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Evidence for the extracellular reduction of α-lipoic acid by *Leishmania donovani* promastigotes: a transplasma membrane redox system

Gautam Datta, Tanmoy Bera *

Division of Medicinal Biochemistry, Department of Pharmaceutical Technology, Jadavpur University, Calcutta 700 032, India

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Abstract

Leishmania donovani cells, capable of reducing certain electron acceptors with redox potentials at pH 7.0 down to −290 mV, outside the plasma membrane, can reduce the oxidised form of α-lipoic acid. α-Lipoic acid has been used as natural electron acceptor probe for studying the mechanism of transplasma membrane electron transport. Transmembrane α-lipoic acid reduction by *Leishmania* was not inhibited by mitochondrial inhibitors as azide, cyanide, rotenone or antimycin A, but responded to hemin, modifiers of sulphhydryl groups and inhibitor of glycolysis. The protonophores carbonyl cyanide chlorophenylhydrazone and 2,4-dinitrophenol showed inhibition of α-lipoic acid reduction. This transmembrane redox system differs from that of mammalian cells in respect to its sensitivity of UV irradiation and stimulation by diphenylamine. Thus a naphthoquinone coenzyme appears to be involved in α-lipoic acid reduction by *Leishmania* cells.

Keywords: Transplasma membrane; Electron transport; Ferricyanide reduction; α-Lipoic acid reduction; 1,2-Naphthoquinone-4-sulfonate reduction; Hemin; *Leishmania donovani*

1. Introduction

Leishmania, dimorphic parasitic protozoa that live as flagellated promastigotes in the digestive tract of their insect vectors and as non-flagellated amastigotes in the phagolysosome of mammalian macrophages, cause the potentially fatal disease visceral leishmaniasis [1,2]. The intracellular amastigote form proliferates in the acid pH of secondary lysosomes of human macrophages [3,4]. The extracellular promastigote stage is introduced into subcutaneous tissue in the human host during the bite of an infected sand fly vector. It is phagositosed by a mononuclear phagocyte, after which it converts into the obligate intracellular amastigote form [5]. The receptor mediated ingestion of promastigotes into the mononuclear phagocyte is accompanied by an oxidative burst of the phagocyte, during which oxidants such as superoxide and hydrogen peroxide are

Abbreviations: LDC, *Leishmania donovani* cells; NQSA, 1,2-naphthoquinone-4-sulphonic acid; CCCP, carbonyl cyanide chlorophenylhydrazone; DNP, 2,4-dinitrophenol; DPA, diphenylamine; NBDC, 7-chloro-4-nitrobenz-2-oxo-1,3-diazole; PBS, phosphate buffered saline; BSA, bovine serum albumin; TCA, trichloroacetic acid; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; ALA, α-lipoic acid; PCMBS, *p*-chloromercuribenzenesulphonate; HCS, hepatocyte cell suspension

* Corresponding author. Fax: +91-33-473-1484;
E-mail: beratanmoy@hotmail.com

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formed [6–8]. Hydrogen peroxide can be converted to hydroxyl radical ($^{\bullet}OH$) through the Fenton reaction in the presence of a source of iron: $H_2O_2+Fe^{2+} \rightarrow {}^{\bullet}OH+OH^{-}+Fe^{3+}$. Therefore, in order to successfully establish an infection, promastigotes must survive despite the local production of toxic oxidant species by the mononuclear phagocyte.

Given the necessity for avoiding the toxic effects of macrophage generated H_2O_2 and $^{\bullet}OH$ in order to cause infection, several hypotheses might account for the differential survival of the different forms of promastigotes. First, the survival of the parasite within the macrophage might be due to the formation of a lesser amount of $^{\bullet}OH$. Secondly, increased resistance to $^{\bullet}OH$ might be due to scavenging of this free radical by extracellular antioxidant compounds.

