The Electron-Transport System of Mitochondria from the Slime Mould *Physarum polycephalum*

By ROGER BARNES, EMER M. COLLERAN* and O. T. G. JONES
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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A method is described for the preparation of mitochondria from the slime mould *Physarum polycephalum*; the mitochondria were not coupled. *P. polycephalum* mitochondria oxidized added NADH via a rotenone-insensitive pathway, but the oxidation of malate plus glutamate was rotenone sensitive; both of these substrates reduced much less cytochrome *b* than did succinate, in both aerobic and anaerobic steady states. Spectroscopy at 77°K separated three absorption maxima in the α -band region, at 560 nm, 553 nm and one at 547 nm due to cytochrome *c*. The absorption at 553 nm was increased in the aerobic steady state by the addition of 2-heptyl-4-hydroxyquinoline *N*-oxide, suggesting that it was due to a *b*-type cytochrome. All three absorption maxima appeared in the aerobic steady state after the addition of a range of substrates. The respiratory activity with different substrates and the response to inhibitors of respiration were similar to those previously described for fungus mitochondria (Weiss *et al.*, 1970; Erickson & Ashworth, 1969). When grown under conditions of haem limitation the mitochondria contained a lower concentration of cytochromes than normal.

Physarum polycephalum has an unusual and specific requirement for protohaem for growth (Daniel *et al.*, 1962); this requirement may be used in studying the mechanism and regulation of cytochrome synthesis during the assembly of mitochondria. We have described how [⁵⁹Fe]protohaem is efficiently incorporated into a soluble cytochrome *c* purified from this organism (Colleran & Jones, 1973); in the present paper we describe the preparation and some properties of mitochondria from *P. polycephalum* grown in both normal medium and in conditions of haem limitation.

Methods

Growth of *P. polycephalum*

Liquid shake cultures of this slime mould (strain i+1029), obtained from Dr. M. J. Carlile, Department of Biochemistry, Imperial College of Science & Technology, London, U.K. were grown and harvested as described previously (Colleran & Jones, 1973). Stock cultures were maintained on agar plates of semi-defined medium (Carlile, 1971).

Normal cultures of slime mould were grown on medium containing 5 mg of protohaem/litre. For low-haem growth conditions, the haem concentration was decreased to 0.5 mg/litre.

* Present address: Regional Technical College, Athlone, Co. Westmeath, Irish Republic.

Preparation of mitochondria

Slime-mould plasmodia, grown in liquid culture for 3 days, were harvested by centrifuging at 15000g for 5 min and the top layer of slime was removed. The plasmodia were homogenized in 300 ml of 0.5M-sucrose containing 10 mM-Tes,† pH 7.5, with a blender (MSE Atomix) for 30 s. The homogenate was passed twice through a Regal milk filter (Johnson & Johnson Ltd., Slough, Bucks., U.K.) to remove slime and then centrifuged twice at 500g for 5 min to remove cell debris. The supernatant was centrifuged at 4000g to sediment the mitochondria. The mitochondrial pellet was resuspended, by using a 'cold finger', in buffer containing 0.5M-sucrose, 5 mM-K₂HPO₄, 2 mM-MgCl₂ and 10 mM-Tes, pH 7.5, then centrifuged for a further 10 min at 4000g, and the pellet was resuspended in a minimal volume of the same buffer. The mitochondria prepared were generally highly coloured owing to the presence of the characteristic yellow pigment produced by *P. polycephalum*.

Spectrophotometric measurements

Absorption spectra at room temperature and at 77°K were obtained with a split-beam spectrophotometer constructed according to the principles of

† Abbreviation: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

Yang & Legallais (1954). Cytochrome concentrations were determined from the difference spectra obtained after reduction with respiratory substrates or dithionite, by using the constants given by Chance (1957). A dual-wavelength spectrophotometer (Chance, 1951) was used in making kinetic measurements.

Cytochrome *c* reductase

The reduction of added horse heart cytochrome *c* by slime-mould mitochondria with NADH, NADH-linked substrates or succinate as substrate was followed in the dual-wavelength spectrophotometer by measuring the increased extinction at 550 nm minus 540 nm. Reoxidation of the reduced cytochrome *c* was prevented by the addition of KCN to the cuvette.

