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Trichomonas vaginalis: NADH and NADPH Dehydrogenases in the Hydrogenosome-Rich Fractions

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Trichomonas vaginalis is a facultaitve anaerobe which lacks mitochondria (Nielsen *et al.*, 1966), cytochromes (Wellerson *et al.*, 1959) and a functional tricarboxylic acid cycle (Read, 1957). Yet it can readily consume oxygen when available. Previous investigations on this aerobic metabolism indicated that respiration of this parasite was enhanced by NADH (Coombs, 1978) or by malate (Ninomiya and Suzuoki, 1952). In a recent investigation (Tanabe *et al.*, 1980), it was suggested that malate-supported respiration was attributable to the sequential functioning of NAD^+-linked malate dehydrogenase (decarboxylating) and NADH oxidase. However, a different metabolic pathway has been proposed to explain malate-supported respiration in the hydrogenosome of *Trichomonas foetus*. Čerkasov *et al.* (1978) attributed the respiratory activity in *T. foetus* to the functioning of pyruvate oxidase in place of NADH oxidase. In contrast to malate-supported respiration, NADH-supported respiration seems to be catalyzed by a single enzyme. Tanabe (1979) partially purified and characterized NADH oxidase of *T. vaginalis*. This enzyme appeared to be different from NADH dehydrogenase of this parasite, which has been detected by Coombs (1978). Although both enzymes were reported to be present in the cytosol, only NADH dehydrogenase had a diaphorase activity, and was labile to oxygen (Tanabe, 1979; Coombs, 1978). These findings suggest that *T. vaginalis* has at least two NADH oxidizing enzymes. In addition, Linstead (1980) found NADPH dehydrogenase activity, which also exhibited a diaphorase activity, in the cytosol of this parasite. A similar enzyme has been detected in *Entamoeba histolytica* (Weinbach *et al.*, 1977), another facultative anaerobe lacking mitochondria, cytochromes and a tricarboxylic acid cycle (see Weinbach *et al.*, 1978). Although Weinbach *et al.* (1977) suggested that NADPH dehydrogenase of *E. histolytica* might function as the scavenger of oxygen which seemed toxic to amoebae, physiological roles of these dehydrogenases in trichomonads still need further clarification as Coombs (1978) and Linstead (1980) pointed out.

During investigation on NADH and NADPH dehydrogenases of *T. vaginalis*, we found these enzymatic activities in sedimentable portions of the extract of this parasite. In addition, we observed that clostridial ferredoxin was functional as the electron acceptor of NADH dehydrogenase. These findings led us to study a detailed localization of NADH and NADPH dehydrogenases in particulate fractions of *T. vaginalis* and their physiological functions in its metabolism.

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Materials and Methods

Parasite: T. vaginalis (strain TS-1:KEIO) was grown in stationary Ehlenmyer flasks containing 120 ml Asami medium (Asami, 1952) as described previously by Tanabe (1979). After cultivating for 48 hours at 37 C, the flasks were chilled in an ice-bath for 5 min. Subsequently, trichomonads were harvested and washed three times by centrifugation in 50 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose at 500 g for 7 min. Finally, trichomonads were suspended in the buffered sucrose so that the protein concentraiton was 4–6 mg/ml. These manipulations were done at 4 C unless otherwise stated.

Reagents: NADH and NADPH were supplied by Oriental Yeast Inc. (Tokyo, Japan). FAD, FMN, riboflavin, menadione, 2,6-dichlorophenol indophenol (DCPIP) and ferredoxins (*Clostridium pasteurianum* type V; spinach type III) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Cytochrome c (horse heart) was obtained from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Other chemicals were of the highest purity commercially available.

Differential centrifugation: Trichomonads, suspended in the Tris-buffered sucrose as mentioned above, were disrupted by homogenization using a glass homogenizer fitted with a Teflon pestle for 8 min. The crude extract was centrifuged at 500 g for 7 min, and the pellet resuspended in 30 ml of the Tris-buffered sucrose. The supernatant fluid was centrifuged at 15,000 g for 20 min, and the pellet also resuspended in the same manner as above. The supernatant fluid of 15,000 g centrifugation was further centrifuged at 110,000 g for 120 min, and the pellet resuspended as above. These pellets were washed once by centrifugation and finally suspended in 0.2 M Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose so that the protein concentration was 1–3 mg/ml. All of these manipulations were done at 4 C.

