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Trichomonas vaginalis: Localization and Characterization of Malate Dehydrogenase and NAD$^+$-linked Malic Enzyme

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Introduction

Although *Trichomonas vaginalis* lacks mitochondria (Nielsen *et al.*, 1966), cytochromes (Wellerson *et al.*, 1959; Tanabe and Hayashi, unpublished data) as well as a functional tricarboxylic acid cycle (Read, 1957; Wellerson *et al.*, 1960), it actively consumes oxygen upon addition either NADH (Tanabe, 1979) or malate and NAD$^+$ (Ninomiya and Suzuoki, 1952; Asami, 1956). Baernstein (1963), on basis of inhibition studies, first proposed that this respiration was catalyzed by a terminal flavoprotein oxidase.

We have been interested particularly in the mechanism of malate oxidation and found that malate was not directly oxidized. We have also demonstrated a NADH oxidase, which catalyzes NADH oxidation in presence of oxygen, in this parasite (Tanabe, 1979), and suggested that malate is first dehydrogenated by malate dehydrogenase (Tanaka, 1971; Brugerolle and Metenier, 1973) and NADH generated is subsequently oxidized by NADH oxidase which utilizes oxygen as a physiological electron acceptor.

In our preliminary characterization of malate dehydrogenase (Tanabe *et al.*, 1976) we detected two types of the enzyme activities, differing in subcellular localization and enzymatic properties, in this organism. This communication deals with purification, subcellular localization and characterization of these two malate dehydrogenases in *T. vaginalis*.

Materials and Methods

Parasite: *Trichomonas vaginalis* (73 strain) was cultivated stationarily in a Erlenmeyer flask containing 120 ml of the Asami medium (Asami, 1952). Detailed procedures of cultivation and harvesting of the parasites have been described previously (Tanabe, 1979).

The parasites were washed twice and finally suspended in 5 mM Tris-HCl, pH 7.4 containing 250 mM sucrose and 1 mM 2-mercaptoethanol at ratio of 1 ml of packed cell per 9 ml of the Tris-buffered sucrose. The parasites suspended in the Tris-buffered sucrose were disrupted by homogenization for 2 min using a glass homogenizer with a Teflon pestle. The homogenate was fractionated by differential centrifugation as follows: a nuclear fraction (600 g×7 min sediment), a large granule fraction (13,000 g×10 min sediment), a small granule fraction (105,000 g×90 min sediment) and a final supernatant fraction (105,000 g×90 min supernatant).

Chemicals: CM-Sephadex C-50, DEAE-Sephadex A-25, Sephadex G-100, Sephadex G-200 and Blue Dextran 2,000 were obtained from Pharmacia Fine Chemicals Japan (Tokyo, Japan). Cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), al-

bumin (hen egg) and aldolase (rabbit muscle) were obtained from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Lactic dehydrogenase from rabbit muscle and diazotate-6-benzamide-4-methoxy-m-toluidine chloride were obtained from Sigma Chemical Co. (St. Louis, MO. USA). NAD^+, $NADP^+$, NADH and NADPH were purchased from Oriental Yeast (Tokyo, Japan). All chemicals were of the highest purity commercially available.

Enzyme assay : The enzymatic activity of malate dehydrogenase was assayed at room temperature by measuring the initial velocity of NAD^+ reduction or NADH oxidation spectrophotometrically with Hitachi-Perkin Elmer 139 type spectrophotometer (Hitachi Co., Tokyo, Japan). The standard assay mixture for NAD^+ reduction contained 10 mM L-malate, 670 μM NAD^+, 2.2 μg of purified enzyme protein and 66.7 mM glycine buffer, pH 10.2 in a final volume of 3.0 ml. The assay mixture of the reverse reaction contained 330 μM oxaloacetate, 330 μM NADH, 0.25 μg of purified enzyme protein and 66.7 mM glycine buffer, pH 9.0 in a final volume of 3.0 ml.

The enzymatic activity of NAD^+-linked malic enzyme was assayed by measuring the initial velocity of NAD^+ reduction in the same manner as above. The standard assay mixture contained 2 mM L-malate, 330 μM NAD^+, 1 mM $MnCl_2$, 0.8 μg of purified enzyme protein and 66.7 mM glycine buffer, pH 8.6 in a final volume of 3.0 ml.

One unit of the enzyme was defined as the amount needed for reduction of 1 micromole of NAD^+ or oxidation of 1 micromole of NADH per min.

