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Characterization of a stable isoenzyme of malate dehydrogenase (MDH1) from blood stream Trypanosoma vivax

Wurochekke, A. U.1*, Nok, A. J.2, Inuwa, H. M2 and Gimba, C. E3

1Biochemistry Department, Federal University of Technology, Yola, Nigeria.
2Biochemistry Department, Ahmadu Bello University, Zaria, Nigeria.
3Chemistry Department, Ahmadu Bello University Zaria, Nigeria.

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MDH1, isoenzyme of malate dehydrogenase from blood stream Trypanosoma vivax was characterized. The enzyme was active over a broad pH and temperature range with optimal values of 5.0 and 35°C respectively. The energy of activation was 20.58kJ/mol and the pKa values were 6.8 and 7.8 implicating ionizable groups at the catalytic site. Kinetic studies conducted in the direction of oxaloacetate reduction gave $K_M$ of 0.56 and 0.3 mM and $V_{max}$ of 4.6 and 3.2 µmol./min/mg for oxaloacetate and NADH respectively. Similarly, in the reverse reaction, malate had $K_M$ of 0.16 Mm and $V_{max}$ of 57 µmol./min/mg, and $K_M$ and $V_{max}$ of NAD$^+$ were 0.1 5 mM and 48 µmol./min/mg. The enzyme was inhibited competitively with NAD$^+$ as a product inhibitor and uncompetitive with malate. The product inhibition studies suggest bi-bi ordered sequential mechanism of catalysis. Some TCA cycle intermediates also inhibited to different extent the activity of the enzyme.

Key words: Malate dehydrogenase, Trypanosoma vivax, isoenzyme.

INTRODUCTION

Trypanosomosis caused by parasitic trypanosomes, continue to remain as one of the major problem to animal production and economic growth in Africa. The parasites in the blood of infected individuals, escape complete elimination by the immune system due to the phenomenon of antigenic variation (Barry et al., 1979). The drugs currently used in the treatment of the disease are unsatisfactory (Barret, 1999). Most of these drugs are targeted at the cellular components, enzymes and metabolic pathways of the parasites (Gutteridge and Coom, 1977; Nok, 2002; Kita et al., 2001)

Blood stream trypanosomes generally depend on glycolysis for energy generation with pyruvate as end product. Most of the glycolytic enzymes are compartmentalize in glycosome. However in some of the trypanosome species, such as Trypanosoma vivax and Trypanosoma congolense, they produce acetate and succinate in addition to pyruvate as end products. These species of trypanosomes are believed to have some Krebs cycle enzymes that functions in the reverse direction. It has been reported during catabolism of glucose by T. vivax, oxaloacetic acid is produced in the cytosol from phosphoenol pyruvate by phosphoenol pyruvate carboxy kinase (PEPCK) in the presence of carbon dioxide. The oxaloacetate produced will be reduced in a reverse reaction by malate dehydrogenase to malate which is readily transported into the mitochondria. Pyruvate and fumarate are then produced from the malate. Fumarate will be reduced to succinate, which is the final step of the pathway, by NADH Fumarate reductase system.

Properties of the malate dehydrogenase in the above pathway are not known. Detail studies on the properties of this enzyme from the parasite will provide means of comparing it with that of the host and if significant differences exist, the enzyme may also be a target for trypanocide. Recently, we reported the existence of two isoforms of the enzyme. Here we present the characteristics of the stable isoenzyme of the malate dehydrogenase from blood stream T. vivax.

*Corresponding author. E-mail: wchekke@yahoo.co.uk.
MATERIALS AND METHODS

Experimental animals
Four apparently healthy brown goats were used. They were kept at the department of veterinary parasitology A.B.U. Zaria. They were given normal feed and water at libitum.

Trypanosome
A stablate of *Trypanosoma vivax* (Agai stain) was provided by the National Institute of Trypanosomiasis (NITR), Jos, Nigeria.

