J Bioenerg Biomembr (2006) 38:299–308
DOI 10.1007/s10863-006-9047-9

ORIGINAL PAPER

Preliminary evidence on existence of transplasma membrane electron transport in *Entamoeba histolytica* trophozoites: a key mechanism for maintaining optimal redox balance

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Received: 6 May 2006 / Accepted: 30 May 2006 / Published online: 13 October 2006

Abstract *Entamoeba histolytica*, an amitochondriate parasitic protist, was demonstrated to be capable of reducing the oxidized form of α-lipoic acid, a non permeable electron acceptor outside the plasma membrane. This transmembrane reduction of non permeable electron acceptors with redox potentials ranging from -290 mV to $+360$ mV takes place at neutral pH. The transmembrane reduction of non permeable electron acceptors was not inhibited by mitochondrial electron transport inhibitors such as antimycin A, rotenone, cyanide and azide. However, a clear inhibition with complex III inhibitor, 2-(n-heptyl)-4-hydroxyquinoline-N-oxide; modifiers of sulphydryl groups and inhibitors of glycolysis was revealed. The iron-sulphur centre inhibitor thenoyltrifluoroacetone failed to inhibit the reduction of non permeable electron acceptors whereas capsaicin, an inhibitor of energy coupling NADH oxidase, showed substantial inhibition. p-triflurometh oxychlorophenylhydrazone, a protonophore uncoupler, resulted in the stimulation of α-lipoic acid reduction but inhibition in oxygen uptake. Mitochondrial electron transport inhibitors substantially inhibited the oxygen uptake in *E. histolytica*. Transmembrane reduction of α-lipoic acid was strongly stimulated by anaerobiosis and anaerobic stimulation was inhibited by 2-(n-heptyl)-4-hydroxyquinoline-N-oxide. Transmembrane redox system of *E. histolytica* was also found to be sensitive to UV irradiation. All these findings clearly demonstrate the existence of transplasma membrane electron transport system in *E. histolytica* and possible involvment of a naphthoquinone coenzyme in transmembrane redox of *E. histolytica* which is different from that of mammalian host and therefore can provide a novel target for future rational chemotherapeutic drug designing.

Keywords Transplasma membrane electron transport · ALA, NQSA, Ferricyanide reduction · NADH/NADPH · Naphthoquinone coenzyme

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Abbreviations

ALA:	α-lipoic acid
NQSA:	1,2-naphthoquinone-4-sulphonic acid
transPMET:	transplasma membrane electron transport
HQNO:	2-(n-heptyl)-4-hydroxyquinoline-N-oxide
BSA:	bovine serum albumin
HCS:	hepatocyte cell suspension
PCMBS:	p-chloromercuribenzenesulphonate
TTFA:	thenoyltrifluroacetone
FCCP:	p-triflurometh oxychlorophenylhydrazone

Introduction

The enteric protozoan parasite *Entamoeba histolytica* is the causative agent of human amoebiasis, a disease that is

surpassed only by malaria and trichomoniasis as a parasitic cause of death (World Heath Organisation, The world health report 1998). Normally, resident of large bowel *E. histolytica* persists for months or even years as an asymptomatic luminal gut infection. However occasionally, the parasite penetrates the intestinal mucosa and disseminate to other organs, most commonly to the liver where they induce abscess formation (Ravdin et al., 1988).

E. histolytica, a microaerophilic organism, does not usually tolerate elevated oxygen concentration. However, during tissue invasion, trophozoites are exposed to an increased concentration of oxygen (Martinez-Palomo et al., 1998). Generation of reactive oxygen species (ROS) such as superoxide radical and peroxide is a characteristic of oxidative stress in living systems. Peroxide can also be converted to hydroxyl radical through the Fenton reaction in the presence of a source of Fe^{2+}. Therefore, in order to successfully establish an infection, trophozoites must survive despite the local production of ROS in liver and other organs.

E. histolytica could avoid the oxidative burst through an iron containing superoxide dismutase (SOD) that can convert superoxide generated to H_2O_2 (Bruchhaus et al., 1994) and this H_2O_2 can be subsequently eliminated by a cysteine rich 29-kDa protein (thiol dependent peroxidase) (Flores et al., 1993; Bruchhaus et al., 1993; Bruchhaus et al., 1997). The increased resistance to $^{\cdot}OH$ might be due to scavenging of this free radical by antioxidant compounds.

Electron transport across the plasma membrane has been described in many eukaryotic cells (Crane et al., 1989), such as erythrocytes, liver, heart, transformed liver cells, HeLa cells, neutrophils, yeast and plant cells but no such information is available with *E. histolytica*. Functions described for this activity include (a) reduction of Fe (III) to supply Fe (II) for transport to the cell (Leuisse et al., 1992), (b) alteration of the redox state of the cytoplasm (Navas et al., 1986) or activation of nuclear genes (Wenner et al., 1988) to stimulate cell growth (Larm et al., 1994), and (c) reduction of extracellular antioxidant compounds, e.g. coenzyme Q hydroquinone (Crane et al., 1997), tocopherol (Stocker et al., 1993), and ascorbic acid (Roy et al., 1997), after they have been oxidized by free radicals.