Electron transport across the plasma membrane has been described in many eucaryotic cells [9,10], such as erythrocytes [11], liver [12], heart [13], transformed liver cells [14], HeLa cells [15], neutrophils [16], yeast [17] and plant cells [18]. Functions described for this activity include (a) reduction of Fe(III) to supply Fe(II) for transport to the cell [19], (b) alteration of the redox state of the cytoplasm [20] or activation of nuclear genes [21] to stimulate cell growth [22], and (c) reduction of extracellular antioxidant compounds, e.g., coenzyme Q hydroquinone [23], tocopherol [24], and ascorbic acid [23], after they have been oxidised by free radicals.

α-Lipoic acid (ALA) and its reduced form, dihydrolipoate, reacts with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen. It also protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E [25]. In this study, we will describe a system in *Leishmania* cells which keeps extracellular ALA reduced. This is, to our knowledge, the first study of the properties of a plasma membrane redox system mediated extracellular ALA reduction in a protozoan parasite. A dihydrolipoamide dehydrogenase system in *Trypanosoma* species has also been identified on the cytoplasmic face of the plasma membrane, but not found in any other cellular fraction [26,27]. Here, we provide evidence for a basic difference between the mammalian and the *Leishmania* cell plasma membrane mediated ALA reduction system. The distinguishing characteristics of the plasma membrane redox system in *Leishmania* is its sensitivity to UV irradiation, 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 transplasma membrane electron transport. The transplasma membrane mediated extracellular ALA reduction also provides a new approach to understand the mechanism of survival of *Leishmania* species within the macrophage.

2. Materials and methods

All biochemicals unless otherwise mentioned were from Sigma (St. Louis, MO, USA). Panmede was purchased from Paines and Byrne (Greenford, Middlesex, UK).

2.1. Cell culture

Leishmania donovani promastigote strain MHOM/IN/1978/UR6, a clinical isolate from a confirmed kala-azar patient, was grown at 24°C on blood-agar medium, pH 7.5 [28]. The cells were washed at $500\times g$ thrice in cold Tris-sucrose-salt solution (250 mM sucrose, 20 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris, pH 7.2) and kept in it at 4°C until use. Viability of the harvested cells was monitored microscopically.

2.2. Measurement of ferricyanide reduction by L. donovani cells (LDC)

Ferrocyanide quantitation was performed using 1,10-phenanthroline complex as described by Avron and Shavit [29]. The incubation mixture contained 140 mM phosphate buffered saline (PBS), pH 7.0, 3 mg LDC, 5 μmoles D-glucose and 5 μmoles $K_3(Fe(CN)_6)$ in a final volume of 1 ml. The incubation was carried out at 25°C. The reaction was terminated by the addition of 0.1 ml 30% (w/v) trichloroacetic acid (TCA) followed by centrifugation at $10\,000\times g$ for 15 min. Ferrocyanide in supernatant was measured by 1.5 ml of 1,10-phenanthroline reagent containing 1.5 mmoles sodium acetate, 0.1 mmoles citric acid, 0.75 μmoles ferric chloride and 12.6 μmoles 1,10-phenanthroline at 510 nm. The blanks were carried out with all reagents except LDC.

2.3. Measurement of ferricytochrome c reduction by LDC

Ferricytochrome *c* reduction by LDC was measured at 550 nm using 20 μM ferricytochrome *c* and 5 mg LDC in PBS, 140 mM, pH 7.0. Incubates were centrifuged at $15\,000\times g$ for 15 min and supernatants were kept for estimation of ferricytochrome *c* reduction.

2.4. Iron reduction assays

Iron reduction by *L. donovani* promastigote cells was assayed as the formation of red complex between reduced iron and 1,10-phenanthroline, using the difference in absorbance between 510 and 600 nm at 25°C. The reaction mixture consisted of 3 mg LDC, 60 μmoles Tris-HCl, pH 7.2, 750 μmoles sucrose, 60 μmoles KCl, 60 nmoles 1,10-phenanthroline and one of the following: ferric EDTA, ammonium Fe(III) citrate in the concentration of 0.3 μmoles and 12 mg, respectively, in a total volume of 3.0 ml. The millimolar extinction coefficient for ferrous 1,10-phenanthroline is 9.5 ($\Delta A_{510-600} = 9.5$ mM^{-1} cm^{-1}). In all the cases, a preincubation period of 5 min was allowed with cells in Tris-sucrose-salt solution before the addition of ferric EDTA, ammonium Fe(III) citrate and 1,10-phenanthroline.

