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Electron transport components of the parasitic protozoon *Giardia lamblia*

Jayne E. Ellis[a], Ruth Williams[b], Deborah Cole[a], Richard Cammack[b] and David Lloyd[a]

[a]*Microbiology Group, School of Pure and Applied Biology, University of Wales College of Cardiff, Cardiff, CF1 3TL, UK* and [b]*Division of Life Sciences, King's College, London, W8 7AH, UK*

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The energy metabolism of the intestinal parasite, *Giardia lamblia*, involves the iron–sulphur protein, pyruvate:ferredoxin oxidoreductase. Cell fractionation studies showed that this enzyme is associated with the membranes. NADH and NADPH dehydrogenases were found in both the membrane and cytosolic fractions. EPR spectroscopic studies showed the presence of iron–sulphur clusters in the membrane fraction and in the cytosolic fraction, non-sedimentable at 6×10^6 $g \cdot$ min. An acidic, soluble protein fraction was separated from the cytosol. It had an EPR spectrum in the reduced state, characteristic of the 2[4Fe–4S] type of ferredoxin, with g-factors at 2.04, 1.93 and 1.89, and the midpoint redox potential was estimated to be −360 mV. This species is probably a ferredoxin, like those of anaerobic bacteria such as *Clostridium* and *Desulfovibrio* spp. and also that of *Entamoeba histolytica*. The protein was readily and irreversibly oxidized to give [3Fe 4S] clusters.

Giardia lamblia; Ferredoxin; Pyruvate:ferredoxin oxidoreductase; Electron transport; Iron–sulphur protein; EPR spectroscopy; NAD(P)H oxidase

1. INTRODUCTION

Giardia lamblia is a common water-borne flagellate protozoon which causes serious infection of the upper small intestine of man. Trophozoites have a predominantly anaerobic metabolism, in which energy is generated by fermentative pathways, both in the presence and absence of oxygen; carbohydrates are incompletely oxidized to ethanol, acetate, alanine and CO_2 [1]. Organelles identifiable as mitochondria are absent and inhibitors of cytochrome-mediated electron transport do not affect oxygen consumption [2–4]. *G. lamblia* thus belongs to a group of protozoa commonly termed 'anaerobic'. Despite the lack of normal aerobic respiratory characteristics, *G. lamblia* consumes oxygen rapidly at the low oxygen tensions [3,5] typically found in its microaerobic environment [6].

In the 'anaerobic' protozoa previously studied, electron transport is dominated by iron-sulphur proteins [7,8]; cytochromes are undetectable [9]. Pyruvate oxidation involves enzymes quite different from the pyruvate dehydrogenase complexes typical of mitochondria-containing eukaryotes. The systems are similar to those present in anaerobic bacteria, in which acetyl CoA is formed from pyruvate by pyruvate:ferredoxin oxidoreductase (PFOR) [10]. Ferredoxins, the electron acceptors for pyruvate oxidation in both the parasitic trichomonads and *Entamoeba histolytica* have been purified and characterized [11–13]. In the former, the ferredoxins are of the [2Fe–2S] type, while in the latter the ferredoxin has [4Fe–4S] clusters [13]. These types of ferredoxins are readily detected by EPR spectroscopy, and distinguished by the temperature dependence of their EPR signals. The 'anaerobic' trichomonads contain PFOR, hydrogenase, and other iron–sulphur proteins which are involved in the formation of hydrogen, in subcellular organelles known as hydrogenosomes. A rich variety of iron–sulphur clusters in these organelles have been detected by EPR spectroscopy, and distinguished by their g-factors, temperature dependence and redox potentials [7,8,14].

No electron transport component has been characterized or purified from *G. lamblia*. The organism has been demonstrated to contain an activity analogous to that of PFOR, though it does not contain hydrogenosomes and does not produce hydrogen. Electron transfer from pyruvate to spinach ferredoxin, FMN, FAD or methyl viologen, but not $NAD(P)^+$ was shown under anaerobic conditions [2]. However the physiological electron acceptor awaits identification. Studies on the respiratory components of *G. lamblia* suggest flavins and iron–sulphur clusters as potential candidates. Although it was previously shown that oxygen consumption was associated mainly with the particulate fraction from this organism [4], Lindmark found that PFOR was non-sedimentable at 3×10^6 $g \cdot$ min in extracts prepared by a harsh homogenization procedure [2].

