Oxygen affinity of the respiratory chain of *Acanthamoeba castellanii*

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1. Apparent *Kₘ* values for O₂ for the soil amoeba *Acanthamoeba castellanii* determined polarographically and by bioluminescence gave similar values (0.37 and 0.41 µΜ respectively). 2. Mitochondria oxidizing succinate or NADH in the presence or absence of ADP gave values in the range 0.21–0.36 µΜ-O₂. 3. Oxidation of respiratory-chain components to 50% of the aerobic steady states in intact cells was observed at the following O₂ concentrations: cytochrome aa₃, 0.1–0.25 µΜ; cytochrome c, 0.3–0.6 µΜ; cytochrome b, 0.35–0.45 µΜ; flavoprotein, 2 µΜ. 4. In isolated mitochondria corresponding values for *a*-, *c*-, and *b*-type cytochromes were 0.007, 0.035–0.05 and 0.06–0.09 µΜ-O₂. 5. It is concluded that an O₂ gradient exists between plasma membrane and mitochondria in *A. castellanii*.

The soil amoeba *Acanthamoeba castellanii*, grown under conditions providing organisms in which the major terminal oxidase is cytochrome *aa₃*, has a high affinity for O₂. Both polarographic measurements (Lloyd *et al.*, 1979) and those based on the O₂-dependence of bacterial bioluminescence (Lloyd *et al.*, 1982) indicate an apparent *Kₘ* < 1 µΜ-O₂, and the latter, more reliable, method gives a value of 0.42 µΜ-O₂. Determinations of the O₂ affinities for other intact eukaryotic micro-organisms have given apparent *Kₘ* values in the range 0.4–1.5 µΜ for baker’s yeast (Longmuir, 1954; Schindler, 1964; Chance, 1965), 2.9 µΜ for the ciliate protozoon *Tetrahymena pyriformis* (Lloyd *et al.*, 1980, 1982), and 0.1 µΜ for the tsetse-fly vector form of a trypanosome (Hill, 1978).

It has been suggested that intact mammalian cells (e.g. hepatocytes and neurons) have lower O₂ affinities than those of isolated mitochondria (Jones, 1978; Jobbsis & Rosenthal, 1978), and the presence of O₂ gradients within mammalian cells has been demonstrated directly (Benson *et al.*, 1980; Podgorski *et al.*, 1981).

The present paper shows that the apparent *Kₘ* values for O₂ for intact amoebae are higher than those for their mitochondria after extraction and that the O₂ concentrations that give 50% oxidation of respiratory-chain components are an order of magnitude higher for the organelle *in vivo* than those *

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**Experimental**

*Maintenance and growth of the organisms*

*Acanthamoeba castellanii* was maintained, grown axenically with shaking at 30°C, and counted as described previously (Edwards *et al.*, 1977).

*Harvesting and breakage of the organism and preparation of mitochondrial fractions*

Methods for harvesting and homogenization of organisms and preparation of mitochondria-enriched fractions were as described by Edwards & Lloyd (1978). Organisms were harvested after 48 h growth (cell concentration 2 x 10⁶–4 x 10⁶ organisms/ml).

*O₂-affinity measurements*

These involved measurements of bioluminescence of *Photobacterium fischeri* (strain MJ-1) mixed with the mitochondrial suspension in a 5 ml stirred open reactor under defined O₂/argon ratios in the gas phase as described previously (Lloyd *et al.*, 1981, 1982). Gases used were high-purity argon (<3 p.p.m. of O₂) and standard gas mixtures consisting of 1% O₂ in N₂ and 0.05% O₂ in N₂ (Air Products, Cardiff, Wales, U.K.). Low O₂ partial pressures were obtained by using a digital gas mixer (Lundsgaard & Degn, 1973), which provides accurate dilution in 5% steps. Absolute O₂ concentrations were calculated from percentage dilutions of stock gas mixtures.

