in the cell-free extracts. In addition to the different proportion of both isoenzymes in the cell-free extract, the yield of MDH1 (40%) was much higher than that for MDH2. The yield of the latter, when corrected for the separation of MDH1, can be at most about 5%.

Both purified isoenzymes differed in a number of properties. The most remarkable difference was that MDH1, as the MDHs from both, eukaryotic and most prokaryotic organisms, is a dimeric enzyme, whereas MDH2 seems to belong to the group of the less known, and probably less frequent, tetrameric MDHs. Recently, Rujano et al have suggested that both MDH isoforms might be tetrameric (Rujano, M. A., Portillo, R., Manzano F., Dubordie, M. and Concepción, J. L. (1998) Abstract BC-15, Mem. Inst. Oswaldo Cruz 93 (Supp. 11), 52).

The MDH1 isoenzyme can be identified with the mitochondrial isoform by several points of evidence. First, the partial peptide sequences obtained from this *T. cruzi* isoenzyme, accounting for about one third of the full molecule, are most closely related to the *T. brucei* mitochondrial isoenzyme [17]. The lower similarity found with the putative *T. cruzi* MDH (identified with MDH2, see below) seems to be in good agreement with the low immunological cross reactivity observed between MDH1 and MDH2, respectively. Second, the basic isoelectric point is quite close to

that determined before for the mMDH [15]. Third, the inhibition of MDH1 by 1mM oxaloacetate (35%) resembles the case of the mitochondrial enzymes from higher eukaryotes, which are usually inhibited by oxaloacetate in the direction of NADH oxidation [29]. Fourth, the $V_{max}/K_m$ ratio for both substrates in the direction of L-malate oxidation, is higher than in the case of MDH2, suggesting that MDH1 is more suitable than MDH2 for the production of oxaloacetate in the tricarboxylic acid cycle.

In the case of MDH2, all the evidence points to its being the glycosomal isoform. First, its sequenced peptides, as well as the mass spectral values obtained, positively identify MDH2 with the putative MDH (GenBank, Accession Number AF051893). This sequence has the highest homology with the glcMDH from *T. brucei* [18], and also contains the C-terminal sequence SKL, characteristic of the enzymes to be imported into the glycosome [30]. Second, its acidic pI is close to the value previously determined for the glycosomal isoform [15]. Third, MDH2 was not inhibited by 1mM oxaloacetate, as are the mMDHs. Fourth, the $V_{max}/K_m$ ratio for both substrates in the direction of L-malate oxidation, that required for the functioning of the tricarboxylic acid cycle, was lower than in the case of MDH1, whereas the same ratio for both substrates in the direction of oxaloacetate reduction (that required for NADH oxidation inside the glycosome, see [16]) was threefold higher for MDH2 than for MDH1.

The lack of immunological cross-reactivity between MDH1 and MDH2, under native conditions, seems to be in good agreement with the lack of significant immunological cross-reactivity previously observed between native tetrameric and dimeric MDHs from Bacilli, suggesting that these enzymes have few surface structural similarities [7,8].

Carbohydrate catabolism by *T. cruzi* epimastigotes presents some relevant differences when compared to the mammalian hosts (see [16] for review): (1) the rate of glucose consumption is considerably higher than that of most cells, and there is a complete lack of Pasteur effect; (2) organic acids, mostly succinate and L-alanine, are produced and excreted into the medium even in the presence of oxygen, during the incomplete glucose oxidation ('aerobic fermentation'); (3) most glycolytic enzymes are localized in a specialized microbody, the glycosome; and (4) the respiratory chain, and probably also the tricarboxylic acid cycle, are less efficient than those in mammals. The mMDH from *T. cruzi* epimastigotes is supposed to play its usual role in the tricarboxylic acid cycle. The glcMDH, on the other hand, has been proposed to act by reducing the oxaloacetate synthesized by the glycosomal phosphoenolpyruvate carboxykinase, thus re-oxidizing the glycolytic NADH and keeping the glycosome in redox balance. Under these circumstances, the presence of the usual cMDH involved in the shuttle system to transfer reducing equivalents from cytosol to mitochondria would not be required, and, in good agreement with this idea, no cMDH activity has been found in *T. cruzi* ([15] and unpublished results).

The results presented herein show for the first time, as far as we are aware, the presence of a tetrameric MDH in a eukaryote, co-existing in the same cell with the more usual dimeric MDH. Further detailed structural studies will be required to disclose the differential structural features that favours the dimeric or the tetrameric structure for each of these isoenzymes.

## Acknowledgements

This work was performed with grants from the Universidad de Buenos Aires, CONICET, Agencia Nacional de Promoción Científica y Tecnológica and the Swedish Agency for Research Cooperation with Developing Countries (SAREC/Sida). CN and JJC are members of the Research Career from the National Research Council of Argentina (CONICET), and GRH has a Research Fellowship from the Agencia Nacional de Promoción Científica y Tecnológica. We are indebted to Berta Franke de Cazzulo for providing the *T. cruzi* cultures. Peptide sequencing was performed in the LANAIS-Pro, UBA-CONICET (National Facility for Protein Sequencing) and at the Protein Structure Laboratory of the Ludwig Institute for Cancer Research, Uppsala Branch.

## References


[1] Musrati RA, Kollaláová M, Mernik N, Mikulásová D. Malate dehydrogenase: Distribution, Function and Properties. Gen Physiol Biophys 1998;17:193–210.

[2] Birktoft JJ, Fernley RT, Bradshaw RA, Banaszak LJ. Amino acid and sequence homology among the 2-hydroxyacid dehydrogenases: mitochondrial and cytoplasmic malate dehydrogenases from homologous system with lactate dehydrogenases. Proc Nat Acad Sci USA 1982;79:6166–70.

