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Resistance of Oxidative Phosphorylation in *Escherichia coli* to Hyperoxia

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Abstract

Exposure to oxygen at 6 atmospheres which was sufficient to stop growth and to reduce respiration rate of *Escherichia coli*, did not significantly alter the oxidative phosphorylation efficiency of various cell-free fractions when measured in air. The P/O ratio was approximately 1·00 ± 0·25 for complete extracts and 0·5 for the dialyzed fraction of greater than approximately 300,000 molecular weight. The fraction containing components of less than 300,000 in molecular weight did not phosphorylate. The rate of phosphorylation in complete extracts was sufficient to account for the production of approximately 34,400 ± 9,700 molecules of ATP from ADP/sec/bacterium. However, intact bacteria in exponential growth had approximately 59-times the oxygen uptake rate of extracts derived therefrom.

Oxygen at high pressure is known to be bacteristatic for various species of bacteria.[1] Previous work[2] had shown that exposure of *Escherichia coli* to hyperoxia resulted in a decreased rate of oxygen uptake from substrates such as glucose, acetate, palmitate or from endogenous substrates. There is considerable evidence for a variety of cellular damage sites in bacteria and in animal tissues, resulting from exposure to hyperoxia (see Haugaard,[3] 1968 for a review). This investigation was designed to determine if reduced efficiency of oxidative phosphorylation resulted from exposure of *E. coli* to toxic levels of oxygen.

Materials and Methods

Escherichia coli, strain E-26, was grown in a medium containing (in grams/liter): sodium acetate, 4·1; NH_4Cl, 2; $KHPO_4$, 7; KH_2PO_4, 7; $MgSO_4 . 7H_2$, 0·05; and $FeSO_4 . 7H_2O$, 0·005. The pH was adjusted to 7·0 with NaOH. Cultures were grown at 37° with an air-flow of 2 liters/min and agitation at 240 revolutions/min in a 14-liter fermentor (Bio-Kulture Model, Fermentation Design, Incorp., Allentown, Pa.) which was modified for pressurized fermentation. During exponential growth at an optical density of 0·8 to 1·0 at 500 nanometers wavelength the system was purged with pure oxygen to remove the air, and was pressurized with pure oxygen to 90 lb/sq. inch over a 10 min period. A purge rate of 10 liters/min was maintained with the oxygen passing through a sparger to provide mixing. After 2 h, the pressure was reduced to 40 lb/sq. inch and culture was passed at 100 ml/min through a cooling coil, which reduced the temperature

from 37° to 0° within 15 sec, directly into continuous flow centrifugation at 4°. Air control cultures were grown and harvested identically, except they were not exposed to hyperoxia. The bacteria were washed twice with [tris (hydroxymethyl) aminomethane] buffer, 0·05 M, pH 7·4. A 20% suspension (w/v) of cells in the same buffer was exposed to ultrasonic energy at 4° at alternate 30 sec intervals for a total of 4 min using the Bronwill cell disintegrator (Bronwill Scientific Co., Rochester, New York). The supernatant obtained after centrifugation at 34,000 × g for 20 min at 4° will be referred to as crude extract. Crude extracts were fractionated at 4° using a Model 52 ultrafiltration cell and XM-300 membranes (Amicon Corp., Allentown, Penn.). Crude extracts were concentrated to half volume under N_2 pressure. The fraction which passed through the membrane will be referred to as XM-300 filtrate. The ultrafiltration cell was converted to the dialysis mode and 5 volumes of tris buffer, 0·05 M, pH 7·4, were passed through the cell. The retentate was adjusted with the same buffer to the original volume of the crude extract and this fraction will be referred to as dialyzed XM-300 retentate. Fractions were stored at 4°. Protein was measured by the method of Lowry *et al.*[4] with bovine serum albumin as the standard.

Oxidative phosphorylation efficiencies were determined for the following fractions prepared from bacterial cultures in exponential growth and from such cultures after exposure to oxygen as previously described: crude extracts, XM-300 filtrates, dialyzed XM-300 retentates, and recombined XM-300 filtrates plus dialyzed XM-300 retentates. P/O ratios were also determined for crude extracts from air-grown bacteria which were incubated with stirring for 5 h at 4° in oxygen at 4 atmospheres overpressure in a model 52 ultrafiltration cell with the outlet closed. Oxidative phosphorylation was measured using the reaction mixtures described by Kashket and Brodie,[5] except the total volume was 6 ml and DL-isocitrate (trisodium salt) at a final concentration of 0·067 molar was the substrate. Reaction temperature was 37°. The components, minus DL-isocitrate were equlibrated for 3 min and the reaction was started by adding D-L isocitrate. Samples were removed immediately and after 10 min and added to $\frac{1}{5}$ volume of 3 M trichloroacetic acid and filtered through a cellulose acetate membrane (0·45 μ pore size, Millipore Corp., Bedford, Mass.). Phosphorus content of the filtrate was measured by the method of Fiske and Subbarow.[6] Oxygen uptake was recorded over a 10 min interval with a polarographic-type probe (model 5301 Biological O_2 Monitor, Yellow Springs Instrument Co., Yellow Springs, Ohio) standardized in tris buffer (0·05 M, pH 7·4) saturated with air at 37°.

