Purification and properties of *Plasmodium falciparum* malate dehydrogenase

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Asexual intraerythrocytic *Plasmodium falciparum* were shown to have a single isoenzyme of malate dehydrogenase. This malate dehydrogenase was purified to apparent homogeneity using a three-step purification protocol. The parasite malate dehydrogenase had an apparent subunit molecular weight of 32 kDa, a pH optimum of 7.0 for the reduction of oxaloacetate, and a sharp thermal transition between 40°C and 45°C. These characteristics distinguish *P. falciparum* malate dehydrogenase from both the cytoplasmic and mitochondrial malate dehydrogenase isoenzymes of humans. In addition, the resistance of the parasite malate dehydrogenase to substrate inhibition by oxaloacetate suggests that it is the cytoplasmic malate dehydrogenase isoenzyme. The apparent absence of mitochondrial malate dehydrogenase from asexual intraerythrocytic *P. falciparum* contributes to evidence indicating that the mitochondrion is undeveloped at this stage of the parasite’s life cycle.

Key words: *Plasmodium falciparum*; Malate dehydrogenase; Asexual intraerythrocytic stage parasite

Introduction

Energy generation during the asexual intraerythrocytic stage of the *Plasmodium falciparum* life cycle is believed to be primarily glycolytic [1,2]. The acristate mitochondria [3,4] which are present lack at least some of the tricarboxylic acid (TCA) cycle enzymes [5]. Activity for only one TCA cycle enzyme, malate dehydrogenase (MDH) has been demonstrated [6].

In most eukaryotic cells, MDH is present as two differentially compartmentalized isoenzymes. The cytoplasmic MDH (cMDH) and mitochondrial MDH (mMDH) both catalyze the NAD(H)-dependent interconversion of malate and oxaloacetate; however, their physiological roles differ. The mMDH is involved in energy generation and biosynthetic pathways via the TCA cycle. The cMDH is important for gluconeogenesis [7] but may have other roles [8–10]. Together, cMDH and mMDH are essential for the malate/aspartate shuttle by which the cell controls the distribution of reducing equivalents between the cytoplasmic and mitochondrial compartments.

Only one MDH isoenzyme could be demonstrated in intraerythrocytic *Plasmodium lophurae* [9]. This suggests that one *Plasmodium* MDH isoenzyme may be developmentally regulated. On the other hand, Sherman also showed four MDH electrophoretic isoforms in *Plasmodium berghei* [9]. These four isoforms could arise from genetic differences in the infecting parasite population or from isoelectric subforms such as those found in individuals of other species [11]. In either case, it is not clear whether intraerythrocytic *P. berghei* contain only cMDH or mMDH, or both isoenzymes.

The present report identifies a single MDH
isoform in the asexual, erythrocytic stages of *P. falciparum*. This *P. falciparum* MDH was purified to a specific activity of about 80 units mg\(^{-1}\) and to apparent homogeneity as analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The physical and enzymatic properties of the purified enzyme clearly distinguish it from mammalian cMDH or mMDH. The regulatory characteristics of this *P. falciparum* MDH are consistent with those of cMDHs from other species.

**Materials and Methods**

**Enzyme assay.** The standard spectrophotometric MDH assay measured the decreased absorption at 334 nm due to NADH oxidation in the presence of oxaloacetate. The reaction buffer contained 25 mM Na-phosphate, pH 7.0/0.25 mM oxaloacetate/0.25 mM NADH/1 mg ml\(^{-1}\) bovine serum albumin. For reactions measuring the activity of MDH with malate as substrate, the reaction buffer contained 50 mM glycine, pH 9.9/100 mM malate/5 mM NAD\(^+\)/1 mg ml\(^{-1}\) bovine serum albumin. All reactions were carried out at room temperature. One unit of MDH activity is defined as the amount of enzyme required to oxidize or reduce 1 \(\mu\)mol coenzyme min\(^{-1}\). To determine specific activity, protein concentration was determined by the method of Bradford [12].

MDH isoforms were separated in a 6% polyacrylamide gel using the phosphate citrate buffer system of Carter [13]. Electrophoresis was for 16 h at 4 V cm\(^{-1}\) at 4°C. The gel was then incubated in reaction buffer for about 1 h at room temperature or at 37°C. The reaction buffer consisted of 100 mM Tris HCl, pH 8.0/500 mM malate/200 mM NAD/300 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma)/200 mM phenazine methosulfate (Sigma). The reaction was stopped by replacing the reaction buffer with 5% acetic acid.