Inoculation of the Trypanosome
The apparently healthy goats were infected with approximately 10^3 parasites/ml of blood intraperitoneally. Parasitaemia level was monitored every day until peak parasitaemia was observed on day 9 post infection.

Parasitaemia determination
The wet blood film technique was used for the parasitaemia determination. Motile parasites were examined under the microscope (Mgx40) and counted as described by Herbert and Iumsden (1976).

Isolation of the Trypanosomes
Trypanosomes were separated from the blood by the method of Lanham and Godfrey (1970). At peak parasitaemia blood was drawn from the infected goat through the jugular vein using a syringe and mixed with heparin as anticoagulant. The blood was diluted with phosphate buffered saline glucose (pH 8.0) and loaded onto a column packed with DEAE-52 cellulose. The parasites were then eluted with the same buffer. Fractions containing parasites were pooled together and centrifuged at 105,000xg for 15min. The pellets, which contain pure parasites, was reconstituted and stored at -4°C.

Preparation of the crude enzyme
The reconstituted pure parasites were subjected to three different cycles of freezing and thawing to solublise the trypanosomes. After the solublisation malate dehydrogenase activity was assayed and protein content determined.

Assay for malate dehydrogenase (EC. 1. 1. 1. 37) activity
Malate dehydrogenase activity was assayed spectrophotometrically in the direction of oxaloacetate reduction, by following the disappearance of NADH at 340 nm. The assay medium contained 0.134 Mm NADH, 0.2 Mm OAA in 25 Mm phosphate buffer pH 7.2 in a total volume of 500 µl.

Ion-exchange chromatography
The enzyme was eluted on DEAE-52 cellulose by linear phosphate gradient (0.02 – 1 M). Five milliliters of the crude enzyme was dispensed in to the equilibrated column. Fifty fractions of 5 ml each were collected and assayed for MDH activity and protein content determined.

Effect of pH on the partially purified enzyme
The activity profile of the partially purified enzyme was determined as function of pH. The following buffers were used in the pH analysis, acetate buffer pH 8.5 - 6.0, phosphate buffer pH 6.5 - 8.0 and tris- glycine buffer pH 8.5 - 9.5.

Effect of temperature on the partially purified enzyme
About 100 µl of the partially purified enzyme was dispensed in to 10 different test tubes and incubated at the following temperatures 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50°C, for 10 min. The enzyme reaction medium was also incubated at the various temperatures for the given time. At the end of the incubation period, the enzyme was quickly transferred in to the reaction medium and the activity measured as earlier described.

Initial velocity studies
The activity of the partially purified enzyme was determined at varying concentration of NADH while OAA was kept as the changing fixed variable. In the reverse reaction conducted at pH 9.5, L- malic and NAD⁺ were used as changing fixed and variable substrates respectively and vice versa.

Product inhibition studies
At different fixed concentrations of NAD⁺ as one of the product, NADH was used as the variable substrate while OAA remained fixed at sub saturating level. On the other hand L-malic acid was used as a product inhibitor at fixed concentration of NADH and variable OAA.

Inhibition by some natural occurring metabolites
Ten milliliters concentration of each of the following metabolites; &-ketoglutaric acid, Isocitric acid, Succinic acid, aspartic acid, glutamic acid was incubated with the enzyme assay medium and the activity of the enzyme determined as described. The percentage inhibition of each of the metabolites was calculated.

RESULTS

After three cycles of freezing and thawing, total and specific activity of the enzyme was determined and is presented in Table 1. Linear phosphate gradient elution on DEAE cellulose resulted into two distinct peaks Figure 1. The active peak gave purification fold of 27.1 and percentage recovery of 56.4%.