In this study we have investigated the iron–sulphur proteins of *G. lamblia* using EPR spectrometry. We

Correspondence address J.E. Ellis, Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221-0006, USA. Fax: (1) (513) 556 5280.

Abbreviations. PMSF, phenylmethylsulphonyl fluoride; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid; MOPS, 3-(*N*-morpholino)propane sulphonic acid; EPR, electron paramagnetic resonance.

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have also examined the location in the cell of PFOR and NAD(P)H dehydrogenases.

2. MATERIALS AND METHODS

G. lamblia trophozoites (strain H-1-P) were obtained from Dr E.L. Jarroll (Department of Biology, Cleveland State University, Cleveland, OH, USA) and grown to late exponential phase in TYS-33 medium, containing bile and supplemented with 10% newborn calf serum [15]. Anaerobic conditions were maintained throughout by conducting all procedures under N_2; thus buffers were prepared using distilled water that had been degassed by boiling and then cooled under N_2. Trophozoites were harvested, after detachment from the wall of the growth vessels by chilling at 4°C for 60 min, and washed twice in buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, pH 7.0) by centrifugation at 750 × *g* for 10 min at 4°C Cell suspensions were broken in disruption buffer (50 mM HEPES, 150 mM NaCl, 225 mM sucrose, 2 mM EDTA, pH 7.4) supplemented with the following proteinase inhibitors: PMSF, 1 mM; leupeptin, 20 μM; benzamidine HCl, 1 mM; phenanthroline, 0.5 mM. Disruption was carried out by shaking trophozoites with acid-washed glass chromatography beads (40 mesh) for 20 s at 4000 Hz using the Braun MSK Cell Disintegrator. After centrifugation at 750 × *g* for 5 min to remove unbroken cells, the homogenate was centrifuged at $10^5 \times g$ for 1 h at 4°C to obtain particulate and soluble fractions. Particulate fractions were washed twice in disruption buffer and recentrifuged. Supernatants from the high-speed centrifugation steps were pooled.

After concentration under N_2 in an ultrafiltration cell (Amicon Centricon 10) the soluble fraction was applied to an ion-exchange column (1 × 18 cm) packed with DEAE-Sephacel (Sigma). Elution of ferredoxin-containing fractions by NaCl (0–0.6 M) in 10 mM Tris-HCl at 10 ml/h was monitored by measuring A_{390nm}. Fractions with > 30% of the maximum absorbance were combined and concentrated using the ultrafiltration cell. Whole cells and cell fractions were stored at 77K under N_2 headspace. EPR analysis was carried out on a Bruker ESP300 spectrometer, cooled by an Oxford Instruments ESR900 helium flow cryostat. Oxidation/reduction potential titrations were performed in the apparatus previously described [16,17], under a flow of argon, using 100 mM MOPS buffer at pH 7.0, with sodium dithionite as reductant and $K_3Fe(CN)_6$ as oxidant, in the presence of the following dye mediators: phenazine methosulphate, methylene blue, indigo disulphonate, phenosafranine, safranine T, benzyl viologen and methyl viologen at a final concentration of 50 μM. Redox potentials were measured at 25°C with a platinum and calomel electrode Potentials are expressed relative to the standard hydrogen electrode.

Oxygen consumption by whole cells and extracts of *G. lamblia* was monitored at 37°C using a Clark oxygen electrode (2 ml final volume) [18]. The solubility of oxygen in air at 37°C was assumed to be 220 μM [19].

The following enzyme activities were assayed. pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) (PFOR), malate dehydrogenase (decarboxylating) (EC 1.1.1.39), malate dehydrogenase (EC 1.1.1.37), NADH (EC 1.6.99.3) and NADPH dehydrogenases (EC 1.6.99.1) [20,21]; the latter two assays used FMN as electron acceptor. Protein was estimated using Coomassie blue [22] with bovine gamma globulin as standard (Bio-Rad).

Optical difference spectra (reduced with dithionite minus oxidized with ammonium persulphate) of whole tropozoites of *G lamblia* were carried out at 77K in a cell of path length 2 mm.