Sucrose density gradient centrifugation: Only 15,000 g $\times$ 20 min pellet was further fractionated on a discontinuous sucrose density gradient centrifugation, because it exhibited the highest specific activities of NADH and NADPH dehydrogenases when examined with cytochrome c as the electron acceptor among three particulate fractions isolated by differential centrifugation. The 15,000 g pellet was washed once and suspended in 3 ml of 10 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose. A discontinuous sucrose density gradient was prepared with 1.8 ml of 65%, 50%, 42%, 35% and 25% sucrose solution (w/w) in 10 mM Tris-HCl buffer, pH 7.4 in a total volume of 9 ml. On the top of the gradient was placed the resuspended 15,000 g pellet. Centrifugation was done in a Hitachi 55P-2 ultracentrifuge using a swinging bucket (SP-25A) for 60 min at 18,000 rpm. Isolated bands were recovered with micropipettes and washed once by centrifugation after diluting with 10 mM Tris-HCl buffer, pH 7.4 so that the concentrations of sucrose were approximately 0.25 M. Finally, the pellets were suspended in 0.2 M Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose, and used for the enzyme assays. All of these manipulations were done at 4 C.

Enzyme assays: NADH and NADPH dehydrogenases were assayed by spectrophotometrically determining the velocity of cytochrome c reduction at 550 nm. The assay mixture contained 80 μM oxidized cytochrome c, 0.5 mM NADH or NADPH, the enzyme preparation (0.15–1.7 mg protein) and 100 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer, pH 7.4 in a final volume of 1 ml. After the endogenous reduction of cytochrome c, which was practically zero, was recorded for a short period, the enzymatic reaction was started by adding NADH or NADPH at 20 C.

NAD^+-linked malate dehydrogenase (decarboxylating) (malic enzyme) was assayed by spectrophotometrically determining the velocity of NAD^+ reduction at 340 nm basic-

ally according to Tanabe *et al.* (1980). The assay mixture contained 0.2 mM NAD^+, 5 mM malate, the enzyme preparation (0.2–1.9 mg protein) and 100 mM HEPES buffer, pH 7.4 in a final volume of 1 ml. This enzymatic activity was also evaluated by determining the concentration of pyruvate formed during the enzymatic reaction as described by Czok and Lamprecht (1974). In particular, this method was applied to determination of the enzymatic activity of the crude extract and the 110,000 g supernatant fluid, because these fractions contain a significant activity of NAD^+-linked malate dehydrogenase. These enzymatic reactions were also conducted at 20 C.

These enzymatic activities were calculated on the basis of molar extinction coefficients of cytochrome c and NAD^+ (reduced minus oxidized).

Protein determination: Protein concencentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

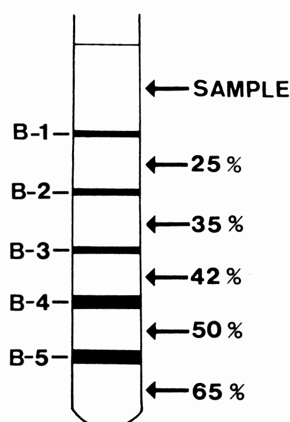

Fig. 1 Discontinuous sucrose density gradient centrifugation of 15,000 g × 20 min pellet of *T. vaginalis*.
Numbers in percent stand for concentrations of sucrose (w/w) in 10 mM Tris-HCl buffer, pH 7.4. Other details as described in the text.

Results

Distribution of NADH and NADPH dehydrogenases in subcellular fractions was examined with cytochrome c as the electron acceptor, and summarized in Table 1. The 110,000 g supernatant fluid exhibited the highest specific activities of these enzymes, while significant activities were also detectable in the 15,000 g pellet.