Initial pH analysis on the enzyme activity determined at pH range of 3.5-9.0 gave optimum pH of 5.0. Dixon plot of log Vmax /Km versus pH gave pKa values of 6.8 and 7.8. The enzyme show broad temperature range (20 - 40°C) of activity with optimum at 35°C. Arrhenius plot of log Vo as function of reciprocal of standard temperature gave activation energy (Ea) of 20.58k/mol

Secondary plots (Figures 2 and 3) of ordinate intercept as a function of fixed substrate concentration of NADH and OAA represents the replots of the primary plot data in the forward reaction. Direct extrapolation from the
Table 1. Purification table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (cm³)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity/min ml</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification fold</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>12</td>
<td>2.0</td>
<td>24.0</td>
<td>0.0051</td>
<td>0.0612</td>
<td>0.00255</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE Cellulose Chromatography</td>
<td>5</td>
<td>0.1</td>
<td>0.5</td>
<td>0.0069</td>
<td>0.0345</td>
<td>0.069</td>
<td>27.1</td>
<td>56.4</td>
</tr>
<tr>
<td>Peak 1 (MDH₁)</td>
<td>5</td>
<td>0.1</td>
<td>0.5</td>
<td>0.0016</td>
<td>0.0080</td>
<td>0.016</td>
<td>6.3</td>
<td>13</td>
</tr>
<tr>
<td>DEAE – Cellulose chromatography</td>
<td>5</td>
<td>0.1</td>
<td>0.5</td>
<td>0.0016</td>
<td>0.0080</td>
<td>0.016</td>
<td>6.3</td>
<td>13</td>
</tr>
</tbody>
</table>

Figure 1. Elution profile of MDH by linear phosphate gradient from DEAE 52 cellulase

secondary plots gave $K_M$ values of 0.38 and 0.56 mM, and $V_{max}$ of 3.2 and 4.6 µmol/min/mg for NADH and OAA respectively.

Similarly, in the reverse reaction, secondary analysis performed with NAD⁺ and L-malate as fixed substrate gave $K_M$ of 0.16 mM and $V_{max}$ of 48 µmol/min/mg for NAD⁺, for L-malate the $K_M$ and $V_{max}$ were 0.16 mM and 57 µmol/min/mg (Figures 4 and 5).

Double reciprocal plot of velocity against NADH at a fixed saturating level of OAA, with NAD⁺ as changing fixed inhibitor, showed competitive inhibition (Figure 6).

Uncompetitive inhibition was observed when L-malate was used as the inhibitor at a fixed saturating level of NADH (Figure 7).

Percentage inhibition by some natural occurring metabolites is presented in Table 2. Aspartic acid inhibited the activity of the enzyme by 56.7%.

DISCUSSION

MDH1 was purified to about 27.1 fold from the crude with 56.4% recovery by one purification step. This implies that a pure enzyme can be resolved from a mixture by one step purification protocol. Adapting suitable and precise purification step will enhance purification fold and minimize contamination and inactivation of enzymes.

The $K_M$ values for the substrates and coenzymes suggest that the enzyme has higher affinity to malate-NAD⁺ than oxaloacetate-NADH. A similar pattern was observed in cytosolic malate dehydrogenase isolated from Taenia
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Figure 2. Secondary Plot with the Intercepts to determine the $K_M$ and $V_{max}$ of NADH. $V_{max} = 3.2 \mu\text{mol .min}^{-1}\text{mg}^{-1}$; $K_M = 0.38 \text{mM}$.

Figure 3. Secondary plot with the intercepts to determine the $K_M$ and $V_{max}$ of OAA. $V_{max} = 4.6 \mu\text{mol .min}^{-1}\text{mg}^{-1}$; $K_M = 0.56 \text{mM}$

$crassiceps cysticerci$ (Zenka and prokopic, 1989). MDH1 have shown considerable amount of activity over a broad range of pH with optimal at 5.0. This result compares favorably with previous reports of malate dehydrogenase isolated from other parasites (Lang-Unnasch, 1992; Zenka and Prokopic, 1989). Analysis of the effect of pH on $V_{max}$ and $K_M$ suggest that the presence of ionizable groups associated with the catalysis of the enzyme. The pKa values found were 6.8 and 7.8 implicating histidine and cystein as the possible amino acids at the catalytic site.