Other assays : Protein was determined by Lowry's Folin-phenol procedure (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Oxaloacetate was determined by the colorimetric method of Katsunuma and Nishii (1975). Pyruvate was determined by enzymatic method using lactic dehydrogenase (Czok and Lamprecht, 1974).

Molecular weight determination : The molecular weights of malic enzyme and malate dehydrogenase were estimated by chromatography on Sephadex G-200 using a 10×500 mm column equilibrated with 30 mM phosphate buffer containing 1 mM 2-mercaptoethanol, pH 7.4. The void volume (Vo) was determined by elution of Blue Dextran 2,000. The column was calibrated by several standard molecules as follows ; cytochrome *c* from horse heart, chymotrypsinogen A from bovine pancreas, albumin from hen egg and aldolase from rabbit muscle.

Purification of NAD^+-linked malic enzyme : The large granule fraction (13,000 g×10 min sediment) was suspended in 30 mM sodium phosphate buffer, pH 7.4 containing 1 mM 2-mercaptoethanol (SPM buffer) and sonicated at 9 kHz for 15 min. The supernatant fraction was isolated by centrifugation at 18,000 g for 20 min, and brought to 50 % saturation by adding solid ammonium sulfate with stirring. After stirred for 60 min, the supernatant fluid was isolated by centrifugation. This fraction was also brought to 75 % saturation and the precipitate was isolated by centrifugation in the same manner as above. The precipitate was dissolved in 10 ml of the SPM buffer and dialyzed against 500 ml of the same buffer overnight. The dialysate was concentrated on a collodion bag and applied to a Sephadex G-100 column (20× 800 mm) previously washed and equilibrated with the SPM buffer. The elution speed was 7 ml per hour. The second protein peak, which contained most activity, was pooled and dialyzed against the SPM buffer overnight. Subsequently the dialysate was concentrated and applied to a DEAE-Sephadex A-25 column (15×250 mm). The sample was eluted on a linear gradient of sodium chloride (zero to 200 mM in the SPM buffer). Most activity was concentrated in the non-adsorbed protein fraction. The active fraction (the first protein peak) was used for the enzyme assay. All procedures were done at 4 C.

Purification of malate dehydrogenase : The final supernatant fluid (105,000 g×90 min) was dialyzed against 2 liters of 30 mM potassium phosphate buffer, pH 6.5 containing 1 mM 2-mercaptoethanol (PPM buffer) for four hours. Solid ammonium sulfate was added

to the dialyzed sample to make 60% saturation with stirring. After stirred for 60 min, the sample was centrifuged and the supernatant fluid was isolated. Ammonium sulfate was again added to the supernatant fluid to make 90% saturation and the precipitate was isolated by centrifugation. The resulting precipitate was dissolved in 10 ml of the PPM buffer and dialyzed against 500 ml of the same buffer overnight. The dialyzed sample was concentrated on a collodion bag and applied to a CM-Sephadex C-50 (20×400 mm) column previously equilibrated with 1 liter of the PPM buffer. The sample was eluted on a linear gradient of sodium chloride (zero to 300 mM in the PPM buffer). The elution speed was approximately 20 ml per hour. The second protein peak, which contained most activity, was dialyzed against 2 liters of the SPM buffer overnight. The dialyzed sample was concentrated in the same manner as above and applied to the column of Sephadex G-100 (20×800 mm). Elution was performed with the SPM buffer. The elution speed was 7 ml per hour. The active fraction (the second protein peak) was used for the enzyme assay. All procedures were done at 4 C.

Results

Enzyme purification : Purification procedures of malate dehydrogenase and NAD^+-linked malic enzyme of *Trichomonas vaginalis* were summarized in Table 1 and 2 respectively. The specific activity of malate dehydrogenase isolated by Sephadex G-100 chromatography increased 20-fold compared with the starting material.

The specific activity of malic enzyme in the first protein peak of DEAE-Sephadex A-25 chromatography increased 23-fold compared to that of 13,000 g sediment.

Product analysis of the purified enzymes : Product analysis of the purified enzyme from the soluble fraction indicated that the concentration of oxaloacetate formed approximates that of reduced NAD^+ (Table 3).

Table 1 Purification of malate dehydrogenase in *Trichomonas vaginalis*

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
105,000g supernatant	100	128	666	5.2	100
Ammonium sulfate (60–90%)	12	26	543	20.9	81
CM-Sephadex C-50	3	4.6	385	83.7	58
Sephadex G-100	3	1.6	173	108.0	26

The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text.