ATP formation

ATP formation was measured by following the incorporation of $^{32}\text{P}_i$ into ATP (Avron, 1960; Chen *et al.*, 1956).

Measurement of O_2 uptake

Respiration rates were measured by using a Clark-type oxygen electrode in a water-jacketed vessel at 25°C.

Proteins

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine plasma albumin [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] as standard.

Results

Mitochondria from *P. polycephalum* oxidize malate plus glutamate slowly in the absence of added NAD^+ ; when NAD^+ is added respiration is greatly stimulated (Fig. 1, curve *a*). Since mitochondria are normally impermeable to nicotinamide nucleotides this suggests that the mitochondria are unlikely to be intact and so the lack of stimulation of O_2 uptake on the addition of ADP is not surprising (P/O ratio of 0.03

Fig. 1. Respiration of *P. polycephalum* mitochondria

The rate of O_2 consumption was measured as described in the Methods section. The incubation medium contained initially sucrose (0.5 M), K_2HPO_4 (5 mM), MgCl_2 (2 mM), Tes (10 mM), pH 7.5, and mitochondria (8 mg of protein). Additions were glutamate (20 μmol), malate (20 μmol), ADP (100 nmol), NAD^+ (2 μmol), rotenone (25 nmol), succinate (20 μmol), pyruvate (20 μmol), citrate (20 μmol), rotenone (25 μmol) and NADH (5 μmol). The total volume was 5.0 ml and the temperature 26°C. For details of curves (a)–(c) see the text.

was found with succinate as substrate). Rotenone effectively inhibits the oxidation of this 'endogenously produced' NADH (Fig. 1, curve *a*), but the subsequent addition of succinate does not re-start respiration. This could be interpreted to show that rotenone acts atypically in these mitochondria by inhibiting electron flow at more than one site, but an experiment where NADH was generated from pyruvate plus citrate shows that this is not the case (Fig. 1, curve *b*). After inhibition of respiration with rotenone, the addition of succinate caused respiration to recommence. It appears likely that succinate did not restore the rotenone-inhibited O₂ uptake caused by malate, because malate oxidation yields oxaloacetate, known to inhibit succinate dehydrogenase (Singer & Kearney, 1963) and that the glutamate-oxaloacetate transaminase of *P. polycephalum* mitochondria is insufficiently active to remove the oxaloacetate produced. Succinate oxidation is inhibited in the usual way by malonate (Fig. 1, curve *b*). When NADH is added to these mitochondria it is oxidized rapidly by a pathway that is insensitive to rotenone (Fig. 1, curve *c*), suggesting that two different routes of electron transfer from NADH are possible, one accessible only to endogenously synthesized NADH that is sensitive to rotenone, the other accessible to added NADH and rotenone-insensitive.

The effects of a range of inhibitors on respiration of mitochondria from *P. polycephalum* are shown in Table 1. KCN gives complete inhibition, suggesting that only one oxidase system is functioning; the cyanide-resistant pathways that have been found both in plant mitochondria (see, e.g., Bendall & Bonner, 1971) and in the *poky* mutant of *Neurospora crassa* (Lambowitz *et al.*, 1972) are absent. Antimycin A is less inhibitory to succinate oxidation than to NADH oxidation and higher concentrations are necessary to inhibit respiration in *P. polycephalum* mitochondria than in mammalian mitochondria.

2-Heptyl-4-hydroxyquinoline *N*-oxide was a more active inhibitor in this organism.