Sucrose density gradient centrifugation of the 15,000 g pellet resulted in isolation of five bands designated B-1 to B-5 from the top of the gradient (Fig. 1). NADH and NADPH dehydrogenase activities were detected in B-

Table 1 Localization of NADH and NADPH dehydrogenases in *T. vaginalis* with cytochrome c as the electron acceptor

	NADH dehydrogenase	NADPH dehydrogenase	NAD^+-malic enzyme
	μ mole of cyt or NAD^+ reduced/mg protein/min		
Crude extract	0.57	0.32	0.49*
500 g pellet	0.02	0.005	0.18
15,000 g pellet	0.16	0.09	0.57
110,000 g pellet	0.02	0.003	0.11
110,000 g supernatant fluid	1.28	0.90	0.01*
B-1	0.005	Nil	Nil
B-2	0.015	0.007	0.02
B-3	0.17	0.028	0.24
B-4	0.52	0.33	1.07
B-5	0.39	0.11	0.74

Data were averages of at least four determinations.
* The activity was represented with μ mole of pyruvate formed/mg protein/min.

Table 2 Effects of various electron acceptors and Triton X-100 on NADH and NADPH dehydrogenases in the 15,000 g pellet of *T. vaginalis*

	Cytochrome c or DCPIP reduced			
	NADH dehydrogenase		NADPH dehydrogenase	
		+ Triton		+ Triton
	μ mole/mg protein/min			
Cytochrome c 80 μM	0.16	0.27	0.09	0.18
FAD 0.2 mM	0.34	0.59	0.17	0.40
FMN 0.2 mM	0.32	0.54	0.19	0.45
Menadione 0.5 mM	0.27	0.50	0.12	0.26
Clostridial ferredoxin 50 μg	0.55		0.10	
Spinach ferrodoxin 50 μg	0.16		0.10	
Riboflavin 0.2 mM	0.34		0.15	
DCPIP 0.2 mM	0.40		0.31	

The concentration of Triton X-100 was 0.1%. These electron acceptors were present in the assay mixtures before the reaction was initiated by adding NADH or NADPH. When electron acceptors other than cytochrome c were employed, activities obtained with cytochrome c as the electron acceptor were not subtracted from experimental data. Reduction of DCPIP was monitored at 600 nm.

Table 3 Effects of transition metal chelators and a flavin antagonist on NADH and NADPH dehydrogenases of *T. vaginalis* with cytochrome c and FMN as the electron acceptor

	Cytochrome c reduced (μ mole/mg protein/min)			
	NADH dehydrogenase		NADPH dehydrogenase	
	Cytochrome c	FMN	Cytochrome c	FNM
None	0.16	0.32	0.09	0.19
+ Bathophenanthroline 1 mM	0.002	0.02	0.008	0.005
+ Salicylaldoxime 5 mM	0.13	0.24	0.08	0.18
+ Salicylhydroxamate 5 mM	0.12	0.24	0.06	0.13
+ o-Phenanthroline 5 mM	0.12	0.20	0.08	0.13
+ Atebrin 5 mM	0.12	0.19	0.03	0.10

The inhibitors were present in the assy mixtures before the reaction was started by adding NADH or NADPH. Salicylaldoxime and o-phenanthroline were dissolved in methanol or ethanol; therefore, control experiments were done in the presence of the same amounts of these alcohols, which did not affect the enzymatic activities. Other details as in the text and in the legend to Table 2.

Table 4 Effects of palmitoyl CoA on NADH and NADPH dehydrogenases of the 15,000 g pellet of *T. vaginalis* with cytochrome c and FMN as the electron acceptor

	Cytochrome c reduced (μ mole/mg protein/min)			
	NADH dehydrogenase		NADPH dehydrogenase	
	Cytochrome c	FMN	Cytochrome c	FMN
None	0.16	0.32	0.09	0.19
+ Palmitoyl CoA 0.2 mM	0.26	0.49	0.16	0.35

Palmitoyl CoA was present in the assay mixture before the reaction was started by adding NADH or NADPH. Other details as in the text and in the legend to Table 2.

3, B-4 and B-5 (Table 1). The highest specific activities were observed in B-4, while other fractions, i.e., B-1 and B-2, had little activities of these enzymes. NAD^+-linked malic enzyme was concentrated in the 15,000 g pellet, and also primarily detectable in B-3, B-4 and B-5 (Table 1). These findings suggest that NADH and NADPH dehydrogenases in the particulate fraction were coenriched with NAD^+-linked malic enzyme. When FMN was employed as the electron acceptor, NADH and NADPH dehydrogenases exhibited the same localizations as those examined with cytochrome c as the electron acceptor (Table 1). In the present experiment, further characterization of NADH and NADPH dehydrogenases in the 15,000 g pellet was attempted. The enzymes in the 110,000 g supernatant fluid have been previously characterized by other investigators (Coombs, 1978; Linstead, 1980).