Table 2 Purification of NAD^+-linked malic enzyme in *Trichomonas vaginalis*

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
13,000g sediment	50	49	66	1.4	100
Sonic extract	48	21	37	1.8	56
Ammonium sulfate (50–75%)	3	5	31	6.2	48
Sephadex G-100	3	1.2	22	18.5	34
DEAE-Sephadex A-25	4	0.4	12	30.9	19

The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text.

NAD^+- and $NADP^+$-linked malic enzymes, lactic dehydrogenase and NADH oxidase were not detected in this fraction. These evidences, therefore, indicate that this enzyme is NAD^+-linked malate dehydrogenase.

As shown in Table 3 the ratio of NAD^+ reduction to pyruvate formation was approximately 1 with the purified enzyme from 13,000 g sediment, indicating that this enzyme catalyzes the oxidation of L-malate to pyruvate with the reduction of NAD^+. Malate dehydrogenase, $NADP^+$-linked malic enzyme and lactic dehydrogenase were also not detected in the purified enzyme fraction. Thus, it is concluded that this enzyme is NAD^+-linked malic enzyme.

Subcellular distributions of malate dehydrogenase and NAD^+-linked malic enzyme : Subcellular localization of malate dehydrogenase and NAD^+-linked malic enzyme was investigated using fractions isolated by differential centrifugation (Table 4). It was found that malate dehydrogenase was present in the 105,000 g supernatant (the soluble) fraction, whereas malic enzyme was largely associated with the 13,000 g sediment (the large granule) fraction.

Characterization of malate dehydrogenase and NAD^+-linked malic enzyme : The purified malate dehydrogenase could oxidize D-malate besides L-malate, although the rate of oxidation was less than 5% of that observed with L-malate. The purified malic enzyme catalyzed oxidation of L-malate, but not D-malate, L-lactic acid, D- or L-tartarate, succinate and tartronate. $NADP^+$ was not functional as an electron acceptor.

The molecular weight of malate dehydrogenase and malic enzyme were estimated by chromatography on Sephadex G-200. When the Kav value of malate dehydrogenase was plotted as a function of the logarithm of

Table 3 Product analysis of malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis*

Enzyme	NAD^+ reduced	Pyruvate formed	Oxaloacetate formed
		μmoles/min/mg protein	
NAD^+-linked malic enzyme	29.4	28.8	Not determined
Malate dehydrogenase	12.1	Nil	12.0

The concentration of NAD^+ reduced was determined spectrophotometrically as in the text. For determinations of oxaloacetate and pyruvate, the neutralized supernatant fluids of perchloric acid extracts of the assay mixture were isolated and used, after enzymatic reactions were conducted for 2 min.

Table 4 Subcellular distribution of malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis* homogenate

Fraction	Total volume (ml)	Total protein (mg)	Malate dehydrogenase	NAD^+-linked malic enzyme
			μmoles/min/mg protein	
Whole homogenate	13.0	39.4	2.15(1.00*)	0.59(1.00*)
600g sediment	5.0	11.3	0.63(0.29)	0.53(0.89)
13,000g sediment	5.0	8.4	0.18(0.09)	1.15(1.95)
105,000g sediment	7.0	4.4	0.60(0.30)	0.06(0.10)
105,000g supernatant	9.8	13.9	5.51(2.56)	0.06(0.10)

* Relative specific activity

Malate dehydrogenase activity was determined by spectrophotometric method. The activity of NAD^+-linked malic enzyme was estimated by the enzymatic determination of pyruvate. Details of the assays are given in the text and the legend to Table 3.

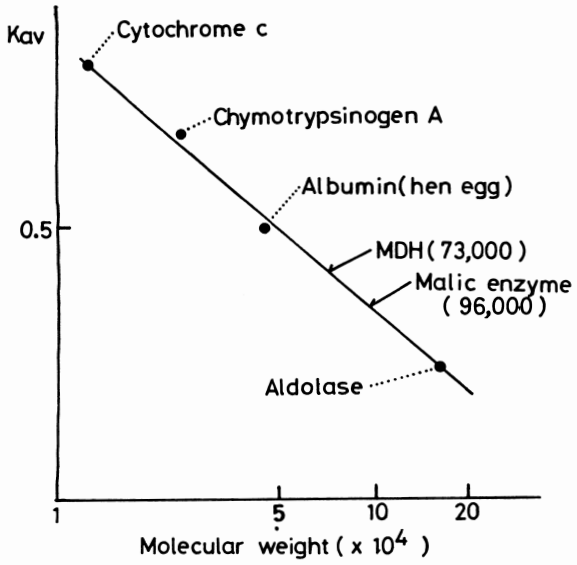

Fig. 1 Molecular weight determination of malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis* by Sephadex G-200 gel filtration.