Addition of succinate to mitochondria caused an increased absorbance at 604 nm owing to reduction of cytochromes *a*+*a*₃, even in the aerobic steady state (Fig. 2, trace *a*), together with some reduction at 560 nm, attributed to cytochrome(s) *b*, and at 550 nm, attributed to cytochrome *c*. (It was not possible to record the γ bands of the cytochromes since some of the yellow pigment that is characteristic of *P. polycephalum* was present in the mitochondrial fraction and gave very large absorbance changes in this region if the oxidation state of the mitochondria was changed.) When the dissolved O₂ in the cuvette was used up there was a big increase in the extent of reduction of cytochromes *a*+*a*₃ and *c* (Fig. 2, trace *b*). Although more cytochrome *b* was reduced on standing or when CN⁻ was added (Fig. 2, trace *c*) cytochrome *c* remained the main absorbing component in these succinate-reduced mitochondria; the addition of dithionite caused a broadening of absorption bands around 555 nm, probably owing to the reduction of several cytochromes of the *b* and *c* type and possibly of some free protohaem (Fig. 2, trace *d*).

When NADH was substrate relatively little cytochrome *b* was reduced, particularly when glutamate plus malate was used to supply 'internal' NADH (Fig. 3). On standing for 20 min some cytochrome *b* appeared to be reduced (Fig. 3*b*, trace C) and slightly more appeared when CN⁻ was added: this prevents the slow loss of electrons from the mitochondrial system that occurs in a cuvette that has been made anaerobic only by respiratory activity. Spectroscopy at 77°K confirmed that more compounds with absorption maxima in the cytochrome *b* region were reduced by succinate than by NADH-linked substrates (Fig. 4*a*). A maximum at 560 nm is clearly visible, together with a pronounced shoulder at 553 nm; in some preparations this latter band

Table 1. *Effect of inhibitors on respiration in P. polycephalum mitochondria*

O₂ uptake was measured as described in Fig. 1.

Addition	Final concn.	Inhibition (%)		
		Glutamate + malate oxidation	Succinate oxidation	NADH oxidation
None		0	0	0
Rotenone	20 μ M	92	0	16
Antimycin A	20 μ M	57	8	*
2-Heptyl-4-hydroxyquinoline <i>N</i> -oxide	20 μ M	65	69	42
KCN	200 μ M	100	100	100
Malonate	2 mM	0	100	0

* Not measured.

Fig. 2. Difference spectra of *P. polycephalum* mitochondria with succinate as substrate

Mitochondria (about 5 mg of protein) were suspended in 5 ml of buffer containing sucrose (0.5 M), K_2HPO_4 (5 mM), $MgCl_2$ (2 mM) and Tes (10 mM), pH 7.5, and the suspension was divided between two cuvettes. Succinate (20 μ mol) was added to the sample cuvette and spectra were recorded at room temperature. Trace (a) was recorded after succinate addition, trace (b) immediately after anaerobiosis, trace (c) after the addition of KCN (10 μ mol), and trace (d) after the addition of dithionite to sample. ----, Base line.

appeared as a definite peak. The absorption at 553 nm is likely to be due to either cytochrome c_1 (Chance, 1957) or to a form of cytochrome b (cf. Lambowitz *et al.*, 1972). There is little doubt that the absorption band at 547 nm is due to cytochrome c , since this absorption maximum coincided with that obtained for purified cytochrome c from this organism (Colleran & Jones, 1973). In an attempt to identify the compound absorbing at 553 nm the inhibitor 2-heptyl-4-hydroxyquinoline *N*-oxide was used to block electron flow between cytochromes b

and c . In the aerobic steady state this caused, as expected, oxidation of cytochrome c (at 548 nm) and reduction of cytochrome b at 560 nm, together with reduction at 553 nm (Fig. 4b). We tentatively suggest that this absorption at 553 nm is due to a b -type cytochrome, and have no clear evidence for the presence of cytochrome c_1 in these mitochondria.

In Fig. 5(a) we demonstrate the kinetics of reduction of cytochromes $a+a_3$. This shows the remarkably high reduction obtained in steady-state aerobic conditions, usually about 20–30% of the total cytochromes $a+a_3$. For cytochrome b measured at 560 nm, the reduction pattern is more complex (Fig. 5b). Some 10 min after the anaerobic change only about 25% of the cytochrome b is reduced and the addition of KCN brings about a big increase in the state of reduction.

