Oxygen affinity of the respiratory chain of *Acanthamoeba castellanii*

David LLOYD,* Helga MELLOR† and Judith L. WILLIAMS

Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales, U.K.

(Received 30 December 1982/Accepted 29 March 1983)

1. Apparent K_m values for O_2 for the soil amoeba *Acanthamoeba castellanii* determined polarographically and by bioluminescence gave similar values (0.37 and $0.41 \mu M$ respectively). 2. Mitochondria oxidizing succinate or NADH in the presence or absence of ADP gave values in the range 0.21 – $0.36 \mu M$ - O_2 . 3. Oxidation of respiratory-chain components to 50% of the aerobic steady states in intact cells was observed at the following O_2 concentrations: cytochrome aa_3 , 0.1 – $0.25 \mu M$; cytochrome c , 0.3 – $0.6 \mu M$; cytochrome b , 0.35 – $0.45 \mu M$; flavoprotein, $2 \mu M$. 4. In isolated mitochondria corresponding values for a -, c - and b -type cytochromes were 0.007 , 0.035 – 0.05 and 0.06 – $0.09 \mu M$ - O_2 . 5. It is concluded that an O_2 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 aa_3 , has a high affinity for O_2 . Both polarographic measurements (Lloyd *et al.*, 1979) and those based on the O_2 -dependence of bacterial bioluminescence (Lloyd *et al.*, 1982) indicate an apparent $K_m < 1 \mu M$ - O_2 , and the latter, more reliable, method gives a value of $0.42 \mu M$ - O_2 . Determinations of the O_2 affinities for other intact eukaryotic micro-organisms have given apparent K_m values in the range 0.4 – $1.5 \mu M$ for baker's yeast (Longmuir, 1954; Schindler, 1964; Chance, 1965), $2.9 \mu M$ for the ciliate protozoon *Tetrahymena pyriformis* (Lloyd *et al.*, 1980, 1982), and $0.1 \mu M$ 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 O_2 affinities than those of isolated mitochondria (Jones, 1978; Jobsis & Rosenthal, 1978), and the presence of O_2 gradients within mammalian cells has been demonstrated directly (Benson *et al.*, 1980; Podgorski *et al.*, 1981).

The present paper shows that the apparent K_m values for O_2 for intact amoebae are higher than those for their mitochondria after extraction and that the O_2 concentrations that give 50% oxidation of respiratory-chain components are an order of magnitude higher for the organelle *in vivo* than those *in vitro*.

* To whom requests for reprints should be addressed.

† Present address: Department of Microbiology, Murdoch University, Murdoch, W.A. 6150, Australia.

Experimental

Maintenance and growth of the organisms

Acanthamoeba castellanii was maintained, grown axenically with shaking at $30^\circ C$, 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 2×10^6 – 4×10^6 organisms/ml).

O_2 -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 O_2 /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 O_2) and standard gas mixtures consisting of 1% O_2 in N_2 and 0.05% O_2 in N_2 (Air Products, Cardiff, Wales, U.K.). Low O_2 partial pressures were obtained by using a digital gas mixer (Lundsgaard & Degn, 1973), which provides accurate dilution in 5% steps. Absolute O_2 concentrations were calculated from percentage dilutions of stock gas mixtures.

Measurements were made at $25^\circ C$ by using a buffer consisting of 0.32 M-sucrose, 10 mM-Tris/ H_2SO_4 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 $260\text{ }\mu\text{M-O}_2$. Calibration curves for light-emission versus O_2 concentration in the absence of mitochondria were obtained for each determination, and respiration rates (V_r) were calculated from the equation:

$$V_r = K(T_G - T_L)$$

where K is the O_2 -transfer constant, T_G the O_2 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 O_2 in the absence of organisms was 3 min, corresponding to an O_2 -transfer constant of 0.23 min^{-1} (from $K = \ln 2/t_{1/2}$). Apparent K_m values are presented as means $\pm$ 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 O_2 was slightly less than 5 min, corresponding to an O_2 -transfer constant of 0.14 min^{-1} .