Dihydrolipoic acid, the reduced form of ALA can act as an antioxidant and interact with reactive O_2 species such as superoxide radicals, hydroxyl radicals, hypochlorus acid, peroxyl radicals, and singlet oxygen. It can also protect membranes by interacting with ascorbic acid and glutathione, which may in turn recycle vitamin E (Roy et al., 1997). In the present study, we have described a system in *E. histolytica* cells that keeps extracellular ALA reduced and demonstrated the existence of transPMET activities in trophozoites of *E. histolytica*. TransPMET is critical for maintaining cellular redox balance, viability and the regulation of cell signaling, cell growth, apoptosis, proton pumping, and ion channels (Medina et al., 1997; Baker et al., 2000). This is, to our knowledge, the first study of the properties of a plasma membrane redox system mediated extracellular non permeable electron acceptor reduction in an amitochondriate protozoan parasite. The distinguishing characteristics of the plasma membrane redox system in *E. histolytica* are its sensitivity to UV irradiation which is not found in mammalian cells. The present study therefore provides a possible selective target in this pathogen for the development of a selective inhibitor of transPMET. The transPMET mediated extracellular ALA reduction also provides a new approach to understand the mechanism of survival of *E. histolytica* trophozoites under high oxygen environment when they disseminate to liver and other organs to cause multiorgan disorder.

Experimental procedures

All biochemicals unless otherwise mentioned were from Sigma Chemicals (St. Louis, MO, USA).

Parasite

Axenically grown pathogenic trophozoites of *E. histolytica* (strain HM1: IMSS) was maintained in TYI-S-33 medium containing sterilized 10% heat inactivated bovine serum and vitamin mixture (Diamond et al., 1978). They were subcultured after every 72 hr. Cells were harvested by centrifugation at $400\times g$ and washed thrice in buffer A (120 mM NaCl, 5 mM KCl, 1 mM $EDTANa_2$ and 20 mM HEPES, pH 7.2) at $400\times g$. The cells were immediately used for assay. Viability of harvested cells was monitored by Trypan Blue exclusion method.

Protein estimation

The amount of protein was determined by the biuret method in the presence of 0.2% deoxycholate. BSA was used as a standard.

Measurement of ferricyanide reduction by *E. histolytica* cells

Ferrocyanide quantitation was performed using 1,10-phenanthroline complex as described by Avron and Shavit (Avron et al., 1963). The incubation mixture contained 120 mM NaCl, 20 mM HEPES, 20 mM potassium acetate, 5 mM $MgCl_2$, pH 6.4, 3 mM $K_3Fe(CN)_6$, 5 mM

D-glucose and 3 mg *E. histolytica* cells in a final volume of 1.5 ml. The reaction mixture was incubated for 10 min at 37 °C followed by incubation in ice and centrifugation at 2000×*g* for 10 min. Ferrocyanide in supernatant was measured by 1.5 ml of 1,10-phenanthroline reagent, which contained 1.5 mmoles sodium acetate, 97 μmoles citric acid, 0.75 μmoles ferric chloride and 12.6 μmoles 1,10-phenanthroline. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except *E. histolytica* cells.

Iron reduction by *E. histolytica* cells

Iron reduction by *E. histolytica* trophozoites was assayed as the formation of red complex between reduced iron and 1,10-phenanthroline, using the difference in absorbance between 510 nm and 600 nm at 37°C. The reaction mixture consisted of 3 mg *E. histolytica* trophozoites, 120 mM NaCl, 20 mM potassium acetate, 20 mM HEPES, pH 6.4, 5 mM $MgCl_2$, 5 mM D-glucose, 20 μM 1,10-phenanthroline and one of the following: ferric-EDTA, ammonium Fe (III) citrate in the concentration of 0.1 mM and 12 mM respectively, in a total volume of 3 ml. The millimolar extinction coefficient for ferrous-1,10-phenanthroline is 9.5 ($\Delta A_{510-600}$=9.5/mM/cm). In all the cases *E. histolytica* trophozoites were preincubated for 5 min in buffer before the addition of ferric-EDTA, ammonium Fe(III) citrate and 1,10-phenanthroline.

Indigo disulphonate reduction by *E. histolytica* cells

Indigo disulphonate reduction by *E. histolytica* trophozoites was measured as decrease in absorbance at 608 nm at 37°C. The reaction mixture was the same as the iron reduction assays with 150 nmol indigo disulphonate added in place of the iron compounds and 1,10-phenanthroline.

ALA and NQSA reduction assay

ALA and NQSA reduction by *E. histolytica* trophozoites was assayed as the formation of ferrocyanide as a result of the reduction of ferricyanide by dihydrolipoic acid and hydroquinone of NQSA. Ferrocyanide was estimated according to the method of Avron and Shavit (Avron et al., 1963). The reaction mixture contained 3 mg *E. histolytica* trophozoites, 120 mM NaCl, 20 mM potassium acetate, 5 mM $MgCl_2$, 20 mM HEPES, pH 6.4, 5 mM D-Glucose and 1 mM ALA or 1 mM NQSA in a final volume of 1.3 ml. The reaction mixtures were incubated for 10 min at 37°C. After incubation, the reaction mixtures were kept in ice and then centrifuged at 4°C at 2000×*g* for 10 min. Supernatant was added to 0.2 ml of 75 mM $K_3Fe(CN)_6$ followed by 1.5 ml 1,10-phenanthroline reagent. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except *E. histolytica* cells. Two molecules of ferricyanide have been considered to react with one molecule of hydroquinone of NQSA or one molecule of dihydrolipoic acid.