Kav was determined by the method described in the text, where Kav=(Ve-Vo)/(Vt-Vo): Vo, void volume; Vt, bed volume; Ve, elution volume. Each point was the average of 2 determinations.

molecular weight of protein eluted from Sephadex (Fig. 1), a value of 73,000 daltons was obtained. The molecular weight of malic enzyme was estimated to be 96,000 daltons.

Kinetic experiments showed that the apparent Km values of malate dehydrogenase for L-malate, oxaloacetate, NAD^+ and NADH were 520, 100, 150 and 25 μM respectively (Figs. 2 and 3). Vmax was calculated to be 10.2 μmoles of NAD^+ reduced/min/mg protein in the forward reaction and was 112.0 μmoles of NADH oxidized/min/mg protein in the reverse reaction. Substrate inhibition of malate dehydrogenase either by L-malate or oxaloacetate was observed at 20 mM L-malate or 3 mM oxaloacetate. Apparent Km values of malic enzyme for L-malate and NAD^+ were 210 and 13 μM respectively. Vmax was approximately 30.0 μmoles of NAD^+ reduced/min/mg protein (Fig. 4).

Effect of pH on malate dehydrogenase and malic enzyme (Fig. 5) indicated that an optimal pH of malate dehydrogenase was 10.2 for the forward reaction and 9.3 for the reverse reaction under the present assay

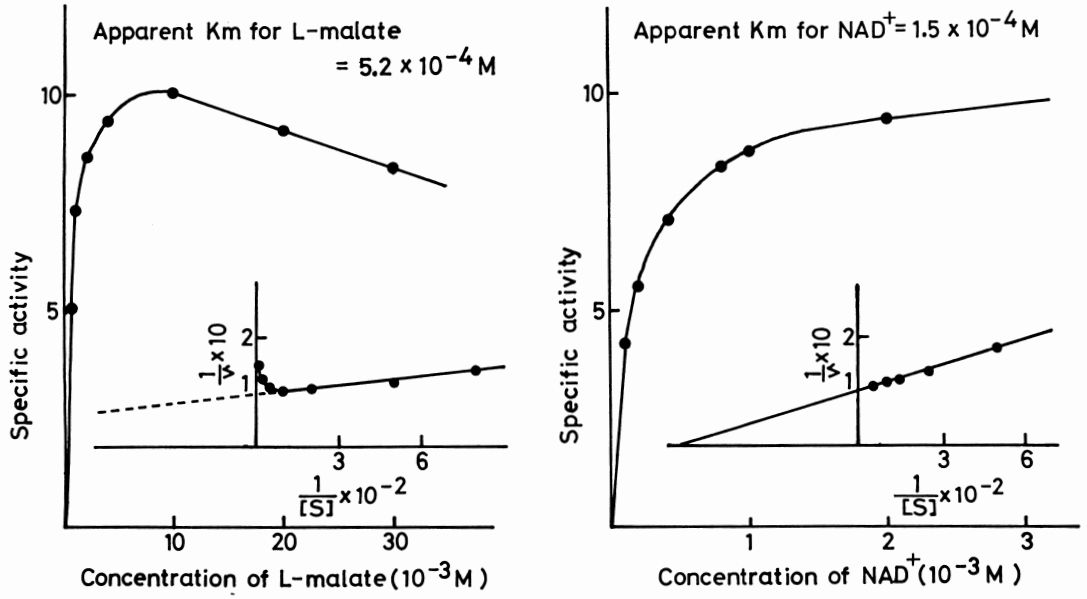

Fig. 2 Kinetic study of malate dehydrogenase in *Trichomonas vaginalis*.
The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text.