Results

The average rate of oxygen uptake measured by 43 determinations on four exponentially growing cultures of *Escherichia coli* at 37° in minimal medium with acetate as the carbon and energy source, was $27{\cdot}39 \pm 0{\cdot}64$ μl/min/10^{10} bacteria ($1{\cdot}23 \pm 0{\cdot}03 \times 10^6$ molecules of O_2/bacterium/sec). Oxygen uptake was reduced by approximately 50% in such cultures after exposure to 6 atmospheres overpressure of oxygen as described in materials and methods. Oxygen uptake was measured in culture medium saturated with air at 1 atmosphere. Growth rate, as measured by change in optical density, was 80 min/generation in air. Growth stopped immediately after pressurization with oxygen.

The oxidative phosphorylation efficiencies of cell-free fractions obtained from air-

TABLE I. Oxygen and phosphorus uptake and P/O ratios for various fractions obtained from air-grown and oxygen-exposed bacteria

Cell fractions*	Oxygen uptake nanogram atoms/min/mg protein	Phosphorus uptake nanogram atoms/min/mg protein	P/O ratio
Crude extract† (3,13)	14·2 ± 2·7	11·4 ± 3·3	0·83 ± 0·26
Crude extract (O_2-exposed cells)‡ (3,9)	12·0 ± 0·2	11·7 ± 3·2	1·05 ± 0·47
Crude extrace (exposed to O_2)§ (2,8)	10·2 ± 1·3	10·1 ± 2·0	1·01 ± 0·23
Filtrate† (2,3)	7·4 ± 2·0	0	—
Filtrate (O_2-exposed cells)‡ (3,5)	5·0 ± 1·7	0	—
Dialyzed retentate† (2,7)	17·4 ± 3·1	8·1 ± 3·8	0·49 ± 0·26
Dialyzed retentate (O_2-exposed cells)‡ (2,7)	15·0 ± 3·0	5·5 ± 1·7	0·42 ± 0·12
Filtrate + dialyzed retentate† (2,7)	11·7 ± 0·8	12·5 ± 3·4	1·07 ± 0·27
Filtrate + dialyzed retentate (O_2-exposed cells)‡ (3,5)	11·3 ± 2·0	8·6 ± 4·0	0·74 ± 0·25

* Crude extracts were the supernatants after sonification and centrifugation; filtrates and retentates were fractions which passed through and were retained, respectively, by ultramembranes (Amicon XM-300). Details are given in the text. Numbers in parentheses are number of experiments, followed by total determinations.

† Bacteria were grown in air in a fermentor at 37° in minimal medium with acetate as the sole carbon and energy source.

‡ Bacteria, incubated as described in (†) to log growth, were exposed to oxygen at 6 atmospheres for 3 h.

§ Bacteria were grown as in (†) and the crude extracts were then exposed to 4 atmospheres of O_2 at 4° for 5 h.

grown and from oxygen-exposed *E. coli* are shown in Table I. The rate of oxygen uptake and the P/O ratio decreased in crude, cell-free extracts upon storage at 4°. Therefore the reported data are from determinations done within 2 days, and normally on the same day that extracts were prepared.

The oxygen uptake of crude extracts (Table I) was approximately 1·7% of the oxygen uptake of exponentially growing bacteria from which the extracts were obtained, i.e. 838 nanogram atoms O_2 were taken up per min from the cells equivalent to 1 mg of soluble protein (2·92 ± 0·06 mg soluble protein per 10^{10} broken bacteria).

The dialyzed fraction retained by XM-300 membranes (retentate) from crude, cell-free extracts (Table I) contained 70·6 ± 10·0% of the protein present in crude, cell-free extracts and 24·3 ± 1·4% of the protein passed through the membrane (filtrate). The percentages are the average ± the standard deviation for five experiments. Recombined, dialyzed retentate and filtrate fractions contained all components which were present in crude, cell-free extracts, but at half the concentration.

The filtrate was less efficient in stimulating oxygen uptake than was the dialyzed retentate and the filtrate could not couple oxygen uptake with phosphorylation (Table I). However, factors present in the filtrate were needed by the dialyzed retentate for oxidative phosphorylation since the P/O ratio was reduced by half in their absence. The data in Table I are reported per mg of protein. To compare oxygen and phosphorus uptake of filtrate or dialyzed retentates with either the crude extract or with the recombined filtrate and dialyzed retentate, the values must be corrected for the equivalent protein concentration of these fractions in the crude extract, 0·243 and 0·706, respectively. The

average oxygen uptake of filtrates and retentates in nanogram atoms/mg equivalent total protein in crude extracts was 1·8 and 12·3, respectively, for a total of 14·1. This is in close agreement with the average value of 14·2 for crude extracts of air-grown cells (Table I).

Discussion

The results indicate that hyperbaric oxygen exposure which completely stops growth of *Escherichia coli* produces no detectable impairment of oxidative phosphorylation efficiency in cell extracts measured in air. The rate of phosphorylation in extracts is sufficient for production of 34,400 $\pm$ 9,700 molecules of ATP from ADP per second per bacterium. If one assumes that phosphorylation efficiency is the same in intact cells as in extracts (an assumption which may not be valid) it follows that approximately 2×10^6 molecules of ATP could be produced from ADP per second per bacterium in exponential growth. The generation time is 80 min and the dry mass of a cell is about 4×10^{-13} g; therefore, approximately 25 g of dry biomass would be synthesized per gram mole of ATP. P/O ratio was not measured in whole cells due to known difficulties in assigning functions to uptake.

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