**Purification protocol.** *P. falciparum* MDH activity was purified from strain NF54 (obtained from P. Nguyen-Dinh) which was grown in culture as previously described [14,15]. To minimize the production of gametocytes by the NF54 strain, the parasitemia was kept at 1–2% of the red blood cells by frequent subculturering.

The first step of the purification was to enrich for trophozoite infected cells using the gelatin flotation method of Pasvol [16]. Parasite-enriched cells were used directly or stored at \(-70°C\). These cells were suspended in an equal volume of Buffer A (50 mM Na-phosphate, pH 7.0/1 mM EDTA/1 mM dithiothreitol/10% glycerol) containing 1.7 mM phenylmethyl sulfonyl fluoride. Mechanical rupture was achieved using a 50% duty cycle and the maximum power for the microtip of a Branson 250 Sonicator for 3 min. Cell debris was pelleted by centrifugation for 1 min in a microfuge (14 000 \(\times\) g). A portion of the supernatant was assayed for MDH using the gel assay described above. The remaining supernatant was diluted 10-fold with Buffer A containing 1.7 mM phenylmethyl sulfonyl fluoride and loaded onto a 2-ml Reactive Blue 72 agarose (Sigma) column at a flow rate of about 4 ml h\(^{-1}\). The column was washed with 10 column volumes of Buffer A. Bound MDH activity was eluted into silenized tubes with Buffer A containing 1 mM NADH. Fractions were assayed spectrophotometrically for MDH activity and then stored at \(-70°C\) until further purification or use. The third purification step used a 2.5 ml polybuffer exchange column (PBE 94, Pharmacia). For the experiment shown in Fig. 4, the enzyme was loaded on the column in 200 mM Tris-acetate, pH 8.0 and was eluted with 25 ml of a 1:10 dilution of Polybuffer 96 (Pharmacia) which had been adjusted to pH 6.0 with glacial acetic acid. Fractions were collected in silenized tubes, and MDH activity was determined using the spectrophotometric assay. A small sample of each fraction was reserved at \(-70°C\) for SDS gel electrophoresis [17]. Fractions containing peak MDH activity were pooled, bovine serum albumin was added to a final concentration of 1 mg ml\(^{-1}\), and the enzyme was dialyzed into Buffer A prior to storage at \(-70°C\).
Two-dimensional gel electrophoresis. Using a modification of the 2-dimensional gel electrophoresis technique [18], the subunit molecular weight of the protein responsible for the trophozoite MDH activity was determined. Affinity-purified MDH was subjected to non-denaturing PAGE together with erythrocyte MDH (Sigma) and pig mMDH (Sigma) as controls. The gel was stained for MDH activity using the typical gel assay for MDH described above. The bands associated with specific MDH activities were excised from the gel equilibrated with SDS sample buffer (62 mM Tris-HCl, pH 6.8/2.3% SDS/5% 2-mercaptoethanol/10% glycerol) for 45 min, and placed about 0.5 cm above a 12% polyacrylamide SDS gel. The gel pieces were sealed in place with 1% agarose in stacking gel buffer [17]. Molecular weight markers (Sigma; Dalton Mark VII-L) were loaded in a well formed in the agarose stack. Following electrophoresis, the polypeptides in the gel were visualized with silver stain [19].

Results

Identification of *P. falciparum* malate dehydrogenase isoenzymes. It was reported that asexual erythrocytic stage *P. falciparum* contain a new MDH activity which is not associated with uninfected blood [6]. To confirm the presence and to determine the number of *P. falciparum* MDH isoenzymes during the asexual erythrocytic life cycle, parasite-enriched blood was prepared from in vitro cultures and subjected to non-denaturing polyacrylamide gel electrophoresis. The gel was incubated in a reaction buffer containing malate, NAD$^+$ and dye substrate (as described in Materials and Methods). A single new band of NAD-dependent MDH activity was visualized in gels containing a parasite-enriched lysate (Fig. 1). Changes in the gel buffering system or in the conditions used to assay MDH activity in the gels did not result in the identification of more than one asexual parasite-associated MDH activity (data not shown). Purified erythrocyte MDH (cMDH) comigrated with the major MDH activity band in both uninfected and parasite infected lysates (data not shown). Other minor MDH activities were seen, but these were also present in human white blood cells (data not shown). These probably represent human mMDH isoforms.