Table 2 demonstrates capacities of various electron acceptors in the enzymatic reactions of NADH and NADPH dehydrogenases. Since cytochrome c can be nonenzymatically reduced by electron acceptors used in this experiment except for DCPIP, capacities of these acceptors were examined with cytochrome c as the final electron acceptors at 550 nm. These studies indicated both enzymes catalyzed reduction of FAD, FMN, riboflavin, menadione and DCPIP. However, only NADH dehydrogenase significantly reduced clostridial ferredoxin. Moreover, it was suggested that spinach ferredoxin was not reduced by these enzymes under the present assay conditions. Among several electron acceptors examined for NADH dehydrogenase of this parasite, clostridial ferredoxin seemed to be most functional. Reduction of some of these electron acceptors by NADH and NADPH dehydrogenases was enhanced by adding 0.1% Triton X-100 or Lubrol WX. Irrespective of the acceptors employed, the enzymatic activity of NADH dehydrogenase was always higher than that of NADPH dehydrogenase.

These enzymatic activities were markedly inhibited by bathophenanthroline, a transitional metal chelator, when examined with cytochrome c or FMN as the electron acceptor (Table 3). Other chelators like salicylaldoxime, salicylhydroxamate and o-phenanthroline were much less inhibitory at the concentrations tested. Bathocuproine was also scarcely inhibitory at 1 mM under the present assay conditions. In contrast, atebrin, a flavin antagonist, inhibited the enzyme activities (Table 3). Inhibitors and uncouplers of mitochondrial oxidative phosphorylation such as amytal (1.0 mM), rotenone (0.02 mM), KCN (1.0 mM), 2,4-dinitrophenol (0.5 mM) and antimycin (10 μg/ml) had little effects on these enzymatic activities.

Addition of palmitoyl CoA enhanced these enzymatic activities (Table 4), whereas none of CoA and other CoA derivatives such as acetyl CoA, succinyl CoA and malonyl CoA was effective.

Discussion

Our presenet investigations revealed that NADH and NADPH dehydrogenases were present in the sedimentable portions as well as in the cytosol of *T. vaginalis*. This seems to be incompatible at least in part with previous findings on localization of these enzymes by other investigators (Lindmark *et al.*, 1975; Linstead, 1980). The reason of this difference is not known at present. Moreover, our study suggests that most of sedimentable NADH and NADPH dehydrogenase activities were present in hydrogenosomes of this parasite, because NAD^+-linked malic enzyme, which was coenriched with these dehydrogenases, is primarily localized in the hydrogenosome of trichomonads (Lindmark *et al.*, 1975; Tanabe *et al.*, 1980). Effects of detergents on the enzymatic activities also support this view.

Of particular interest is that clostridial ferredoxin functioned as the electron acceptor of NADH dehydrogenase of the 15,000 g

pellet. From this finding, we envisage that a ferredoxin-like protein of low redox potential is present in *T. vaginalis* and functions as the physiological electron acceptor of NADH dehydrogenase. The presence of a ferredoxin-like protein in trichomonads seems to be supported by the findings of Reeves *et al.* (1980). They isolated a low-molecular weight iron-sulfur protein from *E. histolytica,* which resembled trichomonads in several essential aspects of the metabolism, and called this protein "amoebal ferredoxin". Lindmark *et al.* (1975) also reported that clostridial ferredoxin was functional as the electron acceptor of pyruvate synthase of *T. vaginalis.* Ohnishi *et al.* (1980) performed an electron paramagnetic resonance study on the hydrogenosome-rich fraction of *T. foetus* and suggested that there were at least five iron-sulfur clusters. These observations led us to envision that the anaerobic metabolism of *T. vaginalis* resembles those of anaerobic bacteria like clostridia at least in part.