Measurement of oxygen uptake

Oxygen uptake by *E. histolytica* trophozoites was determined in a Warburg constant volume manometer with air as gas phase at 37°C, with 0.2 ml 20% KOH in the centre well and shaking at 80 cycles/min (Umbreit et al., 1957). For respiration studies, the cells were washed thrice in HEPES-salt buffer, pH 6.4. The Warburg flask contained 15 mg trophozoite protein in a total volume of 3 ml HEPES-salt buffer; pH 6.4. Water insoluble compounds were given as a solution in dimethylformamide (5 μl/ml buffer). Appropriate vehicle controls were used for each experiment.

Measurement of ALA, NQSA and $K_3Fe(CN)_6$ reduction by *E. histolytica* cells under anaerobic condition

Anaerobiosis was carried out in glass centrifuge tube in presence of oxygen scavenging enzymic system. The tube contained 3 mg *E. histolytica* cells, 120 mM NaCl, 20 mM potassium acetate, 20 mM HEPES, pH 6.4, 5 mM $MgCl_2$, 5 mM D-glucose, 10 units of glucose oxidase, and 2000 units of catalase in a volume of 2.6 ml. Open surface of liquid was covered by 1 ml heavy liquid paraffin. After 10 min incubation, ALA, NQSA or $K_3Fe(CN)_6$ was injected through the paraffin layer in a final concentration of 1 mM, 1 mM and 3 mM respectively. The assay system was incubated for 10 min at 37°C. The reaction was terminated by immersing the tubes in ice and centrifuged at 2000×*g* for 10 min at 4°C. 1.3 ml of supernatant was removed by syringe and added to 0.1 ml of 70 mM $K_3Fe(CN)_6$, followed by 1.6 ml 1,10-phenanthroline reagent. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except *E. histolytica* trophozoites.

UV irradiation of *E. histolytica* and HCS

Preparation of HCS for UV irradiation: Hepatocytes were isolated from male Sprague-Dawley rats by the collagenase perfusion method (Moldeus et al., 1978). Incubations

were performed at 37°C in Krebs-Henseleit medium, supplemented with 12.6 mM HEPES (pH 7.4), at a concentration of 10^6 cells/ml. At the end of the incubation, the hepatocytes were sedimented by centrifugation at $1000\times g$ for 10 min at 4°C and washed twice in 140 mM PBS, pH 7. Consistently over 94% of the isolated hepatocytes excluded Trypan Blue. The pellet was kept at 4°C until use.

HCS and *E. histolytica* trophozoites were UV irradiated by modifying the procedure as described by Brodie et al (Brodie et al., 1960). The treatment was performed as follows: HCS in 140 mM PBS, pH 6.4 and *E. histolytica* trophozoites in a buffer containing 120 mM NaCl, 20 mM potassium acetate, 5 mM $MgCl_2$, 5 mM D-glucose, 20 mM HEPES, pH 6.4 and both at a protein concentration of 10 mg/ml were placed side by side in separate petridishes at 4°C. A 60 cm long Philips lamp (15 W; maximum emission 360 nm) was placed at a distance of 3 cm from the petridishes and the petridishes containing cells were UV irradiated for 30 min. Control cells were treated similarly without UV irradiation.

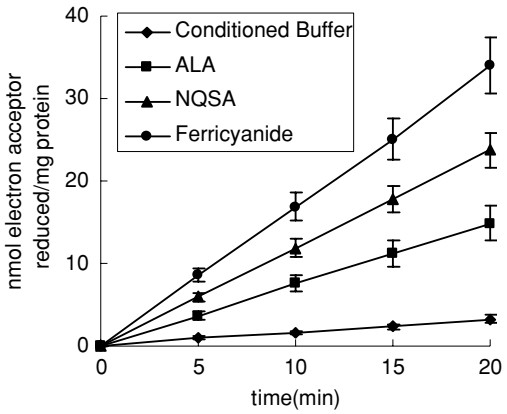

Fig. 2 ALA, NQSA and $K_3Fe(CN)_6$ reduction in cells and conditioned buffer. The conditioned buffer was obtained by incubating 2 mg *Entamoeba histolytica* trophozoites in 120 mM NaCl, 20 mM potassium acetate, 5 mM $MgCl_2$ and 20 mM morpholinopropane sulphonic acid, pH 6.4 without ALA, NQSA and $K_3Fe(CN)_6$ for the desired period of times. Cells were removed by centrifugation and 1 mM ALA, 1 mM NQSA or 3 mM $K_3Fe(CN)_6$ was added to the supernatant and incubated for 10 min. The assay was made as described in Materials and Method section ($n = 3$, S.D. $\pm$ 10%)

Results

Criteria for ALA reduction

Relationship between ALA reduction and the *E. histolytica* trophozoite protein content was linear (Fig. 1). ALA reduction by *E. histolytica* trophozoites was again linear during a 20 min period (Fig. 2). Excretion of reducing agents by *E. histolytica* trophozoites, such as phenols or thiols, could also be a basis for extracellular non permeable electron acceptor reduction. However, conditioned buffer prepared from *E. histolytica* trophozoites suspension (supernatants removed from the cells after 20 min incubation) produced insignificant ALA reduction (Fig. 2), which clearly showed that transPMET is solely responsible for extracellular reduction of non permeable electron acceptors rather than the excreted reducing agents, and was subtracted as a blank.