Purification of *P. falciparum* malate dehydrogenase. *P. falciparum* MDH was isolated using a 3-step purification protocol (Table I). First, infected blood was enriched 50–70 fold for parasites by gelatin flotation [16]. Second, mechanically lysed extracts were subjected to affinity chromatography on Reactive Blue 72 agarose (Fig. 2). Although both parasite and human MDHs bind to this ligand at pH 8.0, at pH 7.0 only the parasite MDH activity remained bound. The bound MDH activity
TABLE 1
Summary of the purification of *P. falciparum* malate dehydrogenase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Trophozoite-enriched blood</td>
<td>15.3</td>
<td>142</td>
<td>0.1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>(2) Reactive Blue agarose chromatography</td>
<td>6.1</td>
<td>0.19</td>
<td>32</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>(3) Chromatofocusing chromatography⁴</td>
<td>1.1</td>
<td>0.014</td>
<td>78</td>
<td>11⁴</td>
<td>780</td>
</tr>
</tbody>
</table>

*Only 4.1 units of the activity eluting from the Reactive Blue agarose column was loaded on the chromatofocusing column. Net yield determined by extrapolation of the yield for this step.*

could be eluted with either NAD or NADH. Both elution procedures resulted in one major protein product with a subunit molecular weight of 32 kDa (Fig. 3B and data not shown). Furthermore, in each case, the elution profile of this polypeptide matched the elution profile for MDH activity (data not shown).

When NADH was used as the eluent, a small amount of human MDH activity often coeluted with the parasite MDH activity from the Reactive Blue 72 agarose column (Fig. 3A). The contaminating human MDH activity comigrated with purified erythrocyte MDH activity in non-denaturing gels.

A control experiment was devised to confirm the nature and extent of human MDH contamination of the Reactive Blue 72 agarose purified parasite enzyme. Uninfected blood was mechanically lysed and subjected to affinity chromatography following the protocol for parasite MDH purification. Only a small amount of human MDH activity eluted in the NADH, suggesting that not more than 25% of the MDH activity from parasite infected blood results from human MDH. The affinity purified fraction containing human MDH activity was further analyzed by SDS-PAGE. As seen in Fig. 3B, there was a 36-kDa polypeptide present in this fraction. This polypeptide comigrated with MDH which had been purified from human erythrocytes (data not shown). There were no visible bands at 34 kDa, the apparent subunit molecular weight of mammalian mMDH, or at 32 kDa, the apparent subunit molecular weight of the parasite MDH. Together, these results indi-

Fig. 2. The elution profile from the Reactive Blue 72 agarose column is shown as measured by protein (□) and by MDH activity (●). The arrow indicates the start of elution with 1 mM NADH.
cated that following affinity chromatography on Reactive Blue 72 agarose, the parasite MDH was contaminated by a small number of other proteins including some human cMDH.

The third step in the purification of the *P. falciparum* MDH was by chromatofocusing. As shown in Fig. 4, the MDH activity profile corresponded to the elution profile of the 32-kDa polypeptide. No other polypeptides were evident in fraction 4 which contained the peak MDH activity though nonspecific staining was seen at 60–64 kDa which appeared to be an artifact of the silver stain procedure. The purified enzyme had a specific activity of about 80 units mg\(^{-1}\) (Table I, and data not shown). There was no evidence for human MDH activity (data not shown) or for a 36-kDa polypeptide in any of the chromatofocusing fractions tested. This suggested that the human MDH contaminating the affinity-purified parasite MDH had not eluted from the column or was too dilute to detect.

As an independent means of identifying the parasite polypeptide responsible for MDH activity, a modified two-dimensional gel electrophoresis technique was devised as described in Materials and Methods. A single polypep-
A tide of approximately 31 kDa was associated with the parasite MDH activity (Fig. 3C). Under the same conditions, mammalian cMDH and mMDH activity migrated at 35 and 33 kDa, respectively. Since the mammalian MDHs migrated as if they were about 1 kDa smaller than their known subunit molecular weight, this suggests that the subunit molecular weight associated with the parasite MDH activity is about 1 kDa more than measured. This result is consistent with the identity of the parasite MDH polypeptide as determined by conventional purification techniques.