Measurements were made at 25°C by using a buffer consisting of 0.32 M-sucrose, 10 mM-Tris/H₂SO₄ and 0.2 mM-EGTA, or in 0.4 M-NaCl/0.1 M-potassium phosphate (pH 7.0); when air-saturated
these buffers contain 260 μM-O₂. Calibration curves for light-emission versus O₂ concentration in the absence of mitochondria were obtained for each determination, and respiration rates (Vᵣ) were calculated from the equation:

\[ V_r = K(T_G - T_L) \]

where \( K \) is the O₂-transfer constant, \( T_G \) the O₂ partial pressure in the gas phase, and \( T_L \) that in the liquid (Degn et al., 1980). The half-time (\( t_1/2 \)) for the equilibration with O₂ in the absence of organisms was 3 min, corresponding to an O₂-transfer constant of 0.23 min⁻¹ (from \( K = \ln 2/t_1/2 \)). Apparent \( K_m \) values are presented as means ± S.D. with numbers of determinations in parentheses.

**Difference spectra and steady-state oxidation states of cytochromes**

Difference spectra and measurements of cytochrome redox states were obtained from suspensions of intact organisms or mitochondria under a gas phase of precisely defined composition in a scanning dual-wavelength spectrophotometer (Hitachi–Perkin–Elmer, model 557). A cuvette, moulded from epoxy resin, was hexagonal in cross-section, with quartz windows and 16 mm path-length. Suspensions (7.5 ml) were maintained at 30°C; stirring was by a cross-shaped stirrer fixed to a stainless-steel shaft entering through a hole in the lid and driven by a synchronous motor at 750 rev./min. The membrane-covered oxygen electrode (Radiometer, Copenhagen, Denmark) was sealed into a port lying below the level of the vortex (Degn et al., 1980). The response time of the system was limited by the rate of stirring; the half-time for equilibration with O₂ was slightly less than 5 min, corresponding to an O₂-transfer constant of 0.14 min⁻¹.

Simultaneous measurements of bioluminescence of *P. fischeri* (Oshino et al., 1972) in this system gave O₂ determinations at concentrations where the O₂-dependent current produced by the oxygen electrode became small compared with the 'residual' current (still observed at zero O₂). A liquid-filled light-lead (4 mm diam.) was used with an interference filter (\( \lambda_{max} = 495 \) nm, half band-width 10 nm) to monitor bioluminescence in the cuvette; the photomultiplier and associated circuit were identical with that described for O₂-affinity measurements (Lloyd et al., 1980). A Wratten 47B filter was used to protect the spectrophotometric photomultiplier from bioluminescence.

**Protein assays**

Protein was measured by the method of Lowry et al. (1951), with bovine serum albumin as standard.

**Results**

**O₂ affinity of intact organisms**

The dependence of respiration rate on O₂ partial pressure over a concentration range of 5.0–0 μM-O₂

![Fig. 1. Dependence of respiration rate of A. castellanii mitochondria on O₂ concentration](image)

Bioluminescence of *P. fischeri* exposed to gas mixtures of various compositions was measured *(a)* in the absence and *(b)* in the presence of *A. castellanii* mitochondria (2 mg of protein/ml). Measurements were made in an open system in a buffer containing (final concentrations) 0.32 M-sucrose, 10 mM-Tris/H₂SO₄, 0.2 mM-EGTA, 10 mM-potassium phosphate and 0.1% bovine serum albumin (pH 7.4) in the presence of 10 mM-sodium succinate and 0.8 mM-ADP at 25°C. Emission intensity in the absence of mitochondria gave a calibration for the concentration of dissolved O₂ (\( T_L \)); this was used to measure values for \( T_L \) at a series of fixed values of O₂ partial pressure in the gas phase (\( T_G \)) in *(b)*, and hence the respiration rate [from \( V_r = K(T_G - T_L) \)].
was determined for growing organisms (suspended in growth medium). Lineweaver–Burk plots of $1/V$ against $1/[O_2]$ at different cell densities (adjusted by using conditioned growth medium) gave linear relationships irrespective of whether the oxygen electrode or *Photobacterium* luminescence was used for measurement. Both methods gave similar values for the apparent $K_m$ for $O_2$: $0.37 \pm 0.16 \mu M$-$O_2$, (13) with the electrode, and $0.41 \pm 0.05 \mu M$-$O_2$ (12) with the bioluminescence assay. Determinations made by stepwise increases of $O_2$ partial pressure gave upward-concave Lineweaver–Burk plots; similar observations made with a number of lower eukaryotes have been described previously (Lloyd et al., 1980).