2.5. Indigo disulphonate reduction assay

Indigo disulphonate reduction by *L. donovani* promastigote cells was measured as the decrease in absorbance at 608 nm at 25°C. The reaction mixture was the same as for the iron reduction assays with 150 nmoles indigo disulphonate added in place of iron compounds and 1,10-phenanthroline.

2.6. ALA and 1,2-naphthoquinone-4-sulphonic acid (NQSA) reduction assay

ALA and NQSA reduction by *L. donovani* promastigote cells 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 [29]. The reaction mixture consisted of 5 mg LDC, 140 mM PBS, pH 6.4, 1 mM ALA or 0.4 mM NQSA in a final volume of 3.0 ml. The reaction mixtures were incubated for 10 min at 25°C. After incubation, the reaction mixtures were kept in ice and then centrifuged at 4°C at $10\,000\times g$ for 10 min. 1.4 ml of the supernatant was removed and added to 0.1 ml of 75 mM $K_3(Fe(CN)_6)$, 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 LDC. Two molecules of ferricyanide have been considered to react with one molecule of dihydrolipoic acid or one molecule of hydroquinone of NQSA.

2.7. Preparation of hepatocyte cell suspension (HCS)

Liver from a healthy rat was removed and minced in a mortar and pestle to make a homogeneous paste and suspended in PBS, 140 mM, pH 6.4 at 4°C. The suspension was centrifuged at $1000\times g$ for 10 min at 4°C. The supernatant was discarded and the pellet again resuspended and the same operation was repeated twice as before and the pellet was kept at 4°C until use.

2.8. UV irradiation of HCS and L. donovani promastigote cells

HCS and *L. donovani* promastigote cells were UV irradiated by modifying the procedure as described by Brodie et al. [30]. This treatment was performed as follows: HCS and LDC at a protein concentration of 10 mg/ml of PBS, 140 mM, pH 6.4, were placed side by side in separate petri dishes at 4°C. A 30 cm long Eye G8T5 lamp (8 W; maximum emission 360 nm) was placed at a distance of 3 cm from the petri dishes. Control cells were treated similarly without UV irradiation.

2.9. Protein estimation

The amount of protein was determined by the biuret method in the presence of 0.2% deoxycholate [31]. Bovine serum albumin (BSA) was used as standard. 1 mg whole-cell protein corresponds to 1.4×10^8 cells.

3. Results

3.1. ALA concentration

ALA reduction by *L. donovani* cells shows a typical saturation kinetics with increasing ALA concentration. A double reciprocal plot of the data (Fig. 1) yielded a K_m value of 0.66 mM for ALA.

3.2. pH optimum for assays

ALA reduction by *Leishmania* cells gives optimum rates between pH 6.1 and pH 6.4 (Fig. 2).

3.3. Reduction of other electron acceptors

Leishmania cells can also reduce other nonpermeant electron acceptors besides ALA, such as ferricyanide and NQSA. Table 1 lists the concentrations of these compounds which give a maximum reduction rate with *Leishmania* cells. The tested compounds that are active have standard redox potentials at pH 7.0 ranging from +360 mV to −290 mV.