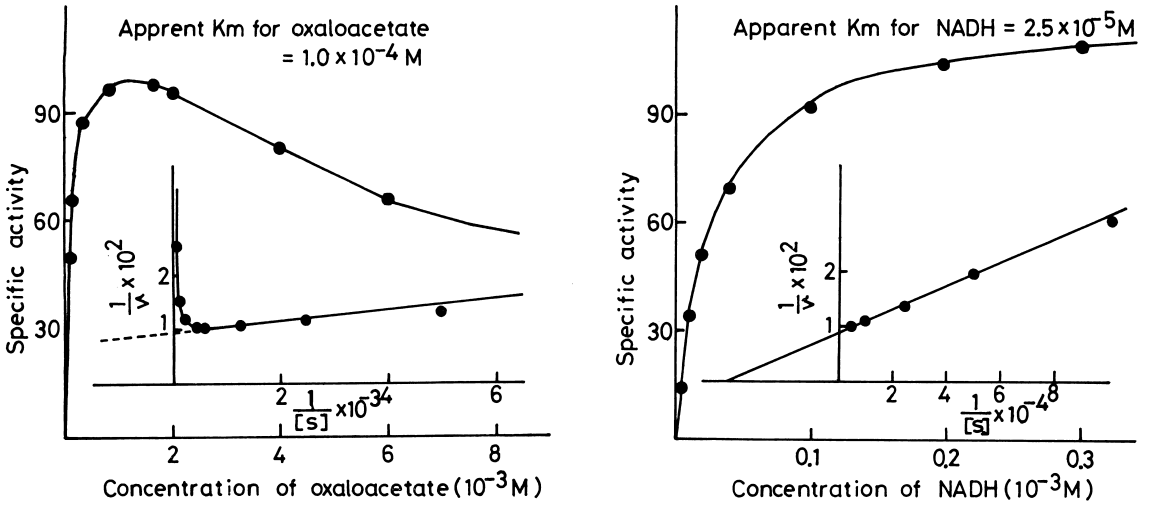

Fig. 3 Kinetic study of malate dehydrogenase in *Trichomonas vaginalis*.
The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text.

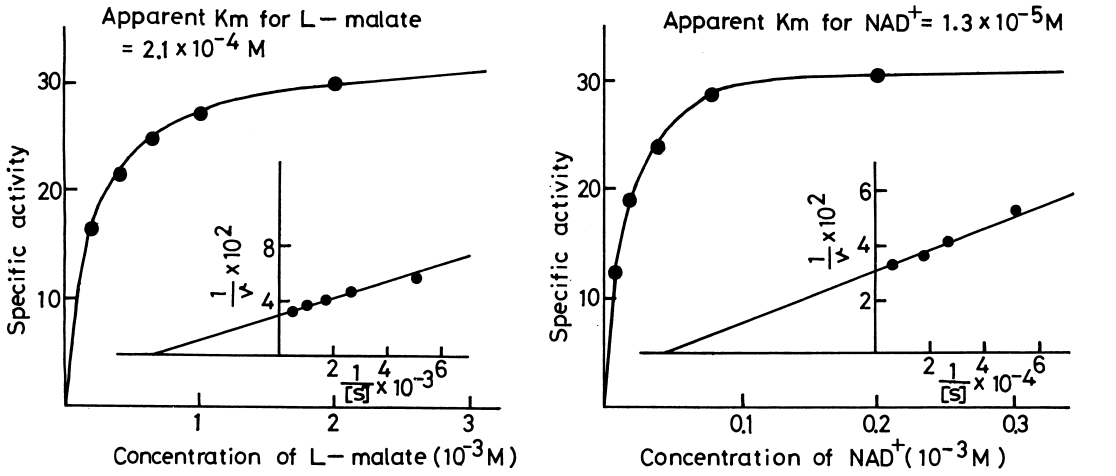

Fig. 4 Kinetic study of NAD^+-linked malic enzyme in *Trichomonas vaginalis*.
The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text.

conditions. An optimal pH of the forward reaction of malic enzyme was 8.3.

Effect of divalent cations on malate dehydrogenase and malic enzyme is summarized in Table 5 showing that the activity of malic enzyme was markedly increased by addition of $MnCl_2$. Mg^{++} and Co^{++} were, however, considerably less effective. These cations had no effect on malate dehydrogenase at the concentrations tested.

