International Journal of Physical Sciences Vol. 3 (9), pp. 224-230, September 2008
Available online at http://www.academicjournals.org/IJPS
ISSN 1992 - 1950 © 2008 Academic Journals

Full Length Research Paper

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

Wurochekke, A. U.[1]*, Nok, A. J.[2], Inuwa, H. M[2] and Gimba, C. E[3]

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

Accepted September 5, 2008

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 phenolmenon 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. Parasiteamia level was monitored every day until peak parasiteamia was observed on day 9 post infection.

Parasiteamia determination

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

Isolation of the Trypanosomes

Trypanosomes were separated from the blood by the method of Lanham and Godfrey (`1970). At peak parasiteamia 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^{o}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 solublization 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^{o}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; &-ketogluteric 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 V_{max}/K_M versus pH gave pKa values of 6.8 and 7.8.

The enzyme show broad temperature range (20 - 40^{o}C) of activity with optimum at 35^{o}C. Arrhenius plot of log Vo as function of reciprocal of standard temperature gave activation energy (E_a) of 20.58kj/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.

Step	Volume (cm^3)	Protein (mg/ml)	Total protein (mg)	Activity/min ml	Total activity (units)	Specific activity (units/mg)	Purification fold	% yield
Crude	12	2.0	24.0	0.0051	0.0612	0.00255	1	100
DEAE Cellulose Chromatography Peak 1(MDH_1)	5	0.1	0.5	0.0069	0.0345	0.069	27.1	56.4
DEAE – Cellulose chromatography Peak 2 (MDH_2)	5	0.1	0.5	0.0016	0.0080	0.016	6.3	13

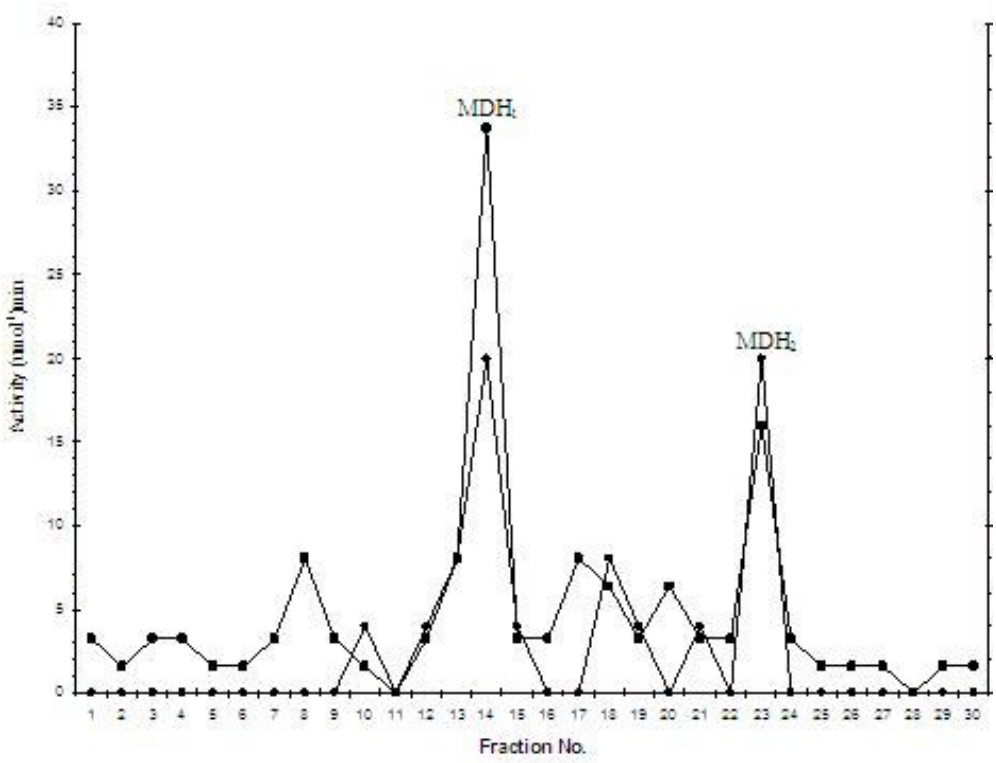

Figure 1. Elution profile of MDH by linear phosphate gradient from DEAE 52 cellulase