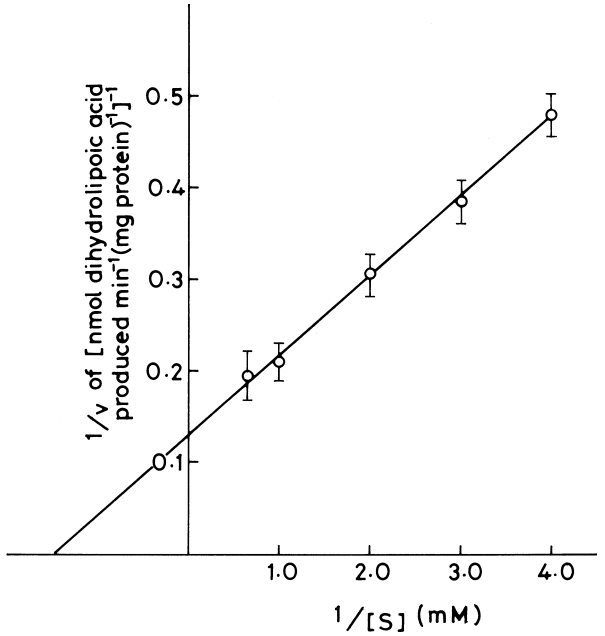

Fig. 1. Lineweaver-Burk plot of LDC-ALA reductase activity. Appropriate blanks for all concentrations of ALA were corrected.

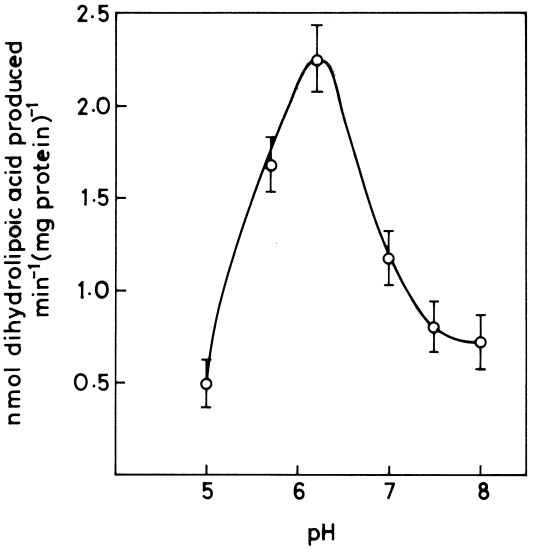

Fig. 2. Effect of pH on the rate of ALA reduction by LDC. LDC were prepared and incubated as described in Section 2. The pH of the final contents were determined by glass electrode.

3.4. Criteria for extracellular ALA reduction

If ALA is reduced at the outer surface of *Leishmania* 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 2.

Excretion of reducing agents from cells, such as phenols or thiols, would be a basis for external ferricyanide reduction. The supernatants removed from the cells after 10 min incubation produced no significant ferricyanide reduction (Fig. 3), showing that transmembrane electron transport by whole cells is necessary for ALA reduction. The relationship between ALA reduction and the amount of protein added was linear with LDC (Fig. 4).

3.5. Specific inhibitors and stimulators

The mitochondrial terminal electron transport chain inhibitors cyanide, azide, complex III inhibitor antimycin A and complex I inhibitor rotenone failed to inhibit extracellular ALA reduction in LDC. With LDC the addition of 50 μM carbonyl cyanide chlorophenylhydrazone (CCCP) and 2 mM 2,4-dinitrophenol (DNP) resulted in 70% and 43% inhibition, respectively (Table 3). 0.5 mM diphenylamine (DPA)

Table 1
Comparison of the rates of nonpermeable electron acceptors reduced by *L. donovani* cells[a]

Electron acceptors	Concentration (mM)	Rate of reduction (nmol/min/mg protein)	Redox potential $E°$ pH (7.0) (mV)
Ferricyanide	5.00	0.650 ± 0.030	+360
Ferricytochrome *c*	0.10	0.030 ± 0.008	+225
NQSA	0.40	2.650 ± 0.300	+187
Ferric EDTA	0.10	0.020 ± 0.006	+90
Fe(III) ammonium citrate	4.00[b]	0.020 ± 0.006	–
Indigo disulphonate	0.05	0.001	−125
ALA	1.00	2.240 ± 0.260	−290

[a]Activities were measured on one cell culture using four aliquots of cells computed ± standard deviation. 3-day-old cell cultures were used in all experiments. Methods for the reduction of various electron acceptors are given in Section 2.
[b]mg/ml.

showed substantial stimulation in *Leishmania*, but had no effect on ALA reduction by hepatocytes (data not shown). 5 μM hemin was found to be the most potent inhibitor of ALA reduction. The sulphhydryl group inhibitors 7-chloro-4-nitrobenz-2-oxo-1,3-diazole (NBDC), *N*-ethylmaleimide (NEM), phenylarsine oxide and *p*-chloromercuribenzenesulphonate (PCMBS) showed potent inhibition, and anaerobiosis had no effect on ALA reduction.