As shown in Table 6 the purified malic enzyme was inhibited by EDTA-2Na (0.5 mM) and heavy metal ions such as $AgNO_3$ (0.1 mM), $HgCl_2$ (0.01 mM) and $CuSO_4$ (1.0 mM). Parachloromercuribenzoate and monoiodoacetate, alkylating agents, potently

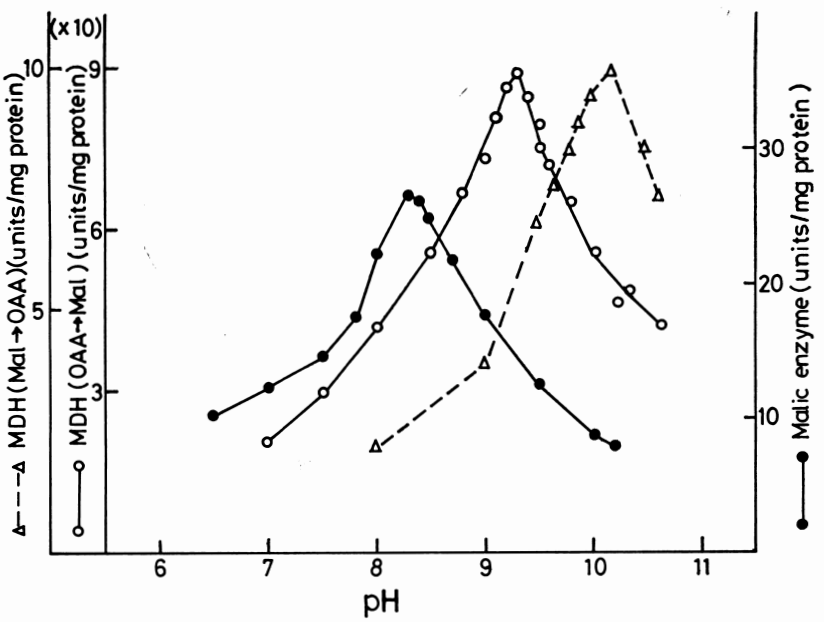

Fig. 5 Optimal pH of malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis*.

The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text. Deta are the average of two independent determinations at each pH.

Table 5 Effect of divalent cations on malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis*

	Concentration in reaction mixture	NAD^+-linked malic enzyme	Malate dehydrogenase
		μmoles/min/mg protein	
	(mM)		
Control assay mixture	....	19.8	98.9
+ $MnCl_2$	1.0	31.8	102.8
+ $MgCl_2$	1.0	24.4	95.8
+ $CoCl_2$	0.5	25.7	Not determined

The enzymatic activity was determined by spectrophotometric method. Details of the assay for malate dehydrogenase are given in the text. Control assay system for malic enzyme was the same as the standard assay mixture given in the text except for omitting $MnCl_2$. Divalent cations were present before the reaction was started by addition of substrate.

inhibited the malic enzyme activity. Only silver nitrate inhibited malate dehydrogenase, although other heavy metal ions, chelator and alkylating agents had little effect on malate dehydrogenase.

Heat inactivation experiments indicated that malate dehydrogenase was slightly more thermolabile than malic enzyme (Fig. 6).

Discussion

Previous findings indicated that *Trichomonas vaginalis* contained at least two types of the activity of NAD^+ reduction elicited

Table 6 Effect of inhibitors on malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis*

Added substance	Concentration in reaction mixture	NAD^+-linked malic enzyme	Malate dehydrogenase
		Inhibition (%)	
	(mM)		
$AgNO_3$	0.1	100	100
$HgCl_2$	0.1	100	5
	0.01	72	0
$CuSO_4$	1.0	67	12
	0.1	13	0
EDTA-2Na	0.5	100	0
Monoiodoacetate	1.0	100	0
p-Chloromercuribenzoate	0.7	100	21
	0.07	93	7
	0.007	62	0

The enzymatic activity was determined by spectrophotometric method. Details of the assay are given in the text. Inhibitors were incubated with the assay mixture except for substrate at 25 C for 15 min, and then the reaction was started by addition of substrate. Data are the average of at least two independent determinations.

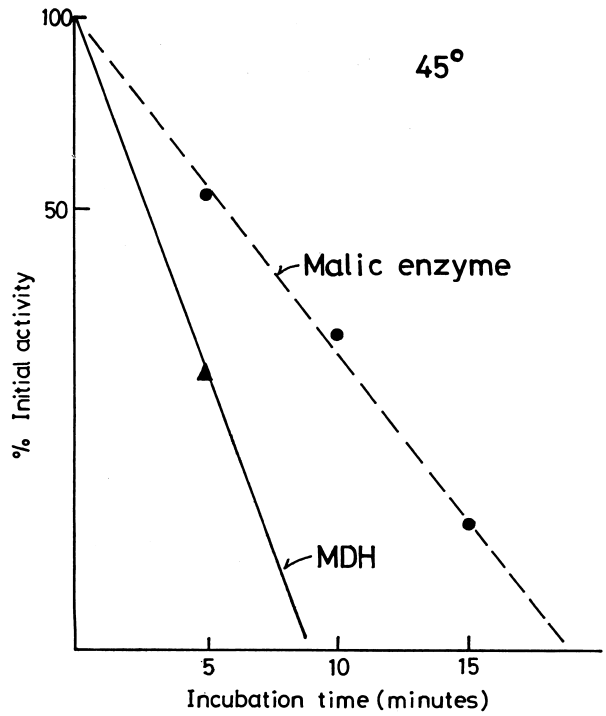

Fig. 6 Thermal inactivation of malate dehydrogenase and NAD^+-linked malic enzyme in *Trichomonas vaginalis*.