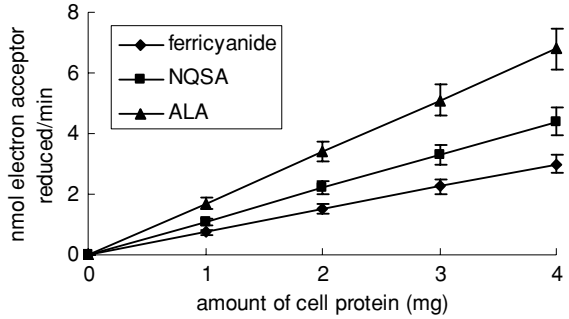

Fig. 1 Effect of cell density on the rate of ALA, NQSA, and $K_3Fe(CN)_6$ reduction by *Entamoeba histolytica* trophozoites. The assay was made as described in Materials and Method in section 2 ($n = 3$, S.D. $\pm$ 10%)

ALA concentration

ALA reduction by *E. histolytica* trophozoites suspension displayed typical saturation kinetics with increasing ALA concentration. A double reciprocal plot of the data yielded a K_m value of 0.5 mM for ALA (Fig. 3).

pH optima

ALA reduction by *E. histolytica* trophozoites give optimum rates at pH 6.4 (Fig. 4).

Reduction of other electron acceptors

E. histolytica cells can also reduce other non-permeable electron acceptors besides ALA, such as ferricyanide and NQSA, whereas ferricytochrome C, ferric-EDTA, ferric ammonium citrate and indigo disulphonate were not reduced (Table 1). The reduction of NQSA and ferricyanide was also linear with *E. histolytica* trophozoite protein content and within a time period of 20 min (Fig. 1 & 2). Table 2 lists the K_m and V_{max}, pH optimum of those non permeable electron acceptors which give a maximum reduction rate with *E. histolytica* trophozoites. The tested compounds that were actively reduced have standard redox potentials ranging from +360 mV to −290 mV at pH 7.0. Lack of ferricytochrome C, ferric-EDTA, ferric ammonium citrate, and indigo disulphonate reduction by *E. histolytica* trophozoites signifies the specificity of electron acceptors in transPMET system.

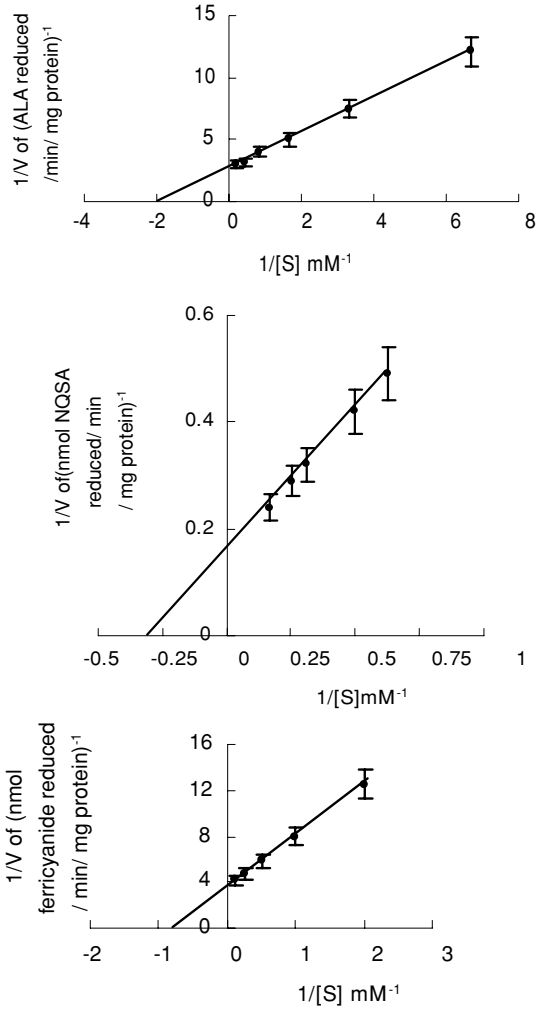

Fig. 3 Lineweaver-Burk plot of *Entamoeba histolytica* trophozoites-NQSA, *Entamoeba histolytica* trophozoites-ferricyanide, *Entamoeba histolytica* trophozoites-ALA reductase activity. The assay was made as described in Materials and Methods ($n = 3$, S.D. $\pm$ 10%). Appropriate blanks for all concentrations of NQSA, ferricyanide and ALA were corrected