3.6. Effect of UV irradiation on LDC and HCS

Sixty minute UV exposure on LDC resulted in substantial inhibition of ALA reduction, whereas UV irradiation on HCS failed to inhibit ALA reduction (Table 4).

Table 2
Recovery of ALA in the medium after removal of *L. donovani* cells

Treatment	Dihydrolipoic acid in supernatant[a] (μmol)
Cells+ALA (zero time)	4.930 ± 0.500
Cells+ALA (10 min incubation)	4.810 ± 0.500
Cells only (10 min incubation)	0.008

[a]Cells (15 mg) in the centrifuge tubes and 1 mM ALA were incubated in 140 mM PBS (10.0 ml), pH 6.4 at 25°C. At the end of incubation, cells were immersed in ice and centrifuged in the cold (4°C) at 10 000 × *g* for 15 min. Supernatants were treated with 10 ml 2% (w/v) metaphosphoric acid and 40 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.

4. Discussion

The extracellular reduction of ALA by *Leishmania* cells resembles the reduction of the various nonpermeable artificial electron acceptors (Table 1). Transmembrane electron transport is well established in many eucaryotic cells of both plant and animal origin [9,10], but this is to our knowledge the first demonstration with a protozoan cell *Leishmania*. In this study, we have shown that ALA acts as the natural electron acceptor [32] of transplasma mem-

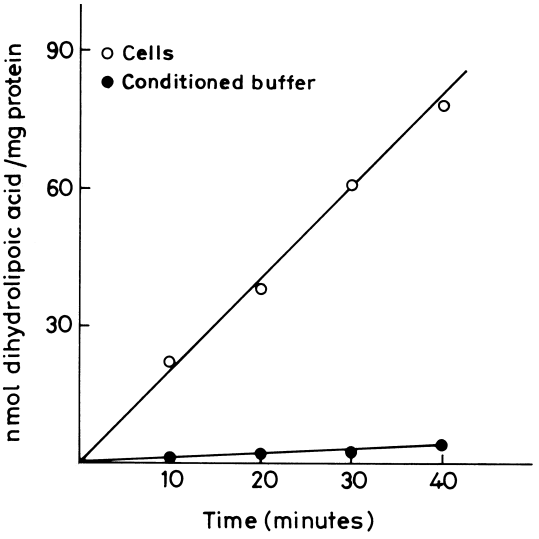

Fig. 3. ALA reduction in cells and conditioned buffer. The conditioned buffer was obtained by incubating 5 mg LDC in 140 mM PBS, pH 6.4 without ALA for the desired period of time. Cells were removed by centrifugation and 1 mM ALA was added to the supernatant and incubated for 10 min. The assay was made as described ($n = 4$, S.D. ≤ 7%).

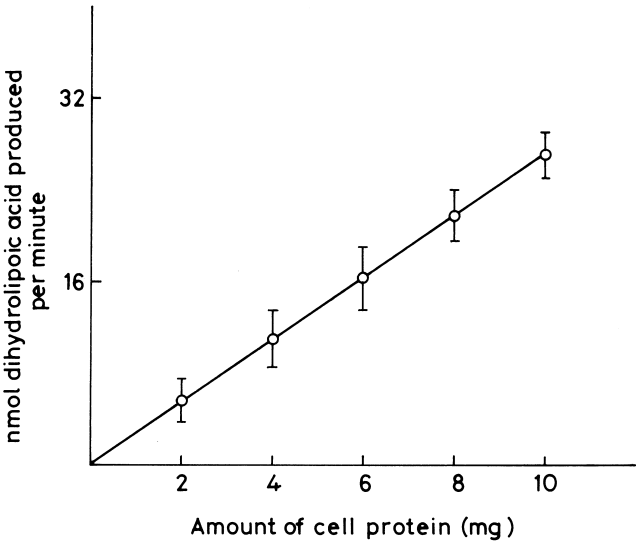

Fig. 4. Effect of cell density on the rate of ALA reduction by LDC. LDC were prepared and incubated as described in Section 2.