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*

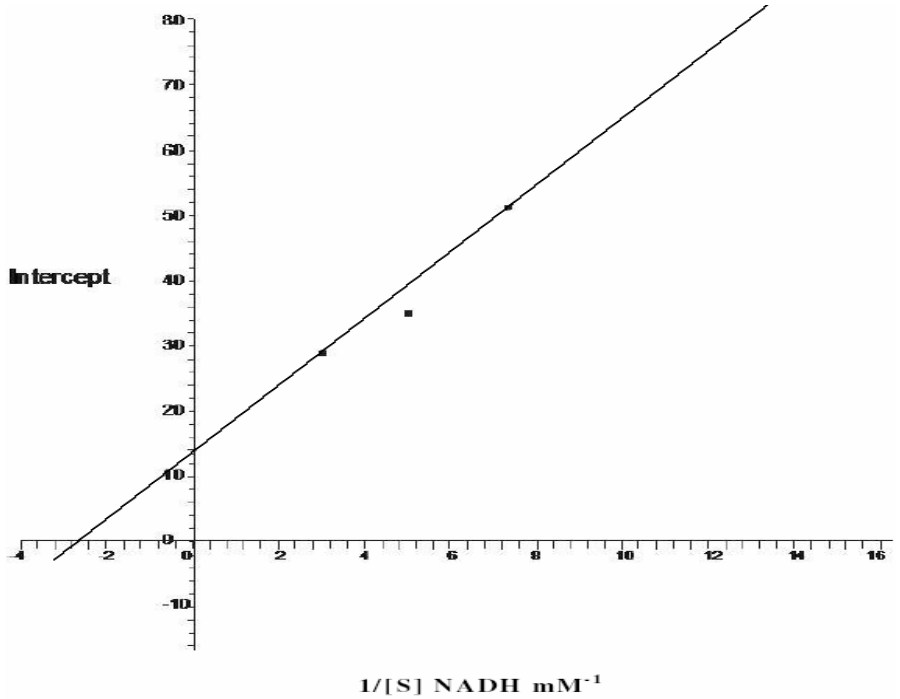

Figure 2. Secondary Plot with the Intercepts to determine the K_M and V_{max} of NADH. V_{max} = 3.2 µmol .min^{-1}mg^{-1}; K_M = 0.38mM.

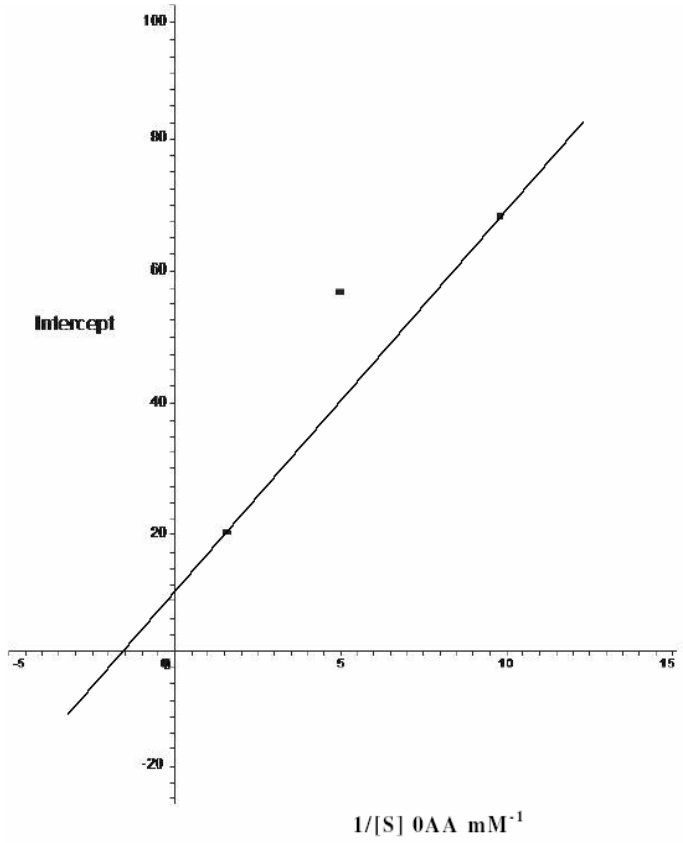

Figure 3. Secondary plot with the intercepts to determine the K_M and V_{max} of OAA.V_{max} = 4.6 µmol .min^{-1}mg^{-1}; K_M = 0.56 mM