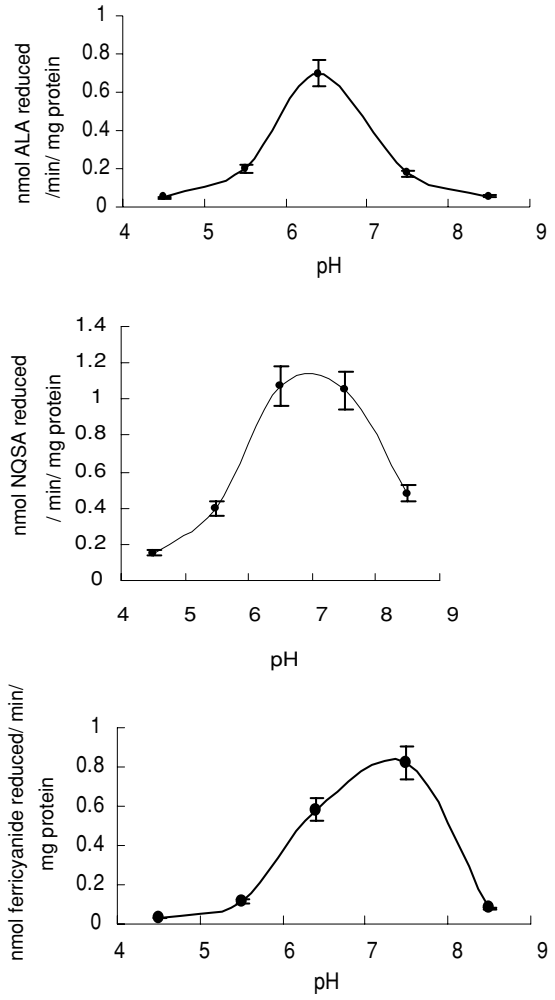

Fig. 4 Effect of pH on the rate of NQSA, ferricyanide and ALA reduction by *Entamoeba histolytica* trophozoites. *Entamoeba histolytica* trophozoites were prepared and incubated as described in Materials and Methods. The assay was made as described in Materials and Methods ($n = 3$, S.D. $\pm$ 10%). The pH of the final contents were determined by glass electrode. Appropriate blanks for all concentrations of ALA, NQSA and ferricyanide were corrected

Extracellular site for ALA reduction

If ALA is reduced at the exoplasmic surface of the *E. histolytica* cells; it should be recovered when the supernatant is reduced by Zn and metaphosphoric acid for the presence of dihydrolipoic acid. The results of such experiments are shown in Table 3.

Specific inhibitors and stimulators

Action of lipase on *E.histolytica* abolished extracellular reduction of ALA, NQSA and ferricyanide and oxygen uptake (Table 4). Elimination of glucose (5 mM) and addition of glycolytic inhibitor iodoacetamide (3 mM) substantially diminished the rate of ALA, NQSA and ferricyanide reduction and oxygen uptake (Table 4). The mitochondrial complex I inhibitor rotenone (20 μM) and complex III inhibitor antimycin A (2.5 μg/ml) showed substantial stimulation of ALA, NQSA and ferricyanide reduction, whereas they showed inhibitory effect on oxygen uptake (Table 4, Fig. 5, Fig. 6). Mitochondrial complex IV inhibitor cyanide (10 mM) showed insignificant inhibitory effect on ALA, NQSA and ferricyanide reduction and moderate inhibitory effect on oxygen uptake (Table 4). Mitochondrial complex III inhibitor HQNO (10 μM) showed substantial inhibition on ALA, NQSA and ferricyanide reduction and O_2 uptake (Table 4). The nonpermeable sulphhydryl group inhibitor PCMBS (100 μM) strongly inhibited ALA, NQSA and ferricyanide reduction and oxygen uptake (Table 4). With *E.histolytica* trophozoites the addition of 1 μM FCCP, a protonophore uncoupler, resulted in the stimulation of ALA

Table 1 Comparison of the rates of nonpermeable electron acceptors reduced by *E. histolytica* trophozoites[a]

Electron acceptors	Conc. (mM)	Rate of reduction (nmol/min/mg protein)	Redox potential E° pH(7.0) (mV)
Ferricyanide	5.00	1.85 ± 0.41	+360
Ferricytochrome C	0.10	0.046 ± 0.01	+225
NQSA	0.40	1.31 ± 0.24	+187
Ferric EDTA	0.10	0.027 ± 0.07	+90
Fe(III)ammonium citrate	4.00[b]	0.028 ± 0.07	–
Indigo disulphonate	0.05	0.006 ± 0.02	−125
ALA	1.00	0.34 ± 0.07	−290

[a] Activities were measured on one three-day-old cell culture using four aliquots of cells computed ± standard deviation. Methods for the reduction of various electron acceptors are given in Section 2.
[b] mg/ml.

reduction, inhibition of $K_3Fe(CN)_6$ reduction and no effect on NQSA reduction (Table 4). TTFA (400μM), an inhibitor of mitochondrial complex II iron-sulphur protein, has no effect on ALA, NQSA and ferricyanide reduction and O_2 uptake (Table 4). Capsaicin (50 μM), an inhibitor of energy coupling NADH dehydrogenase showed substantial inhibition on ALA, NQSA and ferricyanide reduction and O_2 uptake (Table 4). Anaerobiosis resulted in strong stimulation of ALA, NQSA and ferricyanide reduction and anaerobic stimulation was inhibited by HQNO (Table 4).

Effect of UV irradiation on *E. histolytica* cells and HCS

30 min UV exposure on *E. histolytica* cells resulted in substantial inhibition of $K_3Fe(CN)_6$ and O_2 reduction but stimulation in ALA and NQSA reduction. However, UV irradiation on HCS failed to alter ALA, ferricyanide and O_2 reduction (Table 5).

