REACTIVITY WITH OXYGEN OF BACTERIAL CYTOCHROME OXIDASES a_1, aa_3 AND o

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1. Introduction

The occurrence of two or more, spectrophotometrically distinguishable cytochrome oxidases in several species of bacteria has been known for a long time [1–3]. However no comparative study of the physiological properties of these oxidases (a_1, a_2, aa_3 and o) has been published.

In this paper the reactivity towards oxygen of oxidases a_1, aa_3 and o, each present as the sole or major oxidase in membrane suspensions, is determined. Cytochrome oxidase a_2 could not be so studied, since no organism could be found which contained a_2 as the sole or major oxidase. The results indicate that cytochrome o, in three bacteria, and cytochrome aa_3 in a bacterium and a Keilin–Hartree prepn. from pig heart had similar rates of reaction with oxygen (second order constant, K_1 : 2×10^7 M^{-1} sec^{-1} approx. at 30°) in fairly close agreement with previous data for mitochondrial cytochrome aa_3 from various sources [4]. The preparation containing a_1 as the major oxidase yielded a second order constant an order of magnitude greater.

These observations, taken together with data on the capacity for oxidative phosphorylation of these organisms [5], allows the rationalisation of the presence of cytochrome a_1 in various bacteria which naturally grow in conditions of low, or fluctuating oxygen tension.

2. Methods

2.1. *Organisms used*

The following species were used in this study: *Acetobacter* [T-71] and *Kurthia zopfii* [C-5] from the culture collection of the M.R.C. Microbial Systematics Research Unit, The University of Leicester; *Acinetobacter lwoffi* [4B-P.D.J. Weitzman]; *Xanthomonas hyacinthi* [NCPPB 519], and *Microbacterium thermosphactum* [ATCC 10018].

2.2. *Preparation of respiratory membranes*

Bacteria were cultured at 30° on rich broth media and with high aeration, as described elsewhere [5]. Cells, in either logarithmic (log) or stationary (stat) phase of growth, were harvested by centrifugation at 4°, homogenised in distilled water, recentrifuged and resuspended by homogenisation in 25 mM sodium phosphate buffer pH 7.4 to an extinction at 680 nm of 50–80. Cell suspensions, in 10 ml aliquots, were disrupted by approx. 4 min sonication at 7.8 microns (peak to peak).

Whole cells and cell walls were removed by centrifugation at 7000 *g* for 10 min, and the respiratory membranes were sedimented by further centrifugation at 157,000 *g* for 1–2 hr. The membranes were finally suspended in the phosphate buffer to a protein concentration (as measured by a modified Biuret technique [6]) of approx. 35 mg/ml.

Keilin–Hartree sub-mitochondrial membranes were prepared from fresh pig heart by a modified procedure [7].

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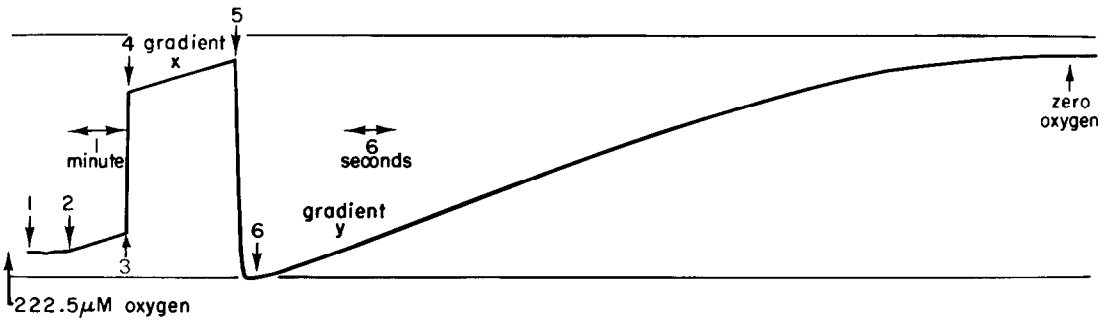

Fig. 1. Desaturation curve obtained using *K. zopfii* membranes (50% actual size). Manipulations as follows: 1, addition of buffer and 0.63 mg protein (2.4 ml); 2, addition of NADH + malate (0.1 ml); 3, stop chart; 4, restart chart; 5, amplify; and 6, speed up chart 12-fold. Amplification = $\frac{12y}{x}$.

2.3. *Determination of concentration of cytochrome oxidases*

Dithionite-reduced *minus* oxidised, and dithionite-reduced plus carbon monoxide *minus* dithionite-reduced difference spectra [8] were obtained using either a Shimadzu MPS-50L or Unicam SP1800 spectrophotometer.

The concentration of cytochrome a_3 was calculated using the data of Cheah [9], and that of cytochrome *a* was then calculated using the data of Van Gelder [10]. The concentration of cytochrome *o* was calculated according to Daniel [11], and that of cytochrome a_1 was estimated by assuming a similar $\Delta\epsilon$ (for the peak to trough in the CO-difference spectrum) to that of a_3; an assumption which seemed reasonable from their qualitatively, very similar difference spectra.