P. polycephalum requires protohaem for growth and cytochrome synthesis (Colleran & Jones, 1973) and in low-protohaem media growth is slow. Mitochondria were prepared from such haem-limited cultures in low yields compared with haem-sufficient cultures (approx. 10–20% of normal yield) and appeared to contain lower concentration of cytochromes (Table 2). To avoid errors that might arise from the reduction of free protohaem within the mitochondria by dithionite, cytochrome concentrations have been calculated from both dithionite reduction and from succinate reduction. By either method of assay it appeared that the cytochrome concentration in haem limitation was about 50% of normal.

In a separate series of experiments with extracts from normal *P. polycephalum* we have failed to detect any of the following enzymes concerned in protohaem synthesis: aminolaevulinatase (assayed by the sensitive [^{14}C]succinate method of Irving & Elliott, 1969); conversion of aminolaevulinatase into porphyrinogens and porphyrins; ferrochelatase (assayed by dual-wavelength spectroscopy; Jones & Jones, 1969).

Discussion

The slime mould *P. polycephalum* is a member of the Myxomycetes in which the life cycle includes a characteristic phase, the plasmodium, containing many nuclei but not subdivided into cells. The Myxomycetes have been regarded by some taxonomists as akin to protozoa, by others as fungi (Carlile, 1971), whereas the cellular slime moulds such as *Dictyostelium discoideum* are commonly regarded as fungi. The mitochondrial electron-transport chain of *P. polycephalum* does not show any of the unusual cytochrome components that have been found in some protozoa, e.g. *Tetrahymena pyriformis*, which apparently lacks cytochromes $a+a_3$ (Turner *et al.*, 1971), or *Crithidia oncopelti* and *Trypanosoma rhodesiense*, which lack normal $a+a_3$ (Srivastava, 1971),

Fig. 3. Difference spectra of *P. polycephalum* mitochondria with NADH and NAD⁺-linked substrates

Sample and reference cuvettes were set up as described in Fig. 2. (a) NADH (2.5 μmol) was added to the sample cuvette and a spectrum measured immediately after anaerobiosis. (b) Glutamate (10 μmol), malate (10 μmol) and NAD⁺ (2 μmol) were added to the sample cuvette. Trace A was recorded immediately, trace B immediately after anaerobiosis and trace C 20 min after anaerobiosis. ----, Base line.

Table 2. Cytochrome contents in mitochondria from *P. polycephalum* grown under normal and low-haem conditions

Difference spectra were obtained as described in Figs. 2 and 3. After reduction with substrates or dithionite, the concentrations of cytochromes *a*, *b* and *c* were calculated from the extinction changes at 605 minus 630 nm, 560 minus 570 nm, or 550 minus 540 nm, by using ΔE_{nm} of 16, 20 and 20 respectively (Chance, 1957).

Method of reduction	Growth conditions	Concn. of cytochrome <i>a</i> + <i>a</i> ₃ (nmol/mg of protein)	Concn. of cytochrome <i>b</i> (nmol/mg of protein)	Concn. of cytochrome <i>c</i> (nmol/mg of protein)
Succinate (anaerobic)	Normal	0.0713 ± 0.01	0.061 ± 0.01	0.165 ± 0.045
Succinate (anaerobic)	Low haem	0.036 ± 0.012	0.031 ± 0.012	0.091 ± 0.05
Dithionite	Normal	0.072 ± 0.013	0.215 ± 0.021	0.260 ± 0.008
Dithionite	Low haem	0.055 ± 0.022	0.102 ± 0.06	0.123 ± 0.06
NADH (anaerobic)	Normal	0.0622 ± 0.005	0.036 ± 0.009	0.177 ± 0.054

and *Paramecium aurelia*, in which mitochondria have an absorption band at 588 nm (Kung, 1970). Indeed, cytochromes *a*+*a*₃, *b* and *c* were present in *P. polycephalum* at concentrations rather similar to those reported for *D. discoideum* (Erickson & Ashworth, 1969). Low-temperature spectroscopy suggests that *P. polycephalum* lacks the cytochrome *c*₁ found in mitochondria from the fungus *Neurospora crassa* (Lambowitz *et al.*, 1972) and in yeast mitochondria (Sherman & Slonimski, 1964) as well

as mammalian mitochondria, but it is possible that its absorption bands coincide with those of some other cytochrome. The absorption band appearing at 553 nm at 77°K does not behave like that of a *c*-type cytochrome; it becomes reduced on the addition of 2-heptyl-4-hydroxyquinoline *N*-oxide to respiring mitochondria, a property expected of *b*-type cytochromes in normal mitochondria. Cytochromes of the *b* type with absorption maxima at 554 nm at 77°K have been found in *N. crassa* mitochondria by