brane electron transport and may play an important role for the survival of *L. donovani* promastigotes within the macrophage. Evidence is presented for extracellular ALA reducing activity in viable intact LDC. These findings are consistent with the concept of a transplasma membrane electron transport system in which the enzyme can react with the artificial nonpermeable electron acceptor ferricyanide. Reduction does not appear to be accomplished by movement of a reducing agent through the membrane, since conditioning of the medium by adding and removing cells does not produce ALA reduction. *Leishmania* cells are also able to reduce ferricyanide and NQSA appreciably (Table 1). The transmembrane enzyme(s) can reduce compounds with a negative redox potential down to −290 mV. Excretion of the reducing compounds does not account for the transmembrane ALA reduction rates observed with *Leishmania*, because the supernatant which has been in contact with the cells for 40 min does not reduce ALA. The recovery of 98% of added ALA after reduction of the unreacted oxidised ALA is consistent with an extracellular site of ALA reduction.

The integrity of the cells is necessary to provide a substrate for the reduction of ALA. Broken cells do not provide a reducing equivalent, generated via glycolysis. Studies on the effect of D-glucose on ALA

Table 3
Effect of several effectors on extracellular reduction of ALA by LDC

Incubation	ALA reduction rate[a] (nmol/min/mg) protein	Relative rate
Control[b]	2.240 ± 0.260	100
Control[c]	0.067 ± 0.008	3
– ALA (1 mM)	0.180 ± 0.020	8
– D-glucose (5 mM)	1.882 ± 0.242	84
CCCP (50 μM)	0.672 ± 0.080	30
DNP (2 mM)	1.277 ± 0.153	57
Azide (2 mM)	2.235 ± 0.255	100
Cyanide (5 mM)	1.926 ± 0.260	86
Rotenone (0.5 mM)	1.860 ± 0.200	83
Antimycin A (10 μM)	2.173 ± 0.242	97
DPA (0.5 mM)	7.170 ± 0.680	320
NBDC (0.1 mM)	0.157 ± 0.019	7
NEM (0.5 mM)	0.224 ± 0.026	10
PCMBS (0.2 mM)	0.022 ± 0.006	1
Phenylarsine oxide (0.5 mM)	0.134 ± 0.016	6
Iodoacetamide (3.0 mM)	0.900 ± 0.108	40
Hemin (5 μM)	0.112 ± 0.014	5
Anaerobiosis[d]	2.060 ± 0.226	92

[a]ALA reduction was assayed according to procedure as given in Section 2. The effectors were added to LDC 10 min before the addition of ALA. Incubation time with ALA was 10 min. Control experiments which received an equal volume of solvents given along with the effectors had no effect on ALA reduction in LDC. The values represent averages of four experiments.
[b]Control incubation contained LDC, ALA and D-glucose as described in Section 2.
[c]Control incubation contained freeze-thawed LDC [28]. ALA and D-glucose as described in Section 2.
[d]Anaerobiosis of LDC was carried out by evacuation and flushing with N_2/CO_2 (95:5).

Table 4
Comparison of the effect of UV radiation on extracellular ALA reduction by LDC and HCS

Incubation	Time of UV exposure (min)	Rate[a] (nmol of ALA reduced/min/mg protein)	Relative rate
UV unexposed LDC+ALA (1 mM)	–	2.240 ± 0.200	100
UV exposed LDC+ALA (1 mM)	60	0.336 ± 0.040	15
UV unexposed HCS+ALA (1 mM)	–	0.247 ± 0.030	100
UV exposed HCS+ALA (1 mM)	60	0.265 ± 0.033	107

[a]ALA reduction was assayed according to the procedure as given in Section 2. The values represent averages of four experiments.

reduction by LDC (Table 3) suggest that glycolysis is responsible for generating the reducing equivalents as electron donor for transplasma membrane electron transport. In the present study, the identity of the reductant is unknown. In plant and animal cells, transmembrane electron transport depends on the production of an internal reducing agent, such as NADH or NADPH [17,20,33], the oxidation of which liberates electrons which travel to the outside of the cell via a transplasma membrane electron transport chain.