2.4. *Measurement and analysis of oxygen desaturation curves*

Oxygen desaturation curves were obtained in a similar fashion to Longmuir [12], White [13], Schindler [14] and Taniguchi et al. [15]. Oxygen concentration was monitored at 30° using a Clark YSI 4004 electrode fitted with the thinnest available teflon membrane (Yellow Springs Instrument Co.). The time taken for 80% reduction by dithionite was approx. 4 sec. Zero order rates were proportional to protein concentration up to approx. 250 μM min^{-1}. The reaction chamber contained respiratory membranes; an excess of oxidisable substrate as follows: NADH (Sigma grade III) 1 mM, DL-malate and succinate, sodium salts (British Drug Houses) 12 mM or sodium D-iso-ascorbate (B.D.H.) 12 mM plus N,N,N^1,N^1-tetramethyl-*p*-phenylene diamine hydrochloride (Fisons) 7.6 mM (hereafter referred to as asc-TMPD), and 25 mM sodium phosphate buffer, pH 7.4 to a final volume of 2.5 ml. The concentration of oxygen in aerated buffer at 30° was taken to be 222.5 μM (from the data of Chappel [16]). The chamber was sealed with parafilm (Gallenkamp) to prevent leakage in of oxygen. The mixture was stirred with a teflon-covered magnet at 350 rpm.

At about 15 sec before the critical oxygen concentration ie. the point at which the rate of oxygen reduction ceases to be zero order, after White [13], was reached, the sensitivity was increased up to 20-fold and the chart speed was increased 12-fold (fig. 1). The expanded desaturation curves so produced were analysed by measuring the activity (through drawing tangents to the curve) at several oxygen concentrations. The data were then analysed as a Lineweaver–Burk plot [17] to yield values for K_m and V. The maximum velocity, V, here refers to the terminal reaction not the zero order rate.

2.5. *Reagents*

Chemicals used were of analytical grade unless otherwise specified. Glass-distilled, deionised water was used throughout the preparation and examination of respiratory membranes.

3. Results and discussion

With all preparations examined, linear Lineweaver–

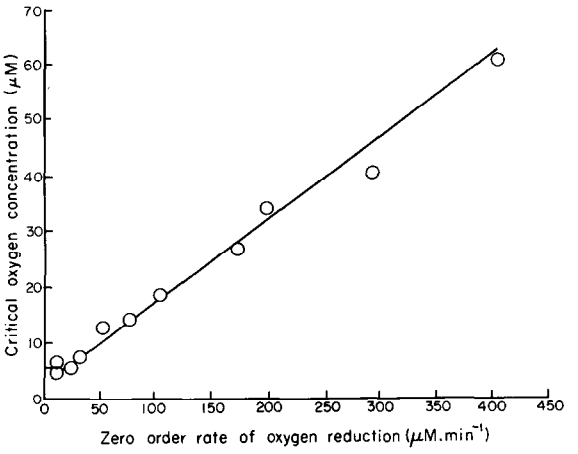

Fig. 2. Dependence of critical oxygen concentration on the zero order rate of oxygen reduction by *A. lwoffi* membranes.

Burk plots were only obtained when the zero order rate was less than approx. 25 μM/min. At higher zero order rates sigmoidal kinetics were observed. These features were closely associated with a variation in the critical oxygen in direct proportion to the zero order rate above approx. 25 μM/min, possibly reaching a constant, minimum value at lower rates, see fig. 2. Note that the accuracy with which the critical oxygen could be measured decreased as the zero order rate decreased owing, firstly to an increasingly gradual deviation from linearity, and, secondly to the slight curvature of the 'zero order' rate below approx. 12 μM/min.

These observations were consistent with the hypothesis that the transition from the aerobic steady state to the anaerobic state begins prior to reaching the critical oxygen; and, as the zero order rate is decreased (by using less protein) to approx. 25 μM/min there is already a high degree of reduction of respiratory carriers when the trace deviates from zero order. In this case there is, presumably, an almost constant amount of reactive enzyme i.e. reduced cytochrome oxidase, present throughout the desaturation curve, and simple first order kinetics are observed. These data and considerations are not in accord with those of White [13] for *Haemophilus parainfluenzae*, however, the presence of more than one oxidase in this organism is probably responsible for the discrepancy.

The min. critical oxygen (see fig. 2) is a value of physiological significance since it is that concentration which will, in practice, start to limit respiration.

According to the above hypothesis, the largely linear relationship between critical oxygen and zero order rate (fig. 2) must be caused by a linear increase in the reduction level of the terminal respiratory carrier during the transition from the aerobic steady

Table 1
Summary of data obtained from oxygen desaturation curves.