Discussion

ALA, NQSA and ferricyanide being impermeable to the cell, were assumed to act at the level of the plasma membrane (Table 3). In this study, we have shown that oxidized form of ALA acts as natural electron acceptor of transPMET and may play an important role for the survival of *E. histolytica* trophozoites under high oxygen environment when they disseminate to liver and other organs to cause multiorgan disorder. Evidence is presented for extracellular ALA reducing activity in viable intact *E. histolytica*. These findings are consistent with the concept of a transPMET in which the redox enzymes can transfer electrons from cytosolic electron donors to artificial non permeable electron acceptors. The transmembrane enzyme(s) can reduce compounds with a negative redox potential down to -290 mV (Table 1). Excretion of the reduced compounds does not account for the transmembrane ALA, NQSA and ferricyanide reduction because the supernatant which has been in contact with the cells for 20 min does not reduce ALA, NQSA and ferricyanide (Fig. 2). The recovery of 97% of added ALA after reduction of the unreacted oxidised ALA is consistent with an extracellular site of ALA reduction (Table 3).

The integrity of the cells is necessary to provide a substrate for the reduction of ALA, NQSA and ferricyanide. Lipase treated broken cells do not provide a reducing equivalent generated via glycolysis. Studies on the effect of D-glucose on ALA reduction by *E. histolytica* cells suggest that glycolysis is responsible for generation of reducing equivalents as electron donor in transPMET. This observation is also consistent with the fact that glycolytic inhibitor iodoacetamide showed substantial inhibition of transmembrane reduction (Table 4). In plant and animal cells, transmembrane electron transport depends on the production of an internal reducing agent, such as NADH or NADPH (Navas et al., 1986; Sijmons et al., 1984) the oxidation of which liberates electrons which travel to the outside of the cell via a transPMET chain and reduces the non permeable electron acceptors. Extracellular ALA, NQSA and ferricyanide reduction is not inhibited by mitochondrial electron transport inhibitors

Table 2 Comparison of K_m, V_{max} and pH optimum values of nonpermeable electron acceptors reduced by *E. histolytica* trophozoites[a]

Electron acceptors	K_m (mM)	V_{max} (nmoles/min/mg protein)	pH optimum
ALA	0.5	0.36	6.3
NQSA	3.39	6.45	6.6
$K_3Fe(CN)_6$	1.25	0.28	7.3

[a] Activities were measured on one three-day-old cell culture using four aliquots of cells computed ± standard deviation. Methods for the reduction of various electron acceptors are given in Section 2.

Table 3 Recovery of ALA in the reaction medium after removal of *E. histolytica* trophozoites

Incubation medium	Dihydrolipoic acid in supernatant[a] (μmol)
Cells + ALA (zero time)	4.920 ± 0.460
Cells + ALA (10 min incubation)	4.818 ± 0.490
Cells only (10 min incubation)	0.047 ± 0.260

[a]Cells (20 mg) in the centrifuge tubes and 1 mM ALA are incubated in 120 mM NaCl, 20 mM potassium acetate, 5 mM $MgCl_2$, 20 mM HEPES, pH 6.4, 5 mM D-glucose (10 ml) at 37°C. At the end of incubation, cells were immersed in ice and centrifuged in the cold (4°C) at 2000×*g* for 10 min. Supernatants were treated with 10 ml 2% (w/v) metaphosphoric acid and 50 mg Zn dust. The mixtures were shaken for 45 min under N_2 atmosphere and the excess Zn was filtered. An aliquot of the filtrate was titrated with standard iodine solution. The values represent averages of four experiments ± standard deviation.

antimycin A, rotenone, cyanide and azide (Table 4). Stimulation of ALA, NQSA and ferricyanide reduction by antimycin A and rotenone may be the result of partial O_2 uptake inhibition which diverted the flow of electrons towards ALA, NQSA and ferricyanide redox site in transPMET system. Increased ALA, NQSA and ferricyanide reduction could be a response to increased NADH or NADPH in the cytoplasm. This observation was further supported by the results of anaerobiosis (Table 4). O_2 uptake was not inhibited completely at high concentration of antimycin A, rotenone, cyanide or azide (Table 4). Thus, antimycin A, rotenone, cyanide and azide resistant residual O_2 uptake supports the presence of multiple O_2 reduction redox site in *E. histolytica* trophozoites. HQNO, a mitochondrial complex III inhibitor, showed substantial inhibition of ALA, NQSA and ferricyanide reduction and O_2 uptake activity (Table 4). Since HQNO is a quinone site inhibitor (Jagow et al., 1986), our data supports the involvment of a quinone coenzyme in transPMET system of *E.histolytica* trophozoites. Capsaicin is a new naturally occurring inhibitor of proton-pumping NADH-quinone oxidoreductases (NDH-1) present in the respiratory chain of various species of bacteria and mitochondria and can be divided into two groups (Yagi, 1987, 1990, 1991, 1993; Yagi et al., 1988). One group of enzymes that bear the energy-coupling site is designated as NDH-1 and the other group that does not is designated as NDH-2. The bacterial NDH-1 enzyme complexes appear to be related to the mammalian mitochondrial NADH-UQ oxidoreductase (complex I) as judged by similarities in prosthetic groups, polypeptide compositions, and specific inhibitors (Yagi, 1987, 1990, 1993; Yagi et al., 1992; Meinherdt et al., 1987). The sensitivity to ALA, NQSA and ferricyanide reduction inhibition (Table 4) in *E.histolytica* trophozoites by capsaicin supports the notion of the presence of an energy-coupling site (NDH-1) in transPMET system.