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 dehydrogenases 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^{o}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-

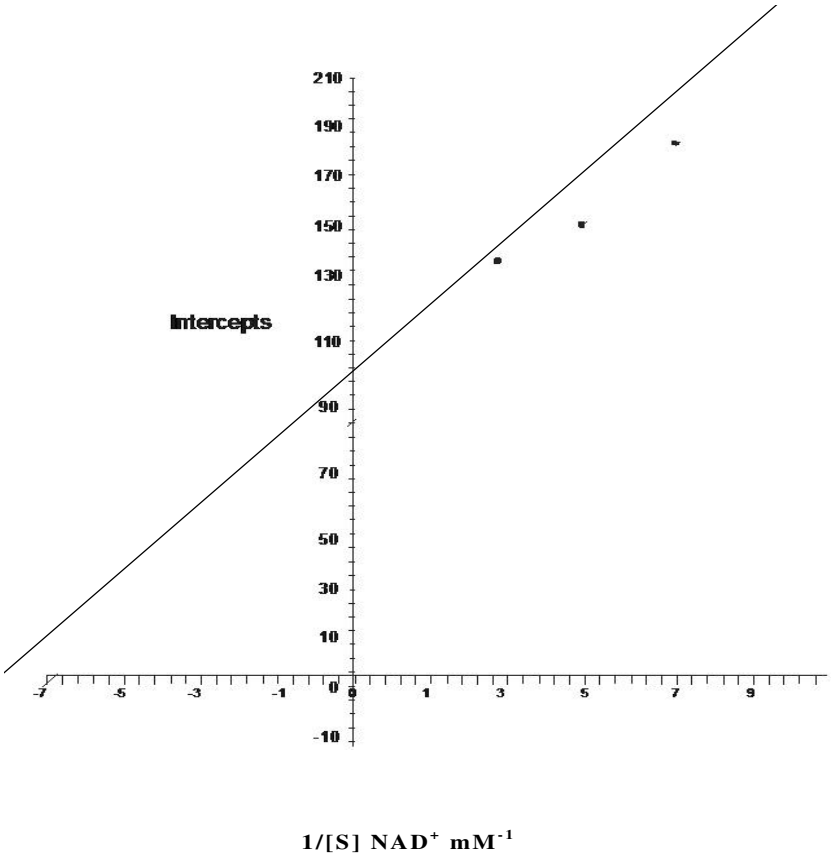

Figure 4. Secondary Plot with the Intercepts to determine the K_M and V_{max} of NAD^+; V_{max} = 4.8 x 10^{-1}µmol .$min^{-1}$$mg^{-1}$; K_M = 0.15 mM

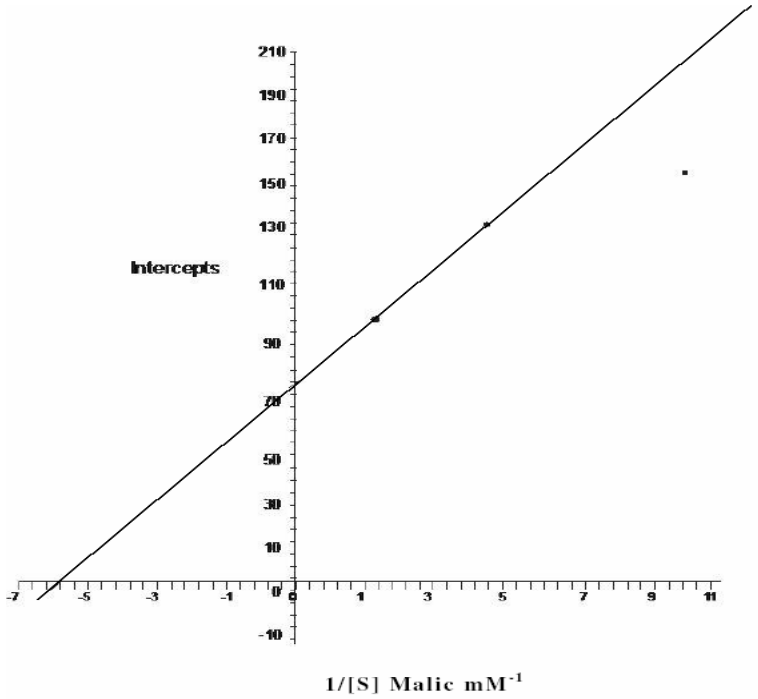

Figure 5. Secondary plot with the intercepts to determine the K_M and V_{max} of malic acid. V_{max} = 5.7 x 10^{-1}µmol .$min^{-1}$$mg^{-1}$; K_M = 0.16 mM