Simultaneous measurements of bioluminescence of *P. fischeri* (Oshino *et al.*, 1972) in this system gave O_2 determinations at concentrations where the O_2 -dependent current produced by the oxygen electrode became small compared with the 'residual' current (still observed at zero O_2). A liquid-filled light-lead (4 mm diam.) was used with an interference filter ($\lambda_{\text{max.}} = 495\text{ nm}$, half band-width 10 nm) to monitor bioluminescence in the cuvette; the photomultiplier and associated circuit were identical with that described for O_2 -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

O_2 affinity of intact organisms

The dependence of respiration rate on O_2 partial pressure over a concentration range of $5.0\text{--}0\text{ }\mu\text{M-O}_2$

Fig. 1. Dependence of respiration rate of *A. castellanii* mitochondria on O_2 concentration

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/ H_2SO_4 , 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 O_2 (T_L); this was used to measure values for T_L at a series of fixed values of O_2 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 $1/V_r$ 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).

O₂ 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 O₂ 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 *aa*₃, *c*₅₄₇ and *b*₅₆₀ and

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 6×10^7 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 $20^\circ C$, 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.

Table 1. Apparent K_m values for O_2 for mitochondria isolated from *A. castellanii*

Mean values $\pm$ s.d. are given for 12 determinations with different mitochondrial preparations. For full details see the text.

Substrate	Apparent K_m for O_2 (μM)	
	No addition	+ADP (0.8 mM)
Succinate (10 mM)	0.21 ± 0.05	0.29 ± 0.04
NADH (0.4 mM)	0.36 ± 0.03	0.34 ± 0.04

Fig. 3. Effect of O_2 concentration on the redox states of respiratory-chain components in intact *A. castellanii*. Organisms were washed and resuspended in 0.13 M-NaCl and incubated at 25°C under argon in a cuvette (1.6 cm light-path) fitted with an oxygen electrode (see the Experimental section) at a cell density of 6×10^6 organisms/ml. Increasing O_2 partial pressure in the gas phase in steps gave a series of redox states for respiratory components. O, Cytochrome aa_3 (441 nm–455 nm); ●, cytochrome c_{547} (547 nm–540 nm); △, cytochrome b_{560} (560 nm–575 nm); ▲, flavoprotein–iron–sulphur protein (465 nm–510 nm).

flavoproteins indicated 50% reduction at 0.1 μM -, 0.29 μM -, 0.37 μM - and 2.0 μM - O_2 respectively.

Redox states of cytochromes at different O_2 concentrations

Whole-cell suspensions of *A. castellanii* were incubated anaerobically in the presence of starved *P. fischeri*. Successive small increments in O_2 of the gas stream gave increases in dissolved O_2 , increased bioluminescence and oxidation of cytochromes. Oxidation to 50% of the aerobic steady-state value was observed at 0.24 μM - O_2 for cytochrome aa_3 , 0.45 μM - O_2 for cytochrome b and 0.59 μM - O_2 for cytochrome c (Fig. 4). When O_2 was decreased in small steps, 50% reduction of cytochromes occurred at 0.08 μM , 0.26 μM and 0.15 μM - O_2 for a -, c -, and b -type cytochromes respectively.

Fig. 4. Effect of O_2 concentration on the redox states of cytochromes in intact *A. castellanii* and in isolated mitochondria

(a) A whole-cell suspension (7.8 ml, 6×10^6 organisms/ml) in 0.4 M-NaCl/0.1 M-potassium buffer (pH 7.0) was incubated under argon with 0.2 ml of the *Photobacterium* suspension (see the Experimental section). Increasing or decreasing the O_2 partial pressure in the gas phase (direction indicated by arrows) gave a series of redox states for cytochromes aa_3 (●), b (▲) and c (■). (b) As in (a), except that mitochondria (7.8 ml, 1.6 mg of protein/ml) were used instead of intact cells; respiratory substrate was 10 mM-sodium succinate in the presence of 0.8 mM-ADP. Results are typical of five experiments.

Similar experiments with mitochondrial suspensions also showed hysteresis in cytochrome responses to changes in O_2 concentration. Decreasing O_2 gave values of 0.007 μM -, 0.035 μM - and 0.06 μM - O_2 respectively for half oxidation of cytochromes aa_3 , c and b ; corresponding values in anaerobic suspensions becoming oxygenated were 0.008 μM -, 0.05 μM - and 0.09 μM - O_2 . 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 O_2 concentration was increasing or decreasing.