**$O_2$ affinity of mitochondrial suspensions**

The dependence of $O_2$ consumption on $O_2$ partial pressure for mitochondria isolated from *A. castellanii* was investigated by using the *Photobacterium* assay (Fig. 1). These mitochondria showed respiratory control ratios of up to 4.0 with 2-oxoglutarate, 1.9 with succinate, 2.0 with malate and 1.8 with NADH. Apparent $K_m$ values for $O_2$ were not significantly different when different respiratory substrates were present (Table 1). These values ranged from 0.21 to 0.36 $\mu M$-$O_2$ both for mitochondria actively respiring in the presence of ADP and $P_i$ (State 3 in the terminology of Chance & Williams, 1956) and for de-energized mitochondria.

**Difference spectra of respiratory pigments in whole organisms under low $O_2$ concentrations**

Anaerobic-minus-hypoxic difference spectra of whole-cell suspensions of *A. castellanii* under different gaseous $O_2$ partial pressures, as measured by the oxygen electrode (Fig. 2), indicate that the respiratory chain has a high affinity. Thus 0.1 $\mu M$-$O_2$ gave measurable absorbance changes at around 440 and 420 nm, corresponding to maxima assigned to a- and c-type cytochromes respectively. At 0.3 $\mu M$-$O_2$ further oxidation of these components was evident, and at 20 $\mu M$-$O_2$ all features characteristic of the respiratory chain in its 'aerobic' steady state were observed, including an absorbance minimum at around 460 nm corresponding to flavoprotein–iron–sulphur proteins. Dual-wavelength measurements on whole-cell suspensions (Fig. 3) with wavelength pairs appropriate for cytochromes $a\alpha_3$, $c_{547}$ and $b_{560}$ and

![Graph](image)

**Fig. 2. Steady-state difference spectra of intact cells of *A. castellanii***

Organisms harvested from the growth medium were washed and resuspended in 0.13 mm-NaCl at $6 \times 10^7$ organisms/ml. Spectrum 1 was scanned after anaerobiosis was attained (gas phase argon) and stored in the digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 452 nm. Subsequent scans were of difference spectra (with the stored spectrum subtracted) of the suspensions held at various steady-state dissolved $O_2$ concentrations (as indicated by oxygen-electrode readings): spectrum 2, 0.1 $\mu M$-$O_2$; spectrum 3, 0.3 $\mu M$-$O_2$; spectrum 4, 20 $\mu M$-$O_2$. The temperature was 20°C, the path-length 1.6 cm, the spectral band-width 2 nm and the scan speed 120 nm/min. Results are typical of six experiments.
flavoproteins indicated 50% reduction at 0.1 μM-, 0.29 μM-, 0.37 μM- and 2.0 μM-O₂ respectively.

**Redox states of cytochromes at different O₂ concentrations**

Whole-cell suspensions of *A. castellanii* were incubated anaerobically in the presence of starved *P. fischeri*. Successive small increments in O₂ of the gas stream gave increases in dissolved O₂, increased bioluminescence and oxidation of cytochromes. Oxidation to 50% of the aerobic steady-state value was observed at 0.24 μM-O₂ for cytochrome aa₃, 0.45 μM-O₂ for cytochrome b and 0.59 μM-O₂ for cytochrome c (Fig. 4). When O₂ was decreased in small steps, 50% reduction of cytochromes occurred at 0.08 μM, 0.26 μM and 0.15 μM-O₂ for aa₃, c- and b-type cytochromes respectively.