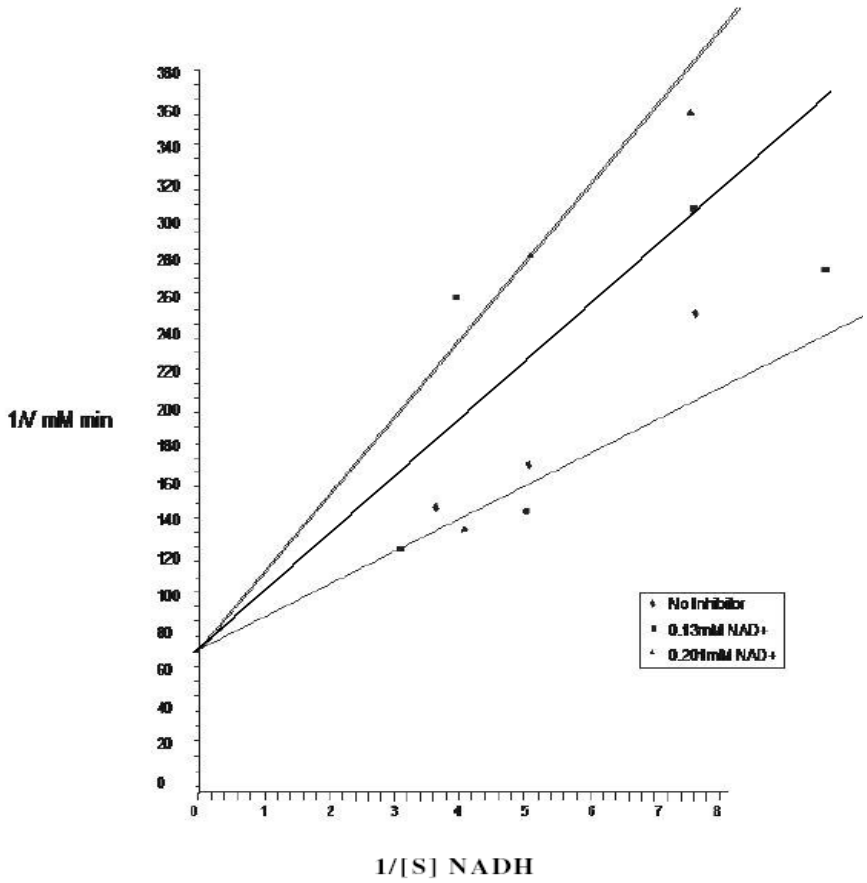

Figure 6. Product inhibition with NAD+ as changing fixed.

Table 2. Percentage inhibition of some naturally occurring metabolites.

Inhibitor	10 mM Pyruvic acid	10 mM Aspartic acid	10 Mm Glutamic acid	10 Mm Succinic acid	10 Mm α-ketogluterate
Percentage inhibition	29.7	56.7	16.2	48.6	27.0

strate 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 + NADH------ E-NADH + OAA ------- (E-NADH-OAA ---- E-NAD^+-MALATE)

E-NAD^+-MALATE--------E-NAD^+ + MALATE-------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-