Fig. 4. Low-temperature spectra of *P. polycephalum* mitochondria

Mitochondria (about 2 mg of protein) were suspended in 1.5 ml of buffer containing sucrose (0.5 M), K_2HPO_4 (5 mM), $MgCl_2$ (2 mM) and Tes (10 mM), pH 7.5, and the suspension was divided equally between two 2 mm perspex cuvettes. (a) Succinate (10 μ mol) (trace A), or malate (10 μ mol), glutamate (10 μ mol) and NAD^+ (2 μ mol) (trace B), were added to the sample cuvette. The suspension was allowed to go anaerobic before being frozen with liquid N_2 . Spectra were then measured. (b) Malate (10 μ mol), glutamate (10 μ mol) and NAD^+ (2 μ mol) were added before the division of the suspension. After the equal distribution 2-heptyl-4-hydroxyquinoline *N*-oxide (0.1 μ mol) was added to the sample cuvette, the cuvettes were frozen immediately and a spectrum was recorded.

Fig. 5. Time-course of cytochrome reduction in *P. polycephalum* mitochondria with succinate as substrate

Mitochondria (7 mg of protein) were incubated in 2.5 ml of buffer containing sucrose (0.5 M), K_2HPO_4 (5 mM), $MgCl_2$ (2 mM) and Tes (10 mM), pH 7.5, at 25°C in a double-beam spectrophotometer. (a) Succinate (10 μ mol) was added and cytochrome *a*+*a*₃ reduction was followed by measuring the change in extinction $\Delta(E_{604} - E_{630})$. (b) Succinate (10 μ mol) was added and cytochrome *b* reduction was followed by measuring the change in extinction $\Delta(E_{560} - E_{575})$. Additions were KCN (5 μ mol) and dithionite.

Lambowitz *et al.* (1972) and one at 553 nm in plant mitochondria (Bonner, 1961). Both *b*-type cytochromes in *P. polycephalum* mitochondria are reduced by succinate or NADH-linked substrates, although succinate leads to a much greater aerobic steady-state reduction of these cytochromes.

In *P. polycephalum* as well as the fungus *N. crassa* (Weiss *et al.*, 1970) and *Aspergillus niger* (Watson & Smith, 1967) and the yeasts *Saccharomyces carlsbergensis* (Ohnishi *et al.*, 1966) and *Torulopsis utilis* (Light & Garland, 1971) there appears to be a rotenone-insensitive 'external' pathway for the oxidation of NADH as well as an 'internal' pathway

that is sensitive to rotenone. This probably reflects the lack of evolution in primitive eukaryotes of a shuttle system for the transport of the reducing power of NADH from the cytosol to mitochondrial matrix. Unfortunately the lack of coupling in our mitochondrial preparation prevents comparison of the P/O ratio for 'external' and 'internal' NADH.

Although *P. polycephalum* is dependent on added protohaem for growth, the properties of the mitochondria and of the purified cytochrome *c* (Colleran & Jones, 1973) do not differ from those of comparable organisms that have no haem requirement. This encourages us to suggest that it will prove a useful organism in studying late stages in the assembly of mitochondria and the association of cytochrome apoprotein synthesis with haem availability. Growth of *P. polycephalum* at low concentrations of protohaem appears to result in the formation of mitochondria with a lowered cytochrome content. Light & Garland (1971) grew *T. utilis* in conditions of iron insufficiency and also noted a decrease in the concentration of mitochondrial cytochromes, but not in the total mitochondrial protein.

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