3. RESULTS

Table I shows typical distributions of enzyme activities and protein after fractionation of cell-free extracts by differential centrifugation. Membranes sedimented at 6×10^6 *g* · min accounted for two-thirds of the total protein. Most of the PFOR was recovered in this fraction which also showed the capacity for O_2 consumption in the presence of pyruvate, NADH or NADPH. Both NADH and NADPH oxidases were stimulated by FAD (up to 8- and 4-fold, respectively), but not by FMN. NADH- and NADPH-oxidoreductases were also present in this sedimentable fraction, as was 28% of the malate dehydrogenase. Most of the latter enzyme, together with almost all the malate dehydrogenase (decarboxylating) was non-sedimentable under these conditions. Difference spectra (reduced − oxidized) of whole cell suspensions (85 mg protein/ml) gave a trough characteristic of flavoproteins (at 450–470 nm); no cytochromes were detected. It may be noted that the reduced-oxidized absorption spectra of iron–sulphur pro-

Table I

	Whole homogenate		Particulate			Soluble			Recovery (%)
	Concentration (mg/ml)	Total (mg)	Concentration (mg/ml)	Total (mg)	Total (%)	Concentration (mg/ml)	Total (mg)	Total (%)	
Protein:	23	64.1	28	42.5	66.0	15.3	19.9	30.9	96.9
	Specific activity (mU · mg protein^{-1})	Total units	Specific activity (mU · mg protein^{-1})	Total units	Units % total	Specific activity (mU · mg protein^{-1})	Total units	Units % total	
Pyruvate:methyl viologen oxidoreductase	2150	138.5	2370	100.6	72.6	360	9.5	6.9	79 5
Malate dehydrogenase (decarboxylating)	593	38.2	89	3.8	9.9	2940	58.5	153.1	163.0
Malate dehydrogenase	863	55.6	367	15.6	28.1	2030	40.6	72 9	101.0
NADH dehydrogenase	149	9.6	128	5.4	56.5	150	3.0	31.1	87.6
NADPH dehydrogenase	195	12.6	203	8.6	68.4	190	3.8	30.0	98.4

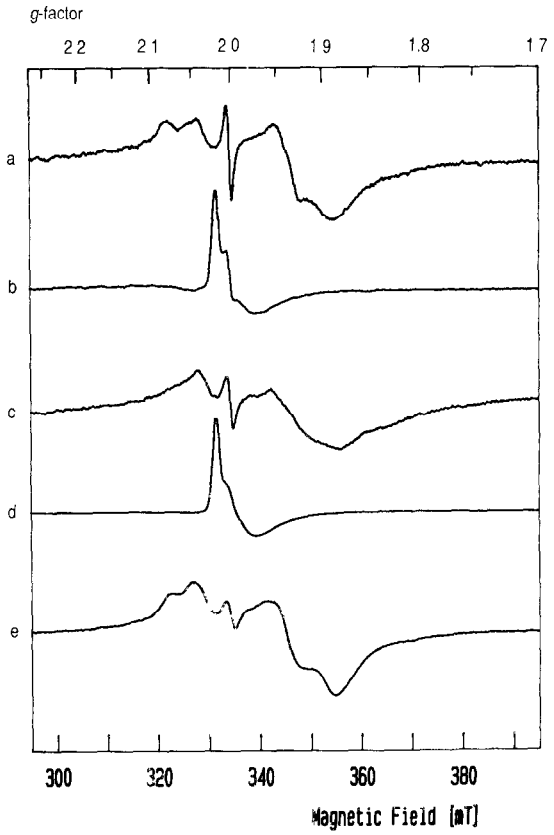

Fig. 1 EPR spectra of *G lamblia* cells and extracts. Conditions of measurement: temperature 8K; microwave power, 2 mW; frequency 9.37 GHz; modulation amplitude, 1 mT (a) whole cell suspension, 45 mg · ml^{-1}, recorder gain 8×10^5; (b) membrane fraction, 50 mg · ml^{-1}, oxidized as prepared, gain 2×10^5, (c) membrane fraction, 50 mg · ml^{-1}, reduced with dithionite, recorder gain 5×10^5; (d) cytosolic fraction, 30 mg · ml^{-1}, oxidized as prepared, recorder gain 3.2×10^5; (e) cytosolic fraction, reduced with dithionite, recorder gain 2.5×10^5.

teins are broad and difficult to distinguish, particularly for [4Fe–4S] clusters.

A range of methods were tried in order to establish a reliable method for the solubilization of PFOR using the appearance of activity in the supernatant after centrifugation at $100{,}000 \times g$ for 1 h, as the criterion of solubilization. PFOR activity remained predominantly (> 80%) associated with the membrane fraction after 30 min incubation in the presence of buffer supplemented with 20 mM mercaptoethanol containing 1 M Na acetate, 1 mM EDTA, or with buffer containing 0.7 M KCl, 0.15 M NaCl, or even in the presence of 2% Triton X-100 or 1% Na deoxycholate. However, inclusion of high concentrations of salt in the buffer significantly reduced the recovery of this enzyme; this suggests that these conditions may lead to inactivation of PFOR.