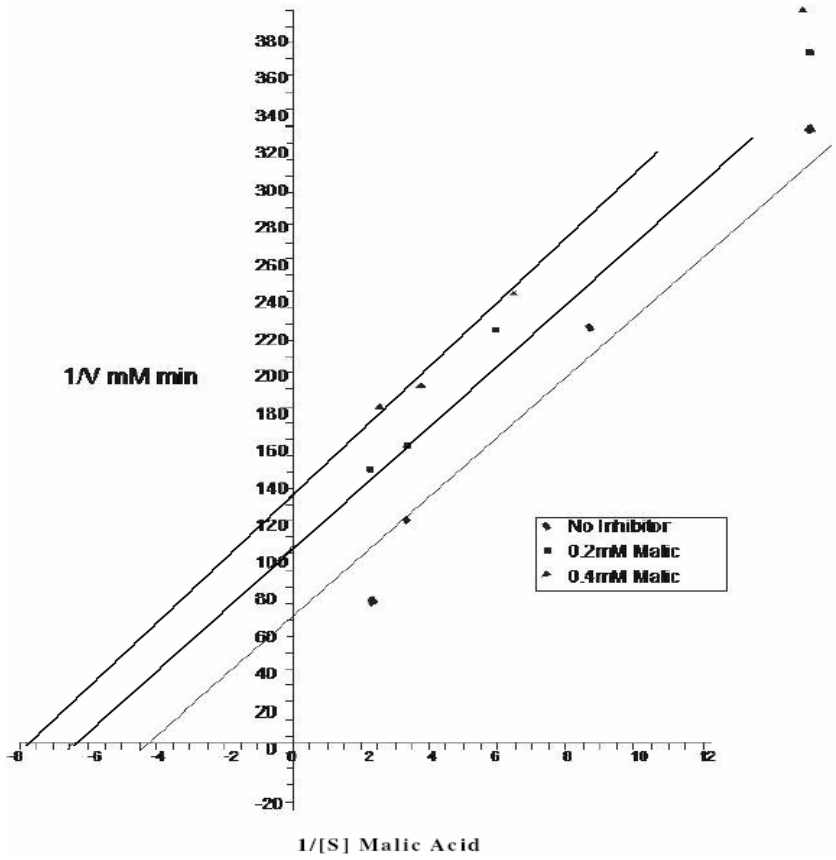

Figure 7. Product inhibition with malic acid as changing fixed

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

Barret MP, Fairlamb AH (1999). The biochemical basis of arsenical diamidine cross resistance in African trypanosomes. Parasitol. 15: 136-140.

Barry JD, Hajduck SL, Vickerman K, Lery D (1979). Detection of multiple variable antigen type in metacyclic population of T. brucei Trans. R. Soc. Trop. Med. Hyg. 73: 205-208.

Bukato G, Kochan Z, Swierczynsk J (1995). Purification and properties of cytosolic and mitochondrial malic enzyme isolated from human brain. Int. J. Biochem. Cell Biol. 27(1): 47-54.

Gutteridge WE, Coomb GH (1977). Catabolism and generation of energy. In Biology of parasitic protozoa. Macmillan Press Ltd., London. Pp. 32-58.

Herbert WJ, Lumsden WAR (1976). Trypanosoma brucei. A rapid "matching" method of estimating host parasiteamia. Experimental parasitology.

Hurdy I (1993). Purification and partial characterization of cytosolic malate dehydrogenase from Trychomonas fetus. Folia parasitol. 40(3): 181-185.

Kita K, Miyadera H, Saruta F, Miyoshi H (2001). Parasite mitochondria as target for chemotherapy. Journal of health sciences. 47(3): 219-239.

Lang- Unnasch N (1992). Purification and properties of plasmodium falciparum malate dehydrogenase. Mol. Biochem. Parasitol. 50(1): 17 25.

Lanham SH, Godfrey DG (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE cellulose. Expl. Parasit. 28: 521-534.

Madero E, Gallego-Iniesta M, Garrido-Pertierra A (1986). Properties and function of mitochondrial malate enzyme from bass liver. Comp. Biochem. Physiol. 83(4): 861-866.

Masini E, Oestreicher EG, Ribeiro LP (1986). Purification and properties of mitochondrial malate dehydrogenase of Toxocara canis muscle. Comp. Biochem. Physiol. 85(1): 223-228.

Nok JA (2002). Azanthraquinone inhibits respiration and invitro growth of long slender blood stream forms of Trypanosoma congolense. Cell Biochem. Funct. 20: 205 – 212.

Webster K, Pingle TR, Osborn M (1972). Measurement of molecular weights by electrophoresis on SDS- Acrylamide gels. In: methods in enzymol, 26, (Fleischer, S. and Parker, L. Eds). Academic Press New York. Pp. 3-18.

Zenka J, Prokopic J (1989). Purification and properties of cytoplasmic malate dehydrogenase from taenia crassiceps (Zeder, 1800) cysticerci. Folia parasitol (praha). 36(1): 59-65.