If NADH dehydrogenase of *T. vaginalis* is in fact similar to bacterial NADH-ferredoxin reductase (EC 1.18.1.1.), reduction of various electron acceptors by NADH (Table 2) may be attributable to a diaphorase activity which NAD(P)H-ferredoxin reductases usually exhibit (see Barman, 1974; Thauer *et al.,* 1977). However, since NADPH dehydrogenase of *T. vaginalis* barely catalyzed reduction of clostridial and spinach ferredoxins (Table 2), reduction of electron acceptors by NADPH appears to need further characterization.

Our present investigation also suggests that transition metal(s) and flavin(s) are involved in the catalysis of NADH and NADPH dehydrogenases of this parasite. These findings seem to be compatible with Coombs (1978) and Linstead (1980). As regards activation of the enzymatic activites by palmitoyl CoA, detailed mechanisms are not known. Our preliminary study, however, suggests that fatty acyl CoA like palmitoyl CoA, oleyl CoA and myristoyl CoA disrupts integrity of the hydrogenosomal membrane of *T. vaginalis* (Takeuchi and Kobayashi, unpublished).

It seems interesting that *T. vaginalis* appears to have at least two NADH oxidizing enzymes. One may function under aerobic condition, and the other under anaerobic condition. Detailed regulatory mechanisms of these two enzymes are still under investigation in our laboratory and will be presented elsewhere.

Summary

The crude extract of *Trichomonas vaginalis* had NADH and NADPH dehydrogenases which catalyzed reduction of an exogenous cytochrome c. Differential centrifugation of the crude extract indicated that these activities were primarily present in the 110,000 g $\times$ 120 min supernatant fluid, while significant activities were also found in the 15,000 g $\times$ 20 min pellet. Subsequent sucrose density gradient centrifugation of the 15,000 g pellet showed most of these enzymatic activities were coenriched with NAD^+-linked malic enzyme activity, a hydrogenosomal enzyme of trichomonads. NADH and NADPH dehydrogenases in the 15,000 g pellet also catalyzed reduction of FAD, FMN, riboflavin, menadione and DCPIP. However, clostridial ferredoxin was reduced only by NADH dehydrogenase, and spinach ferredoxin was reduced by none of these enzymes. In the presence of FMN or cytochrome c as the electron acceptor, these enzymatic activities were inhibited by bathophenanthroline and by atebrin. In contrast, addition of palmitoyl CoA enhanced these enzymatic activities. These findings suggest that NADH and NADPH dehydrogenases are localized in the hydrogenosome-rich fraction of *T. vaginalis,* and that a ferredoxin-like protein of low redox potential may be the physiological electron acceptor of NADH dehydrogenase. It is also suggested that transition metal(s) and flavin(s) may be involved in the catalysis of these dehydrogenases.

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腟トリコモナス：ハイドロゲノソーム分画に局在する NADH-, 及び NADPH 脱水素酵素について

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腟トリコモトスの粗抽出液中にチトクロームCを電子受容体として利用する NADH-, 及ビ NADPH 脱水素酵素を見出した．分別遠沈の結果，これらの酵素の殆んどは 110,000 g 上清に見出されたが，一部は 15,000 g×20 分の沈渣に存在した．更にシヨ糖密度勾配遠沈によつて局在を検討した結果 15,000 g 沈渣の酵素の殆んどは NAD⁺-依存性の malic enzyme と同じ分画に見出された．この 15,000 g 沈渣の酵素の性質を更に検した結果，NADH-, 及び NADPH 脱水素酵素は FAD, FMN, リボラビン等を還元したが，クロストリジウムのフェレドキシンは NADH 脱水素酵素のみによって還元され，ホウレン草のフェレドキシンはいずれの酵素によつても還元されなかつた．又，これらの両酵素ともバソフェナンスロリン，及びアテブリンによつて阻害されたが，パルミチル CoA 添加によつて活性が増大した．

これらの結果より従来の報告に反し腟トリコモナスの顆粒分画，恐らくハイドロゲノソームにもNADH-NADPH 脱水素酵素が存在し，少なくともNADH 脱水素酵素の生理的な電子受容体はフェレドキシン類似の蛋白ではないかという可能性が示された．又，これらの実験より遷移金属—恐らく非ヘム鉄—，及びフラビンが酵素反応に関与していることも示唆された．