[3] Banaszak LJ, Bradshaw RA. Malate dehydrogenases. In: Boyer PD, editor. The Enzymes, vol. 11, Third edition. New York: Academic Press, 1975:369–96.

[4] Rossman MG, Liljas A, Bränden CI, Banaszak LJ. Evolutionary and structural relationships among dehydrogenases. In: Boyer PD, editor. The Enzymes, vol. 11, Third edition. New York: Academic Press, 1975:61–102.

[5] Grau UM, Trommer WE, Rossmann MG. Structure of the active ternary complex of pig heart lactate dehydrogenase with S-lac-NAD at 2.7 A resolution. J Mol Biol 1981;151:289–307.

[6] Clarke AR, Waldman AD, Munro I, Holbrook JJ. Changes in the state of subunit association of lactate dehydrogenase from *Bacillus stearothermophilus*. Biochim Biophys Acta 1985;828:375–9.

[7] Sundaram TK, Wright IP, Wilkinson AE. Malate dehydrogenase from thermophilic and mesophilic bacteria. Molecular size, subunit structure, amino acid composition, immunochemical homology, and catalytic activity. Biochemistry 1980;19:2017–22.

[8] Smith K, Sundaram TKJ. Malate dehydrogenases from actinomycetes: structural comparison of Thermoactinomyces enzyme with other actinomycete and *Bacillus* enzymes. Bacteriology 1984;157:684–7.

[9] Goward RG, Nicholls DJ. Malate dehydrogenase: a model for structure, evolution, and catalysis. Protein Sci 1994;3:1883–8.

[10] Gleason WB, Fu Z, Birktoft J, Banaszak LJ. Refined crystal structure of mitochondrial malate dehydrogenase from porcine heart and the consensus structure for dicarboxylic acid oxidoreductases. Biochemistry 1994;33:2078–88.

[11] Birktoft JJ, Bradshaw RA, Banaszak L. Structure of porcine heart cytoplasmic malate dehydrogenase: combining X-ray diffraction and chemical sequence data in structural studies. Biochemistry 1987;26:2722–34.

[12] Hall MD, Levitt GD, Banaszak LJJ. Crystal structure of a ternary complex of *Escherichia coli* malate dehydrogenase citrate and NAD at 1.9 A resolution. Mol Biol 1992;226:867–82.

[13] Kelly CA, Nishiyama M, Ohnishi Y, Beppu T, Birktoft JJ. Determinants of protein thermostability observed in the 1.9-A crystal structure of malate dehydrogenase from the thermophilic bacterium *Thermus flavus*. Biochemistry 1993;32:3913–22.

[14] Wynne A, Nicholls DJ, Scawen MD, Sundaram TK. Tetrameric malate dehydrogenase from a thermophilic *Bacillus*: cloning, sequence and overexpression of a gene encoding the enzyme and isolation and characterization of the recombinant enzyme. Biochem J 1996;317:235–45.

[15] Cannata JJB, Cazzulo JJ. Glycosomal and mitochondrial malate dehydrogenases in epimastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol 1984;11:37–49.

[16] Cazzulo JJ. Intermidiate metabolism in *Trypanosoma cruzi*. J Bioenergetics Biomembranes 1994;26:157–65.

[17] Anderson SA, Carter V, Parsons M. *Trypanosoma brucei*: molecular cloning and stage-regulation expression of a malate dehydrogenase localised in the mitochondrion. Exp Parasitol 1998;89:63–70.

[18] Anderson SA, Carter V, Parsons M. Molecular cloning of the glycosomal malate dehydrogenase of *Trypanosoma brucei*. Mol Biochem Parasitol 1998;96:185–9.

[19] Montemartini M, Santomé JA, Cazzulo JJ, Nowicki C. Purification and partial structural and kinetic characterization of an aromatic L-alpha-hydroxy acid dehydrogenase from epimastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol 1994;68:15–23.

[20] Cazzulo JJ, Franke de Cazzulo BM, Engel JC, Cannata JJB. End products and enzyme levels of aerobic glucose fermentation in Trypanosomatids. Mol Biochem Parasitol 1985;16:329–43.

[21] Joshi MD, Jagannathan V. Hexokinase. Methods Enzymol 1996;9:371–5.

[22] Fraser RDB, Suzuki E. The use of least squares in the data analysis. In: Leach SJ, editor. Physical Principles and Techniques of Protein Chemistry, vol. 21, Part C. New York: Academic Press, 1973:301–55.

[23] Bradford MM. A rapid quantitative method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1996;72:248–54.

[24] Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.

[25] Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins. RNA and DNA in polyacrilamide gels. Electrophoresis 1987;8:93–9.

[26] Hellman U, Wernstedt C, Gomez J, Heldin CH. Improvement of an 'In-Gel' digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem 1995;224:451–5.

[27] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

[28] Heyde E, Ainsworth S. Kinetic studies on the mechanism of the malate dehydrogenase reaction. J Biol Chem 1968;249:2413–23.

[29] Berenstein LH, Grisham MB, Cole KD, Everse J. Substrate inhibition of the mitochondrial and cytoplasmic malate dehydrogenases. J Biol Chem 1978;253:8697–701.

[30] Sommer JM, Cheng QL, Keller GA, Wang CC. In vivo import of firefly luciferase into the glycosomes of *Trypanosoma brucei* and mutational analysis of the C-terminal targeting signal. Mol Biol Cell 1992;3:749–59.