The enzymatic activity was determined by spectrophotometric method. Details of assay are given in the text. Data are the average of two independent determinations.

by L-malate, differing in subcellular localization and enzymatic properties. The stoichiometric analysis for these two purified enzymes demonstrated that the particulate enzyme was a malic enzyme and the supernatant enzyme was a malate dehydrogenase. Our NAD^+-linked malic enzyme is probably identical with the particulate malate dehydrogenase reported by Brugerolle and Metenier (1973). Müller (1973) and Lindmark (1974, 1975) demonstrated presence of NAD $(P)^+$-linked malic enzyme in the hydrogenosome of trichomonad flagellates. Our biochemical and electronmicroscopical studies (Tanabe *et al.*, 1980) also suggested that NAD^+-linked malic enzyme might be contained in the hydrogenosome-like dense granule of *T. vaginalis*.

Two types of animal L-malate dehydrogenase, i. e., supernatant and mitochondrial types, differing in extractability, kinetic behavior, molecular size, and amino acid composition, have been reported (Siegel and Englard, 1961; Englard and Greiger, 1962; Siegel and Englard, 1962). Compared with these two types of animal enzymes, trichomonad malate dehydrogenase is similar to the supernatant type with respect to substrate inhibition by oxaloacetate or L-malate and insensitivity to p-chloromercuribenzoate. Michaelis constants for the primary substrates are nearly the same with trichomonad enzyme and two types of animal enzymes. The molecular weight of trichomonad malate dehydrogenase is different from those of animal and bacterial enzymes (Yoshida, 1965). Our results except for Km values are similar to those reported previously on *T. vaginalis* malate dehydrogenase (Brugerolle and Metenier, 1973). Although the molecular weight of our enzyme is the same as *Trichomonas gallinae* malate dehydrogenase, these two enzymes are different in the optimal pH, the Km values and the substrate inhibition with L-malate (Dowda and Betterton, 1974).

NAD^+-linked malic enzyme has been demonstrated from several parasites and bacteria. The apparent Km values of trichomonad NAD^+-linked malic enzyme well agree with those of *Lactobacillus arbinosus* enzyme (Korkes *et al.*, 1950). The optimal pH of trichomonad enzyme is the same as NAD $(P)^+$-linked malic enzyme from *Ascaris lumbricoides* (Saz and Hubbard, 1957), but is slightly higher than those of bacterial enzymes (Korkes *et al.*, 1950; London and Meyer, 1969). Inhibition study indicates that the trichomonad NAD^+-linked malic enzyme probably contains an essential sulfhydryl group, and that divalent cation is involved in the catalysis of this enzyme.

Trichomonad flagellates lack mammalian or bacterial type respiratory metabolism, but show an intense respiration in presence of either NADH or malate and NAD^+. Although presence of a terminal flavoprotein oxidase in *T. vaginalis* was first proposed by Baernstein (1963), no quantitative data has been available. Previously we have demonstrated occurrence of NADH oxidase in the soluble fraction of *T. vaginalis*. This enzyme catalyzes the oxidation of NADH in presence of oxygen and thus may function as a terminal oxidase (Tanabe, 1979). Present study indicates a high level of malate dehydrogenase activity is primarily present in the 105,000 g supernatant (the soluble) fraction of *T. vaginalis*. Physiological functions of this enzyme are unknown yet. It is, however, likely that malate dehydrogenase sequentially functioning with NADH oxidase may participate in regulating the redox state of endogenous nicotinamide nucleotides.