Similar experiments with mitochondrial suspensions also showed hysteresis in cytochrome responses to changes in O₂ concentration. Decreasing O₂ gave values of 0.007 μM-, 0.035 μM- and 0.06 μM-O₂ respectively for half oxidation of cytochromes aa₃, c and b; corresponding values in anaerobic suspensions becoming oxygenated were 0.008 μM-, 0.05 μM- and 0.09 μM-O₂. The hysteresis phenomenon was not an artifact of the experimental technique, as control incubations with only starved *P. fischeri* gave similar responses irrespective of whether O₂ concentration was increasing or decreasing.
Discussion

Results presented in this paper give apparent \( K_m \) values for intact \( A.\) castellanii of about 0.4 \( \mu M \text{-}O_2 \) by two independent methods, a value similar to that determined previously (Lloyd et al., 1982). That a slightly lower value (0.21–0.36 \( \mu M \)) was observed with isolated mitochondria suggests that an \( O_2 \) concentration gradient may exist intracellularly between the plasma membrane and the mitochondrial inner membrane at \( O_2 \) concentrations below a critical value. Further evidence comes from experiments in which direct measurements of redox states of cytochromes are compared at similar low \( O_2 \) concentrations in intact organisms with those in isolated organelles. The observations that a 10-fold higher \( O_2 \) concentration is necessary to produce 50% oxidation of the cytochrome \( a_3 \) in \textit{vivo} than that in \textit{vitro} confirms the presence of an intracellular \( O_2 \) gradient.

Comparison of values for \( O_2 \)-dependence of mitochondrial functions in hepatocytes with values for similar functions in isolated organelles led Jones & Mason (1978) to suggest that a substantial \( O_2 \) concentration gradient exists at low extracellular \( pO_2 \) values. Thus half-reduction of cytochromes in mitochondria isolated from pigeon heart muscle occurred between 0.03 \( \mu M \) and 0.27 \( \mu M \cdot O_2 \) (Sugano \textit{et al.}, 1974), and similar values were obtained for rat liver mitochondria (Jones \textit{et al.}, 1977). For hepatocytes, however, much higher apparent \( K_m \) values were observed: those for \( O_2 \) consumption (1.9 \( \mu M \)), for \([ATP]/[ADP]\) half-maximal ratio (7.0 \( \mu M \)) and for \([\text{NAD}^+] / [\text{NADH}]\) half-maximal ratio (12.6 \( \mu M \)) are all about an order of magnitude greater than for organelles in \textit{vitro} (Jones & Mason, 1978). It was suggested that the large hepatic cell size contributes to this diffusion profile. Direct observations within mouse liver cells (Benson \textit{et al.}, 1980) of \( O_2 \)-quenched fluorescence of pyrene-1-butyrates (Vaughan & Weber, 1970) also provides evidence for localized variations in dissolved \( O_2 \) concentrations. Distribution was non-random and appeared to be associated with specific subcellular structures. A further refinement of this technique employed encapsulate pyrene dissolved in paraffin oil (Podgorski \textit{et al.}, 1981). Use of these capsules has shown \( O_2 \) partial pressures within respiring \textit{Amoeba proteus} less than ambient in both air and 100% \( O_2 \), with an \( O_2 \) gradient between cytosol and exterior in the range 2.5–4.9 kPa (19–37 mmHg).

\( A.\) castellanii has a mean cell volume in 0.4M-NaCl of 500 \( \mu m^3 \), on the basis of size determinations with a Coulter Counter (D. Lloyd, unpublished work), corresponding to a mean cell diameter of 10 \( \mu m \) (assuming that cells maintained in suspension are spherical). Thus cells smaller than hepatocytes (approx. 20 \( \mu m \) diam.) or \textit{Amoeba proteus} (75–1200 \( \mu m \) diam.) still have appreciable intracellular \( O_2 \) gradients.

References


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