TransPMET of liver plasma membrane required ubiquinone for activity (Sun et al., 1992). A variety of quinones, other than CoQ, which differ by ring and side chain modifications, can be found to occur not only in bacterial species [e.g. menaquinone, desmenaquinone (Brodie et al., 1963; Lester et al., 1964)], but also in some eucaryotic organisms such as the parasitic helminthes [e.g. rhodoquinone

Table 4 Effect of several effectors on extracellular reduction of ALA, NQSA, $K_3Fe(CN)_6$ and oxygen uptake by *E. histolytica* trophozoites

	Rate of electron acceptor reduction, nmol/min/mg protein[a]							
Addition	Rate of ALA reduction	Relative rate	Rate of NQSA reduction	Relative rate	Rate of $K_3Fe(CN)_6$ reduction	Relative rate	Rate of Oxygen reduction	Relative rate
None	0.30 ± 0.06	100	1.27 ± 0.27	100	1.12 ± 0.23	100	8.14 ± 1.40	100
Lipase (10U)	0.02 ± 0.01	07	0.15 ± 0.06	12	0.11 ± 0.03	10	0.41 ± 0.01	05
-Glucose (5 mM)	0.09 ± 0.03	31	0.45 ± 0.11	35	0.09 ± 0.04	8	2.19 ± 0.14	27
Iodoacetamide (3 mM)	0.07 ± 0.02	24	0.38 ± 0.14	30	0.31 ± 0.08	28	1.13 ± 0.07	14
Antimycin A (2.5 μg/ml)	0.65 ± 0.14	217	2.37 ± 0.43	187	1.92 ± 0.34	171	5.05 ± 0.90	62
Rotenone (20 μM)	0.80 ± 0.16	268	2.15 ± 0.47	169	1.83 ± 0.31	163	5.21 ± 0.98	64
Cyanide (10 mM)	0.26 ± 0.09	87	0.89 ± 0.14	70	0.89 ± 0.15	68	4.88 ± 0.58	60
Azide (2 mM)	0.29 ± 0.07	95	1.09 ± 0.18	86	1.06 ± 0.14	95	3.83 ± 0.65	47
HQNO (10 μM)	0.07 ± 0.01	25	0.58 ± 0.09	46	0.22 ± 0.03	20	2.20 ± 0.42	27
TTFA (400 μM)	0.27 ± 0.06	90	1.14 ± 0.12	95	1.04 ± 0.07	93	6.75 ± 1.26	83
FCCP (1 μM)	0.58 ± 0.10	194	1.22 ± 0.23	96	0.56 ± 0.11	50	3.83 ± 0.57	47
PCMBS (100 μM)	0	0	0.15 ± 0.05	12	0.22 ± 0.07	20	2.77 ± 0.50	34
Capsaicin (50 μM)	0.15 ± 0.05	50	0.76 ± 0.14	60	0.50 ± 0.08	44	4.07 ± 0.72	50
Anaerobiosis	0.70 ± 0.14	232	3.68 ± 0.80	290	2.51 ± 0.45	224	—	—
Anaerobiosis + HQNO(10 μM)	0.08 ± 0.03	27	0.52 ± 0.11	41	0.20 ± 0.06	18	—	—

[a]ALA, NQSA, $K_3Fe(CN)_6$ reduction and O_2 uptake was measured according to the procedure as given in Section 2. The effectors were added to *E. histolytica* trophozoites 10 min before the addition of electron acceptors. Incubation time with electron acceptors was 10 min. Control experiments which received an equal volume of DMF given along with effectors had no effect on electron acceptor reduction in *E. histolytica* cells. The values represent averages of four experiments ± standard deviation.

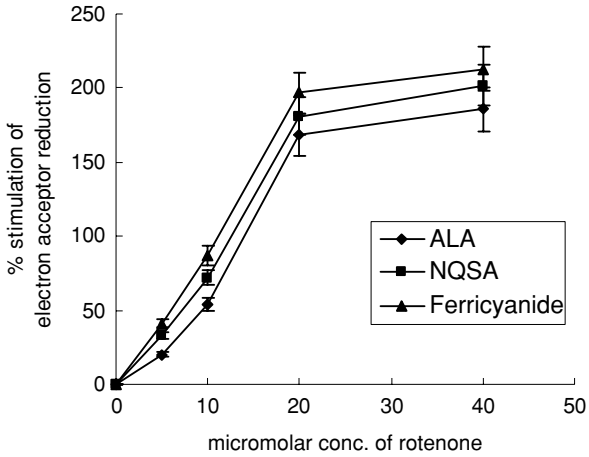

Fig. 5 Percentage stimulation of relative rate of ALA, NQSA and ferricyanide reduction with increasing concentration of mitochondrial electron transport inhibitor Antimycin A. The assay was made as described in Materials and Method in section 2 ($n = 3$, S.D. $\pm$ 10%)

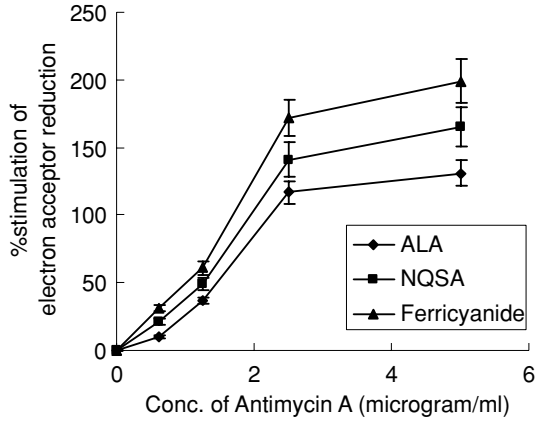

Fig. 6 Percentage stimulation of relative rate of ALA, NQSA and ferricyanide reduction with increasing concentration of mitochondrial electron transport inhibitor Rotenone. The assay was made as described in Materials and Method in section 2 ($n = 3$, S.D. $\pm$ 10%)