Our present study also indicates NAD^+-linked malic enzyme is probably located in the hydrogenosome of *T. vaginalis*. Čerkasovová *et al.* (1973) and Čerkasov *et al.* (1978) demonstrated that the isolated hydrogenosome from *Tritrichomonas foetus* was able to reduce oxygen upon addition of either pyruvate or malate and NAD^+. It is suggested that malate is converted to pyruvate by NAD $(P)^+$-linked malic enzyme and thus pyruvate becomes the final substrate for oxygen uptake (Müller, 1976). Moreover it has been shown that aerobic metabolism of pyruvate appears to be linked with substrate level phosphorylation (Čerkasovová and Čerkasov, 1976;

Čerkasov *et al.*, 1978). These evidences suggest that NAD^+-linked malic enzyme may participate in regulating the pyruvate level functioning in energy conservation in the hydrogenosome of trichomonad flagellates.

Summary

Malate dehydrogenase of *Trichomonas vaginalis* was primarily present in the 105,000 g supernatant fraction, whereas NAD^+-linked malic enzyme was largely associated with the 13,000 g sediment.

Malate dehydrogenase and malic enzyme were purified by differential centrifugation, ammonium sulfate fractionation, ion-exchange chromatography and Sephadex G-100 gel filtration. The specific activities of malate dehydrogenase and NAD^+-linked malic enzyme increased 20- and 23-fold respectively. The molecular weights of malate dehydrogenase and NAD^+-linked malic enzyme were estimated to be 73,000 and 96,000 daltons respectively. The purified malate dehydrogenase had a Vmax of 10.2 μmoles of NAD^+ reduced/min/mg protein in the forward reaction and 112.0 μmoles of NAD^+ oxidized/min/mg protein in the reverse reaction. The apparent Km values of this enzyme for L-malate, oxaloacetate, NAD^+ and NADH were 520, 100, 150 and 25 μM respectively. The purified malic enzyme had a Vmax of 30.0 μmoles of NAD^+ reduced/min/mg protein, and the apparent Km values for L-malate and NAD^+ were 210 and 13 μM respectively. Substrate inhibition of malate dehydrogenase by either 20 mM L-malate or 3 mM oxaloacetate was observed. The optimal pH of malate dehydrogenase was 10.2 in the forward reaction and 9.3 in the reverse reaction. The optimal pH of malic enzyme in the forward reaction was 8.3. The enzymatic activity of malic enzyme was markedly increased by divalent cations. Inhibition studies indicated that malic enzyme is associated with essential cation(s) and sulfhydryl group. Physiological functions of malate dehydrogenase and NAD^+-linked malic enzyme in *T. vaginalis* are discussed.

Acknowledgements

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腟トリコモナスの NAD⁺-linked malic enzyme およびリンゴ酸脱水素酵素の細胞内局在性ならびに性質について

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腟トリコモナスからリンゴ酸の代謝に直接関連する酵素としてリンゴ酸脱水素酵素（以下 MDH と略）およびリンゴ酸を酸化的脱炭酸する NAD⁺-linked malic enzyme（以下 NAD-ME と略）を検出し，以下の成績を得た．

(1) NAD-ME は 13,000 g 沈渣分画，MDH は 105,000 g 上清分画に主として活性が見出された．

(2) NAD-ME および MDH を遠心分画法，硫酸アンモニウム分画法，イオン交換セファデックスカラム法およびセファデックス G-100 ゲル濾過法を用いて，それぞれ 23 倍，20 倍に精製した．

(3) NAD-ME および MDH の分子量はそれぞれ 96,000 および 73,000 と算定された．

(4) NAD-ME のリンゴ酸酸化反応の Vmax は 30.0 μmoles of NAD^+ reduced/min/mg protein で，L-リンゴ酸，NAD^+ に対する Km はそれぞれ 210 μM，13 μM であつた．MDH の L-リンゴ酸，オキザロ酢酸，NAD^+ および NADH に対する Km 値はそれぞれ 520 μM，100 μM，150 μM そして 25 μM であり，リンゴ酸酸化反応の Vmax は 10.2 μmoles of NAD^+ reduced/min/mg protein，また逆反応の Vmax は 112.0 μmoles of NADH oxidized/min/mg protein であつた．

(5) MDH は 20 mM の L-リンゴ酸および 3 mM のオキザロ酢酸により明らかに基質阻害を受けた．

(9) NAD-ME のリンゴ酸酸化反応の至適 pH は 8.3 で，MDH のリンゴ酸酸化反応の至適 pH は 10.2，逆反応のそれは 9.3 であつた．

(7) NAD-ME は 2 価陽イオン，特に Mn^{++} の添加により，その活性が著明に増大した．

(8) NAD-ME は EDTA-2Na，重金属イオン，モノヨード酢酸および p-クロロ水銀安息香酸により顕著に阻害を受けた．