Source of membranes	Cytochrome oxidase*	Substrate	Minimum critical oxygen (μM)	K_m (μM)	V (electron/sec per CO-binding haem)	K_1 $10^7 \times$ (M^{-1} sec^{-1})	Number of determinations
Ac. [T-71] (stat)	a_1 (427,441)†	asc-TMPD	0.4	0.33 ± 0.03	306 ± 34	23.20 ± 3.27	3
A. lwoffi (log)	*o* (417,433)	NADH + succinate	5.5	6.5 ± 1.8	442 ± 109	1.70 ± 0.62	13
X. hyacinthi (log)	*o* (415,431)	NADH + malate	5.0	2.8 ± 0.6	427 ± 81	3.81 ± 1.08	4
K. zopfii (stat)	*o* (417,433)	NADH + malate	3.5	1.8 ± 0.4	112 ± 24	1.56 ± 0.48	3
M. thermosphactum (stat)	a_3 (431,445)	NADH	5.5	7.7 ± 1.3	1080 ± 206	3.51 ± 0.85	3
Pig heart (Keilin–Hartree particles	a_3 (431,445)	NADH + succinate	4.2	2.8 ± 0.6	102 ± 17	0.91 ± 0.23	4

Values are given ± 2 × S.E.

* The Soret peak and trough of the oxidases in the CO difference spectrum are given in parentheses.
† The minor presence of cytochrome *o* was neglected in these calculations.

state to the reduced state.

The data obtained is summarised in table 1, in which are included the second order constants for the oxidase reaction (K_1). These were calculated from the K_m and V according to Chance [18] as follows:

K_m (M) = Max. catalytic centre activity (electron/sec per CO-binding haem)/(4 × K_1 (M^{-1} sec^{-1}))

The maximum catalytic centre activity of the oxidases varies over a 10-fold range, but no class of oxidase is associated with a particularly high or low value. The K_m values are remarkably close to the values for the min. critical oxygen, which indicates that, in each system, the oxidase has roughly twice the capacity for reaction compared to the rate-limiting step of the system under excess oxygen. These features suggest the possibility of a regulatory function for the oxidase.

The K_1 values of aa_3 and *o* are closely similar, and in good agreement with several published values for mitochondrial aa_3 [4]. However, the preparation containing a_1 as the major oxidase has a K_1 an order of magnitude greater. The latter finding was in contrast to the data from *H. parainfluenzae,* which suggested that the a_1 was too slowly oxidised to function significantly in terminal oxidation [19]. Clearly cytochrome a_1 may be a group of enzymes with different properties.

Now, it has recently been shown from studies on growing cultures and anaerobic cell suspensions [5] that bacterial electron transfer system containing either aa_3 or *o* as sole terminal oxidase carry out efficient oxidative phosphorylation (P/O = 3 approx.); but that the electron transfer system of *Ac.* [T-71] is far less efficient (P/O = 1 approx.). Furthermore the production of cytochromes a_1 and a_2 in *Escherichia coli* [W], at low oxygen tensions, is accompanied by a decrease in the P/O from 3 to 1.

From these data it may be calculated, using a simple Michaelis–Menten analysis, that a system utilising *n* molecules of a_1 (P/O = 1, K_m = 0.3 μM and V = 300 sec^{-1}) will synthesise ATP more rapidly than a system utilising *n* molecules of *o* (P/O) = 3, K_m = 3.5 μM and V = 300 sec^{-1}) at any concentration of oxygen below 1.3 μM. This value is the same as the concentration which causes the production of a_1 and a_2 in *E. coli* and *Aerobacter aerogenes* in continuous culture [20, 21].

It is postulated that these two facultatively anaerobic, Gram-negative heterotrophs utilise different oxidase systems under variable oxygen tensions in order to produce ATP, and hence grow, as rapidly as possible, although relatively inefficiently; and that these features represent an adaptation to nutritionally rich but unstable environmental conditions.

The strictly aerobic pseudomonads, of which several species can contain a_1 and a_2 (for example see [22, 23]), are represented in this study by *A. lwoffi* and *X. hyacinthi.* These species only produce significant amounts of a_1 and a_2 under conditions of extremely low aeration (unpublished observation), and it is therefore postulated that they utilise an inefficient a_1 type of system only as a final means of survival, growing with high efficiency as far as is possible. This mode of use of the a_1 system would be compatible with existence in a less competitive habitat.

Ac. [T-71] itself contains similar amounts of cytochromes a_1 and *o* whether grown under low or high aeration, and its growth rate is always slow (mean generation time 2.5 hr approx., unpublished observations). Thus it appears to be adapted to a habitat in which the oxygen concentration is always low.

Now consider the large group of organisms which contain aa_3 and/or *o*. These species will clearly not be as competitive as *E. coli* under conditions of excess nutrients and variable oxygen, and the strict aerobes will not be capable of maintaining life under extremely low oxygen as can *A. lwoffi.* However, this group is known to include species which can form durable spores under unfavourable conditions (*Bacillaceae*); species which can produce antibiotics which increase their competitiveness (*Streptomycetes*), and at least one species which changes its cytochrome content from that of a typical Gram-positive aerobe (aa_3.*o.b.c*) to that of a typical Gram-negative, facultative anaerobe such as *E. coli* (a_1.a_2.*o.b*) as the aeration is decreased [24].

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