Rate of reaction may increase with increase in temperature, but at high temperatures secondary and tertiary structures of enzymes are destroyed and their activity impaired. Isoenzyme of malate dehydrogenase has different susceptibility to thermal inactivation. Some reports indicated that mitochondrial malate dehydrogenase is more susceptible to thermal inactivation as compared to the cytosolic isoenzyme (Masini et al., 1986). In the present work, the optimum temperature of MDH1 was 35°C which is not unexpected since the blood stream parasites must be in equilibrium with physiological body temperature of the animal.

Initial velocity studies on MDH1 suggested sequential or Theorell chance mechanism of catalysis. However, product inhibition studies to determine order of binding of substrates and release of products supported sequential mechanism and rules out Theorell chance mechanism. With NAD$^+$ as a product inhibitor, a competitive inhibition was observed. The inhibitor could bind to one of the sub-
Figure 4. Secondary plot with the intercepts to determine the \( K_M \) and \( V_{\text{max}} \) of NAD\(^+\); 
\( V_{\text{max}} = 4.8 \times 10^{-1} \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}; \ K_M = 0.15 \text{ mM} \)

Figure 5. Secondary plot with the intercepts to determine the \( K_M \) and \( V_{\text{max}} \) of malic acid. 
\( V_{\text{max}} = 5.7 \times 10^{-1} \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}; \ K_M = 0.16 \text{ mM} \)
substrate to form inactive binary complex (that is, enzyme-substrate) or it can form inactive ternary complex (substrate-enzyme-product) there by reducing the activity of the enzyme. On the other hand uncompetitive inhibition was observed when malate was used as a product inhibitor. This means that inactive ternary complex (enzyme-substrate-product) might have been formed. The overall results of initial velocity and product inhibition studies suggest ordered bi-bi sequential mechanism. There could be initial binding of NADH and final release of NAD$^+$ or initial binding of oxaloacetate and final release of malate.

$$E + \text{NADH} \rightarrow \text{E-NADH} + \text{OAA} \rightarrow \text{(E-NADH-OAA \rightarrow \text{E-NAD}^+ - \text{MALATE})}$$

$$\text{E-NAD}^+ - \text{MALATE} \rightarrow \text{E-NAD}^+ + \text{MALATE} \rightarrow \text{E + NAD}^+$$

MDH1 was also inhibited to different extents by some of the TCA cycle metabolites. It is expected that intermediate of the TCA cycle inhibits mitochondrial malate dehydrogenase. Aspartate which can readily cross the inner mitochondrial membrane is formed from oxaloacetate by transamination and it has significantly inhibited the activity of the enzyme. MDH1 could be a cytosolic malate dehydrogenase catalyzing the reduction of oxaloacetate to malate in the cytosol of blood stream T. vivax.

Although the properties of malate dehydrogenase observed in the present work, compares favorably with previous reports of MDHs from other parasites, there exist also some differences in the properties compared to MDHs from plants and animals. For instance, MDH isolated from the bass liver (Madero, 1986) require bivalent cation (Mn$^+$ or Mg$^{2+}$) for its catalysis. Other previous reports include MDH isolated Tritrichomonas fe-

**Table 2. Percentage inhibition of some naturally occurring metabolites.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>10 mM Pyruvic acid</th>
<th>10 mM Aspartic acid</th>
<th>10 Mm Glutamic acid</th>
<th>10 Mm Succinic acid</th>
<th>10 Mm α-ketogluterate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage inhibition</td>
<td>29.7</td>
<td>56.7</td>
<td>16.2</td>
<td>48.6</td>
<td>27.0</td>
</tr>
</tbody>
</table>

**Figure 6.** Product inhibition with NAD$^+$ as changing fixed.
fetus (Hurdy, 1993), human brain (Bukato et al., 1995) and Toxocara canis muscle (Masini, 1996). Significant differences between the parasite MDH and that of the host form the basis for blocking the enzyme without affecting that of the host.

The present work provided basic information on the properties of malate dehydrogenase from blood stream T. vivax and pave way to the formulation of potential inhibitors against its activity.

REFERENCES