(Kohler et al., 1985)]. Our results demonstrate that O_2 uptake and ferricyanide reduction is strongly inhibited by UV irradiation which suggests the involvment of naphthoquinone like compounds for maximal performance, because CoQ was not inactivated by UV irradiation (Brodie et al., 1963; Table 5). Stimulation of ALA, NQSA reduction in UV exposed *E. histolytica* appears to be due to inhibition of O_2 uptake in redox chain. Thus, the naphthoquinone coenzyme involved in transmembrane redox of *E. histolytica,* appears to be positioned before ferricyanide and oxygen reduction site and after ALA and NQSA reduction site. In contrast, UV exposed liver cells that contain CoQ_{10} in the plasma membrane (Sun et al., 1992), failed to inhibit O_2, ALA and ferricyanide reduction. In mammalian cells NQSA is not reduced by transPMET system. The development of new antiamoebic agents can be aided by the identification of these differences in transPMET components between the host and the invading parasite.

The transPMET activity of *E.histolytica* trophozoites was insensitive to TTFA (Table 4), a potent inhibitor of complex II in aerobic tissues such as bovine heart (Kita et al., 1988; Mowery et al., 1977). Since TTFA inhibits the reoxidation of iron-sulphur center (S-3) of bovine complex II (Mowery et al., 1977; Salerno et al., 1980) which is associated with the Ip subunit, the insensitivity of transPMET towards TTFA may indicate a different pathway for electron transfer in Ip subunit. Alternatively, it may reflect structural difference in the vicinity of the S-3 center, which results in different accessibility of TTFA.

The ability of the parasite to cope up with an influx of protons induced by the protonophore FCCP was examined (Table 4). In standard buffer at pH 6.4 FCCP (1 μM) resulted

Table 5 Comparison of the effect of UV irradiation on extracellular ALA, NQSA and $K_3Fe(CN)_6$ reduction and O_2 uptake by *E. histolytica* and HCS

Incubation	Time of UV Exposure (min)	Rate[a] (nmol/min/mg protein)	Relative rate
UV unexposed EHC+ ALA (1 mM)	–	0.32 ± 0.02	100
UV exposed EHC+ ALA (1 mM)	30	0.53 ± 0.09	166
UV unexposed HCS+ ALA (1 mM)	–	0.03 ± 0.03	100
UV exposed HCS+ ALA (1 mM)	30	0.03 ± 0.04	100
UV unexposed EHC+ NQSA (1 mM)	–	1.30 ± 0.21	100
UV exposed EHC+ NQSA (1 mM)	30	1.78 ± 0.32	137
UV unexposed HCS+ NQSA (1 mM)	–	0	0
UV unexposed EHC+ $K_3Fe(CN)_6$(3 mM)	–	1.10 ± 0.26	100
UV exposed EHC+ $K_3Fe(CN)_6$(3 mM)	30	0.55 ± 0.12	50
UV unexposed HCS+ $K_3Fe(CN)_6$(3 mM)	–	0.27 ± 0.04	100
UV exposed HCS+ $K_3Fe(CN)_6$(3 mM)	30	0.27 ± 0.05	100
UV unexposed EHC+ O_2	–	8.14 ± 1.40	100
UV exposed EHC+ O_2	30	2.03 ± 0.30	25
UV unexposed HCS+ O_2	–	20.00 ± 2.82	100
UV exposed HCS+ O_2	30	20.00 ± 2.13	100

[a] ALA, NQSA and $K_3Fe(CN)_6$ and O_2 uptake were assayed according to the procedure as given in Section 2. The values represent averages of four experiments ± standard deviation.

in stimulation of ALA reduction but inhibition in ferricyanide reduction and oxygen uptake.

The inhibition of ALA, NQSA and ferricyanide reduction and O_2 uptake by PCMBS (Table 4), which has no permeability through membranes, indicates outer plasma membrane location of –SH group(s) for transmembrane reduction. Complete inhibition of ALA reduction by the nonpermeable –SH inhibitor PCMBS also suggests predominant extracellular reduction of ALA.

Taken together, ALA, NQSA and the ferricyanide reduction, and O_2 uptake by *E. histolytica* trophozoites may be due to electron transport involving a naphthoquinone coenzyme across the plasma membrane which is placed before ferricyanide and oxygen reduction site and after the ALA and NQSA reduction site. The possibility that there is more than one pathway of electron egress to the electron acceptors cannot be ruled out. However, the mechanism involving cytosolic NADH/NADPH as electron donor and extracellular non permeable ALA as electron acceptor, similar to that described for animal and plant cells, may be responsible for extracellular ALA reduction in such a way that an optimal extracellular redox state is maintained with the consequent protection of parasite against high oxygen concentration in liver and tissues as well as oxidative burst by host immune system. It has also been suggested that virulence of axenically grown *E.histolytica* trophozoites depends to a considerable extent on the cells reducing power (Kumar et al., 1992). Hence, transPMET is an artifice of this parasite for invasion and survival.

Acknowledgments Author like to acknowledge Mr. T. N. Boral for providing technical help during the study.

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