The effects of inhibitors are consistent with electron transport across the plasma membrane (Table 3). Extracellular ALA reduction by LDC is not inhibited by mitochondrial inhibitors as cyanide, azide, rotenone or antimycin A, which shows the reaction is not based on mitochondria from broken cells, because these inhibitors are known to inhibit mitochondrial electron transport in *Leishmania* [34]. Iodoacetamide, an inhibitor of glycolysis, on the other hand, can inhibit ALA reduction by 60% after 10 min incubation with LDC. A decrease in the rate of internal NADH presumed to limit electron donor availability for the transmembrane ALA reduction. Increased ALA reduction with DPA would be a response to increased NADH in the cytoplasm, because of 98% inhibition of glucose dependent oxygen uptake in LDC at the same concentration of DPA (data not shown). Hill and Cross showed that cyanide resistant respiration of *Trypanosoma brucei* was completely inhibited by DPA and they suggested the possibility of extramitochondrial existence of cytochrome *o* as a part of the branched electron transport chain [35,36]. The protonophores CCCP and DNP, which equilibrate proton concentration across the biological membrane [37], showed 60% and 43% inhibition of ALA reduction activity, respectively. From the studies of the effects of different -SH reagents on ALA reduction, information about the localisation of the reactive -SH groups was obtained (Table 3). Inhibition of ALA reduction by NBDC, NEM, PCMBS and phenylarsine oxide [38] strongly suggests the involvement of one or more -SH group(s) for ALA reduction. Inhibition of ALA reduction by PCMBS suggests the outer plasma membrane location of -SH group(s) involved in ALA reduction. Complete inhibition of ALA reduction by the nonpermeable -SH inhibitor PCMBS suggests the predominant extracellular reduction of ALA. The chemical modification with the trivalent arsenical reagent phenylarsine oxide indicates the involvement in extracellular ALA reduction of a reducible disulphide bond [39,40]. Arsenic compounds form very stable five membered rings with 1,2-dithiols [38]. It is obvious from Table 3 that the most striking property is the potent inhibitory effect of hemin on ALA reduction. *Leishmania* species, incapable of haem biosynthesis, acquire haem exogenously from the culture medium or fetal bovine serum, independent of haem synthesised from the macrophages [41]. Galbraith and McElrath observed that the promastigotes of *Leishmania* species possess 4000 hemin binding sites per promastigote in dividing cultured cells [42].

The potent inhibition of ALA reduction due to UV exposure of LDC suggests the involvement of naphthoquinone-like compounds for maximal performance. This observation also suggests the involvement of naphthoquinone-like compounds in transplasma membrane electron transport, because mitochondria of all trypanosomatids require the involvement of coenzyme Q_9 [43] which is not inactivated by UV irradiation [44]. In contrast, UV-exposed liver cells which contain coenzyme Q_{10} in the plasma membrane [23], failed to inhibit ALA reduction (Table 4). The development of new antileishmanial agents can be aided by the identification of this

difference between the host and the invading parasite [45].

In conclusion, ALA reduction by *Leishmania* cells may be due to electron transport, involving a naphthoquinone coenzyme, across the plasma membrane. A mechanism involving cytosolic NADH as electron donor and extracellular ALA as electron acceptor, similar to that described for animal and plant cells, may be responsible for the ALA reduction, in such a way that an optimal extracellular redox state can be maintained with the consequent protection against oxidative damage [25]. Another important function of the transplasma membrane electron transport system in LDC appears to be reduction of iron in hemin, ferrilactoferrin and ferritransferrin and their receptor mediated transport for acquisition of iron [46,47].

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