Fig. 1 shows EPR spectra of *G. lamblia* whole cell suspension, and the membrane and cytosolic fractions.

The sedimentable fraction showed signals at $g = 3$, possibly due to low-spin Fe^{III}, and at $g = 2$ from Cu^{II} (data not shown). In the oxidized state, the most prominent feature at $g = 2.01$ at 8K (Fig. 1b), is indicative of the presence of a fast-relaxing $[3Fe–4S]^+$ cluster. After dithionite reduction, the membrane fraction yielded two overlapping rhombic spectra (Fig. 1c), which were detected only below 30K and therefore probably represent [4Fe–4S] clusters. They could be distinguished on the basis of their temperature dependence: (i) at $g = 2.04$, 1.93, 1.89; (ii) at $g = 2.08$, 1.93, 1.83, detectable only below 20K.

The non-sedimentable fraction showed a signal in the oxidized state at $g = 2.01$ (Fig. 1d), indicative of a [3Fe–4S] cluster; the line shape was different from that in the membrane fraction. In the reduced state a signal at $g = 2.04$, 1.93, 1.89, similar to the membrane fraction (see above) (Fig. 1e), was detectable.

After column chromatography on DEAE Sephacel, the fractions with a high A_{390nm} were combined. EPR spectra were obtained on samples poised at various redox potential values. EPR spectra of the oxidized and reduced samples are shown in Fig. 2. The component with $g = 1.93$ appeared on reduction with a mid-point

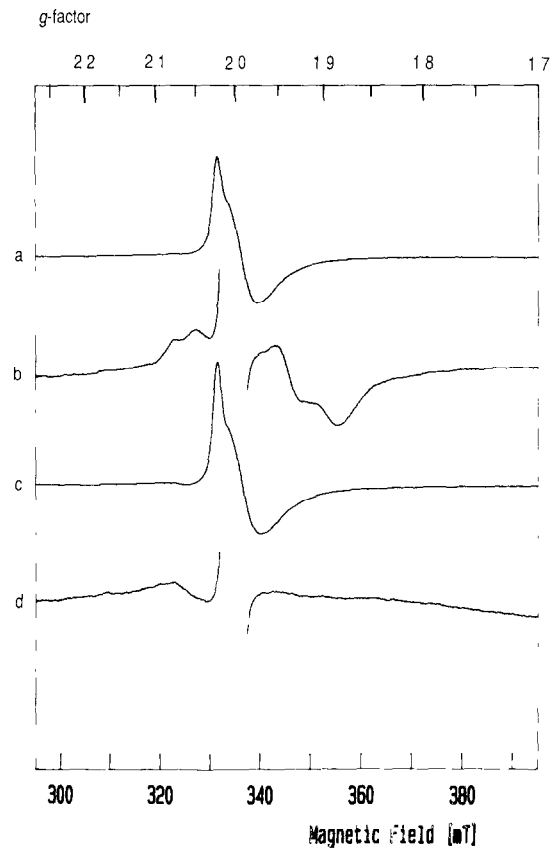

Fig. 2. EPR spectra of the concentrated eluate fraction from DEAE-Sephacel, prepared as for the redox titrations (see section 2). Conditions as for Fig. 1, except temperature 12K. (a) oxidized as prepared; (b) reduced with $Na_2S_2O_4$; (c) reoxidized with 5 mM $K_3Fe(CN)_6$; (d) re-reduced with 5 mM $Na_2S_2O_4$. The gain for spectra (b) and (d) was fivefold greater than (a) and (b). The radical signal at $g = 2.00$ in the reduced samples is due to the presence of viologen mediators.