Characteristics of *P. falciparum* malate dehydrogenase. The thermal stability of the *P. falciparum* MDH was tested by incubating the enzyme in the presence (Fig. 5A) or absence (Table II) of substrate, at various temperatures prior to assay at room temperature. As shown in Fig. 5A, the enzyme is stable to 40°C but there is an abrupt loss of thermal stability between 40°C and 45°C. This thermal transition appears to be an identifying characteristic of the parasite MDH. In contrast, there is very little difference in the thermal stability of human cMDH at these temperatures (Table II). This suggested that at least 90% of the MDH activity measured was generated by the parasite MDH.

Optimal assay conditions for *P. falciparum* MDH were determined using the enzyme which had been purified through the three-step scheme described above. At pH 7.0, the parasite enzyme had maximal activity as

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Fig. 5. The activity of the *P. falciparum* trophozoite malate dehydrogenase eluting from the chromatofocusing column was assayed spectrophotometrically and is expressed in units ml⁻¹ of enzyme. (A) The thermal stability of the purified *P. falciparum* trophozoite MDH was measured by incubating the enzyme for 7 min in the presence of substrate, at the indicated temperature, and then assaying for MDH activity at room temperature. (B) The optimum pH for the purified trophozoite enzyme was determined by measuring enzyme activity in 25 mM each of Tris, sodium phosphate, and Mes adjusted to the indicated pH. (C) MDH activity was determined in assay buffer containing varying concentrations of the substrate oxaloacetate.
TABLE II
Comparison of the *P. falciparum* trophozoite malate dehydrogenase with mammalian cytoplasmic and mitochondrial malate dehydrogenases

<table>
<thead>
<tr>
<th>MDH Source</th>
<th><em>P. falciparum</em> trophozoites&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Human erythrocytes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pig heart&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular localization</td>
<td>–</td>
<td>cytoplasmic</td>
<td>mitochondrial</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>32 kDa</td>
<td>36 kDa</td>
<td>34 kDa</td>
</tr>
<tr>
<td>Activity</td>
<td>1.00</td>
<td>1.07</td>
<td>0.45</td>
</tr>
<tr>
<td>1 mM oxaloacetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 mM oxaloacetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>0.02</td>
<td>0.14</td>
<td>0.32</td>
</tr>
<tr>
<td>NAD → NADH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH → NAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stability&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10</td>
<td>0.87</td>
<td>0.35</td>
</tr>
<tr>
<td>45°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40°C</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a</sup>Purified enzyme eluting from chromatofocusing column.

<sup>b</sup>Purified enzyme from Sigma Chemical Co.

<sup>c</sup>An aliquot of each enzyme was incubated at 40°C or 45°C for 10 min prior to assay at room temperature.

measured by the oxidation of NADH in the presence of oxaloacetate. At pH 9.0, the enzyme appeared to be about 10-fold less active (Fig. 5B). In contrast, when measuring the ability of the parasite enzyme to reduce NAD in the presence of malate, the enzyme appeared inactive at pH 7.0 and had low activity at pH 9.0 or 9.9 (Table II and data not shown).

The effect of increasing substrate concentration on the ability of *P. falciparum* MDH to oxidize NADH is shown in Fig. 5C. The parasite enzyme displays very little substrate inhibition even at 1.5 mM oxaloacetate. This characteristic is summarized in Table II as the ratio of MDH activity measured by NADH oxidation at 1 mM vs. 0.25 mM oxaloacetate.

Discussion

*P. falciparum*, like *P. lophurae* [9], has only one MDH isoenzyme during asexual intraerythrocytic growth. This MDH was purified using a simple three-step purification protocol. The key step in the development of the purification protocol was the identification of conditions under which the parasite MDH but not human MDH bound to the affinity ligand, Reactive Blue 72 agarose. The final purification step, chromatofocusing, removed the final traces of contaminating human MDH activity as well as other non-MDH contaminants.