Discussion

Results presented in this paper give apparent K_m values for intact *A. castellanii* of about $0.4 \mu\text{M-O}_2$ by two independent methods, a value similar to that determined previously (Lloyd *et al.*, 1982). That a slightly lower value ($0.21\text{--}0.36 \mu\text{M}$) 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 aa_3 *in vivo* than that *in 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 $p\text{O}_2$ values. Thus half-reduction of cytochromes in mitochondria isolated from pigeon heart muscle occurred between $0.03 \mu\text{M-}$ and $0.27 \mu\text{M-O}_2$ (Sugano *et al.*, 1974), and similar values were obtained for rat liver mitochondria (Jones *et al.*, 1977). For hepatocytes, however, much higher apparent K_m values were observed: those for O_2 consumption ($1.9 \mu\text{M}$), for $[\text{ATP}]/[\text{ADP}]$ half-maximal ratio ($7.0 \mu\text{M}$) and for $[\text{NAD}^+]/[\text{NADH}]$ half-maximal ratio ($12.6 \mu\text{M}$) are all about an order of magnitude greater than for organelles *in 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 *et al.*, 1980) of O_2 -quenched fluorescence of pyrene-1-butyrate (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 *et al.*, 1981). Use of these capsules has shown O_2 partial pressures within respiring *Amoeba proteus* less than ambient in both air and 100% O_2 , with an O_2 gradient between cytosol and exterior in the range $2.5\text{--}4.9 \text{ kPa}$ ($19\text{--}37 \text{ mmHg}$).

A. castellanii has a mean cell volume in 0.4 M-NaCl of $500 \mu\text{m}^3$, on the basis of size deter-

minations with a Coulter Counter (D. Lloyd, unpublished work), corresponding to a mean cell diameter of $10 \mu\text{m}$ (assuming that cells maintained in suspension are spherical). Thus cells smaller than hepatocytes (approx. $20 \mu\text{m}$ diam.) or *Amoeba proteus* ($75\text{--}1200 \mu\text{m}$ diam.) still have appreciable intracellular O_2 gradients.

References

- Benson, D. M., Knopp, J. A. G. & Longmuir, I. S. (1980) *Biochim. Biophys. Acta* **591**, 187–197
- Chance, B. (1965) *J. Gen. Physiol.* **49**, 163–180
- Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134
- Degn, H., Lundsgaard, J. S., Petersen, L. C. & Ormicki, A. (1980) *Methods Biochem. Anal.* **26**, 47–77
- Edwards, S. W. & Lloyd, D. (1978) *Biochem. J.* **174**, 203–211
- Edwards, S. W., Chagla, A. H., Griffiths, A. J. & Lloyd, D. (1977) *Biochem. J.* **168**, 113–121
- Hill, G. C. (1978) in *Functions of Alternative Terminal Oxidases* (Degn, H., Lloyd, D. & Hill, G. C., eds.), pp. 67–77, Pergamon Press, Oxford
- Jobsis, F. F. & Rosenthal, M. (1978) *Ciba Found. Symp.* **56**, 129–148
- Jones, D. P. (1978) *Biochem. Biophys. Res. Commun.* **80**, 477–483
- Jones, D. P. & Mason, H. S. (1978) *J. Biol. Chem.* **253**, 4874–4880
- Jones, D. P., Mason, H. S., Nakase, Y. & Chance, B. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 566
- Lloyd, D., Edwards, S. W., Kristensen, B. & Degn, H. (1979) *Biochem. J.* **182**, 11–15
- Lloyd, D., Kristensen, B. & Degn, H. (1980) *J. Gen. Microbiol.* **121**, 117–125
- Lloyd, D., James, K., Williams, J. & Williams, N. (1981) *Anal. Biochem.* **116**, 17–21
- Lloyd, D., Williams, J. L., Yarleth, N. & Williams, A. G. (1982) *J. Gen. Microbiol.* **128**, 1019–1022
- Longmuir, I. F. (1954) *Biochem. J.* **57**, 81–87
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lundsgaard, J. S. & Degn, H. (1973) *IEEE Trans. Biomed. Eng.* **20**, 384–387
- Oshino, R., Oshino, N., Tamura, M., Kobilinsky, L. & Chance, B. (1972) *Biochim. Biophys. Acta* **273**, 5–17
- Podgorski, G. T., Longmuir, I. S., Knopp, J. A. & Benson, D. M. (1981) *J. Cell. Physiol.* **107**, 329–334
- Schindler, F. (1964) Ph.D. Dissertation, University of Pennsylvania
- Sugano, T., Oshino, N. & Chance, B. (1974) *Biochim. Biophys. Acta* **347**, 340–358
- Vaughan, W. & Weber, G. (1970) *Biochemistry* **9**, 464–473