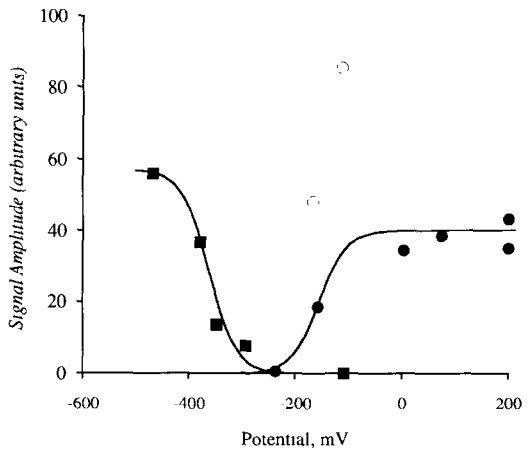

Fig. 3 Oxidation–reduction titrations of the iron–sulphur protein eluted from DEAE-Sephacel. Points ■ represent data from a reductive titration of the $g = 1.93$ signal with $Na_2S_2O_4$; ● from a reductive titration of the $g = 2.01$ signal with $Na_2S_2O_4$; ○ from a titration of the $g = 2.01$ signal by reoxidation with $K_3Fe(CN)_6$.

potential of about −360 mV (Fig. 3). The shape of the spectrum is more complex than a simple rhombic EPR spectrum; the two upward low-field features are particularly noticeable. For this reason it seems likely that the $g = 1.93$ signal represents more than one type of [4Fe–4S] cluster.

The component with a signal at $g = 2.01$, in the protein eluted from DEAE-Sephacel, was similar to that observed in the unreduced cytosolic fraction. It had a mid-point potential of about −155 mV. However it seems likely that this signal is due to a [3Fe–4S] cluster produced by oxidative damage to a [4Fe–4S] cluster. On treatment of extracts with $K_3Fe(CN)_6$, the $g = 2.01$ signal became much larger, without changing its line shape, and on subsequent reduction with dithionite the $g = 1.93$ signal was no longer observed (Fig. 2d). Such irreversible modification of [4Fe–4S] clusters in ferredoxins is well documented [25], but the *G. lamblia* protein appears to be exceptionally sensitive. We conclude that the major iron–sulphur species of the cytosol is most likely to be a [4Fe–4S] or 2[4Fe–4S] ferredoxin which by comparison with most bacterial ferredoxins is highly sensitive to oxidation by O_2 or ferricyanide.

4. DISCUSSION

The identity and subcellular location of components responsible for electron transport in *G. lamblia* has been a matter of conjecture for many years. Now that it seems likely that this organism with neither mitochondria nor hydrogenosomes is a genuinely primitive eukaryote rather than a secondarily adapted one, the question has renewed urgency. A widely accepted view, based on sequence analysis of the 16SrRNA [26] places this organism on a very deep branch of the eukaryotic lineage, although this phylogenetic assignment is not without dissent [27].

G. lamblia has only a rudimentary subcellular organisation with respect to membraneous organelles, although its locomotory and cytoskeletal structures are well-developed [28]. The membranes sedimented at 6×10^6 $g \cdot$ min include fragments of plasma membrane, endoplasmic reticulum and lysosomal vesicles. The data presented here confirm earlier findings [4] that both NADH and NADPH oxidases are partially sedimentable and present evidence that PFOR is membrane-associated. Presence of membrane-bound PFOR has also been reported in the hydrogenosomes of the parasitic flagellate, *Trichomonas vaginalis* [29]. It is also worth noting that Weinbach et al. [4] found carriers mediating electron transfer in *G. lamblia* to be present in the particulate fraction.

Of the four EPR signals from iron–sulphur centres, only two can as yet be attributed to known electron transport functions. The component sedimenting with the membranes with g values at 2.08, 1.93 and 1.83 may correspond to the [4Fe–4S] centre of PFOR; similar features have previously been observed for the analogous enzyme from *Halobacterium halobium* [30]. Although this enzyme has been partially purified from the microaerophilic protozoon *T. vaginalis* [29], EPR studies have not been published, and only tentative assignment of signals from hydrogenosomes purified from this organism are possible (A. Chapman, R. Cammack and K.P. Williams, unpublished data).

The non-sedimentable iron–sulphur protein with g-factors in the reduced state at 2.04, 1.93 and 1.89 and which showed a midpoint redox potential of about −360 mV is a ferredoxin, containing [4Fe–4S] clusters. Therefore the ferredoxin acceptor of PFOR in *G. lamblia* is probably not the well-characterized [2Fe–2S] type found associated with the hydrogenosome of *T. vaginalis*, which has been purified and sequenced [9,10,31]. Indeed, the ferredoxin of *G. lamblia* is more similar to that of *Clostridium* or *Desulfovibrio* species [32,33] and the iron–sulphur species tentatively assigned as a [4Fe–4S] ferredoxin. In this way it resembles another 'anaerobic' protozoon, *E. histolytica*, that infects the lower intestine, and like *G. lamblia*, also lacks both mitochondria and hydrogenosomes [13].

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