The yield of purified parasite MDH activity was typically 11–13% of the total MDH activity in the trophozoite-enriched blood (Table I, and data not shown). Although there are always losses during the purification process, the net yield can be used to make a minimum estimate of the portion of the infected cell MDH contributed by the parasite. Since only 70% of the cells in the trophozoite-enriched blood contained parasites, the parasite MDH must contribute at least 16% of the MDH activity in an infected erythrocyte. The purified parasite MDH had a specific activity of about 80 units mg⁻¹, which represents a 780-fold purification over trophozoite-enriched blood and a 130-fold purification over saponin freed trophozoites (Table I, and data not shown). The specific activity of the purified parasite MDH is within the usual range of specific activities for purified MDHs from other species. For instance, the reported specific activity of purified *Schistosoma mansoni* cMDH was 42 units mg⁻¹ [20] and of purified bovine mMDH was 1493 units mg⁻¹ [21].

The apparent subunit molecular weight of the purified *P. falciparum* MDH is 32 kDa. This indicates that the parasite MDH is one of the smallest identified thus far. It appears to be
about the same size as *Escherichia coli* MDH [22]. Using a modification of the two-dimensional gel electrophoresis technique, it was demonstrated that the *P. falciparum* MDH activity was associated with a polypeptide of approximately 31 kDa. Under the same conditions, mammalian MDH samples also appeared to migrate slightly faster than their known subunit molecular weights. Thus, this experiment provides independent evidence that the parasite MDH activity does in fact correspond to the major 32-kDa polypeptide which was purified and visualized by SDS-PAGE. Conclusive evidence for this awaits the cloning of the *P. jalciparum* gene encoding the 32-kDa polypeptide.

Characterization of the *P. falciparum* MDH indicates that it differs from mammalian MDHs in both its enzymatic and physical properties. For instance, the thermal stability of *P. falciparum* MDH underwent a sharp transition between 40 ° and 45°C (Fig. 5A). In contrast, at these temperatures, human cMDH was fairly stable and pig mMDH, while unstable, did not have a comparably sharp transition (Table II). Similarly, under the assay conditions used, the *P. falciparum* MDH was proportionately much less active than the human cMDH or pig mMDH when malate was the substrate. It should be noted in this regard, that the high malate concentration used in these experiments has been shown to inhibit bovine cMDH [21] and *Schistosoma mansoni* cMDH and mMDH [20].

The most interesting characteristic of the *P. falciparum* MDH is its resistance to substrate inhibition by oxaloacetate. The mMDH from cows, pigs and *Schistosoma mansoni* all have optimal activity at about 0.25 mM oxaloacetate; at higher oxaloacetate concentrations their activity decreases [20,23,24]. In contrast, the cMDHs from these species are not inhibited by oxaloacetate concentrations of 1–2 mM. Thus, the *P. falciparum* trophozoite MDH interacts with oxaloacetate in a manner typical of cMDHs of other species. Further studies are needed to demonstrate that purified enzyme is actually located in the cytoplasmic compartment of *P. falciparum*.

If the *P. falciparum* trophozoite MDH is in fact the parasite cMDH, it is unlikely that its function is gluconeogenesis, since during intraerythrocytic growth glucose is supplied by the human host. The parasite MDH did not appear to have α-keto acid reductase activity as has been described for some cMDHs [10] (data not shown). The most likely function is the regeneration of NAD⁺. Sherman suggested that *Plasmodium* cMDH could reoxidize NADH produced by glycolysis in a manner analogous to lactate dehydrogenase [9]. Further NAD⁺ could be regenerated in the mitochondrion by the NADH-fumarate reductase pathway as proposed for trypanosomes [25]. In this case, cytoplasmic malate enters the mitochondrion where it is converted to fumarate by fumarase. The fumarate is the substrate for NADH-fumarate reductase, which produces succinate and oxidizes NADH to NAD⁺. The fumarate can then be regenerated by succinate dehydrogenase. Recent studies of *P. yoelli* and *P. jalciparum* mitochondria are consistent with the presence of an NADH-fumarate reductase pathway in *Plasmodium* species [26].

Finally, since *P. falciparum* trophozoites apparently contain only the cMDH isoenzyme, the mMDH isoenzyme, if present, must be developmentally regulated. This agrees with the apparently undeveloped nature of the mitochondria during the asexual intraerythrocytic stage of the life cycle [1–5]. It has been suggested that the development of mitochondrial cristae during gametocytogenesis may correspond to functional development of the organelle [3]. If so, it should be possible to use the MDH isoenzymes as markers of mitochondrial biogenesis in *P. falciparum*.

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References