ELECTRON TRANSPORT IN AEROBICALLY GROWN *PARACOCCUS DENITRIFICANS*: KINETIC CHARACTERIZATION OF THE MEMBRANE-BOUND CYTOCHROMES AND THE STOICHIOMETRY OF RESPIRATION-DRIVEN PROTON TRANSLOCATION

Hugh G. LAWFORD*, John C. COX, Peter B. GARLAND, Bruce A. HADDOCK
Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, UK

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1. Introduction

John and Whatley [1] have reviewed the information so far available about the functional organization of the membrane-bound components responsible for electron transport dependent ATP synthesis in aerobically grown *Paracoccus denitrificans* and drew attention to the similarities that exist between these components and those of the inner mitochondrial membrane. The object of the present work was to extend these studies and to characterize further the following properties of the plasma membrane of *P. denitrificans:* (a) the cytochrome components identifiable by low-temperature difference spectroscopy; (b) the fast oxidation–reduction kinetics of the membrane-bound cytochromes; and (c) the stoichiometry of respiration-driven proton translocation during the oxidation of different added substrates.

2. Materials and methods

P. denitrificans (Micrococcus denitrificans NCIB 8944) was obtained from Dr W. A. Hamilton (Department of Microbiology, University of Aberdeen, Aberdeen AB9 1AS, UK) and grown at 30°C in a mineral salts medium [2] containing vitamin-free casamino acids (0.1% w/v) and glycerol (0.5% w/v) unless otherwise stated. For cells used subsequently in proton-translocation studies, growth was in 2-litre unbaffled conical flasks agitated in a rotary shaker [3]; cells were harvested at various stages in the growth cycle, washed and starved as indicated previously for *Escherichia coli* [4]. Particles, used in the cytochrome studies, were prepared in a buffer containing 10 mM HEPES-KOH (pH 7.5), 300 mM KCl and 5 mM $MgCl_2$ [5] from cells that had been grown in a 10-litre fermentor vessel under conditions of vigorous aeration [3] and harvested in the mid-exponential phase of growth.

Oxidase activities [6], protein determinations [6], low temperature spectroscopy [7], rapid oxidation kinetics of cytochromes [8] and proton translocation studies [6,9] were measured or performed as described in previous publications from this laboratory.

3. Results and discussion

3.1. *Low temperature difference spectroscopy of membrane-bound cytochromes*

From fourth-order finite difference analysis of low temperature spectra of aerobically grown *P. denitrificans* harvested in the exponential phase of growth, Shipp has concluded that this organism contains two *c*-type cytochromes, with absorption maxima at 547 nm and 551 nm, and possibly three *b*-type cytochromes, with absorption maxima at

*Permanent address: Department of Biochemistry, University of Toronto, Toronto, M5S 1A8, Ontario, Canada.

Abbreviations: HEPES, 2-(*N*-2 hydroxyethylpiperazin-*N'*-yl) ethane sulphonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TES, *N'*-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

554 nm, 558 nm and 563 nm [10]. In addition cytochromes $a + a_3$, and possibly cytochrome *o*, serve as a terminal oxidase for the respiratory chain [1,11]. Low temperature reduced minus oxidized difference spectra recorded with our spectrophotometer indicated minimally the presence of two *c*-type cytochromes with absorption maxima at 546 nm and 549 nm (or alternatively one *c*-type cytochrome with a split α-peak), two *b*-type cytochromes with absorption maxima at 556 nm and about 562 nm, and cytochrome $a + a_3$ with an absorption maximum at 604 nm (fig.1a). In the presence of antimycin A and O_2 (generated from H_2O_2), the addition of NADH caused the reduction of at least two *b*-type cytochromes with clearly resolved absorption maxima at 556 nm and 563 nm (fig.1b). The improved resolution in the *b*-type cytochrome spectra, compare fig.1a with fig.1b, could be the result of the presence of a third *b*-type cytochrome with an absorption maximum between 556 and 563 nm which lies on the O_2 side of the site of inhibition by antimycin A or alternatively it could be ascribed to an antimycin A-induced, O_2-dependent red-shift in the absorption spectrum of the *b*-type cytochrome with an absorption maximum at 563 nm as with mitochondrial *b*-type cytochromes [12]. As in mitochondria the steady state reduction level of the *b*-type cytochromes was greater in the presence of antimycin A and O_2 than anaerobically in the absence of inhibitor as shown by the troughs at 556 and 563 nm in fig.1c. In the absence of antimycin A, both *b*-type cytochromes were partially but not totally reduced by L-ascorbate in the presence of TMPD (fig.1d and 1e).

Reduced + CO minus reduced difference spectra (data not shown) indicated that the major CO binding pigment, and hence presumably terminal oxidase, was cytochrome a_3. In addition, and in agreement with earlier reports [11], a proportion of the *b*-type cytochrome with absorption maximum at 556 nm also reacted with CO, and therefore, could be considered as cytochrome *o* in the original terminology and definition of Castor and Chance [13]. However as shown in the next section there was no evidence from kinetic data to suggest that a *b*-type cytochrome was acting as a terminal oxidase.

The data presented in fig.1 support the idea that the respiratory chains of the plasma membrane of *P. denitrificans* and of the inner mitochondrial membrane are similar [1], and although the *b*-type cytochromes are somewhat different in their absorption properties it appears likely that they respond in a similar manner to the presence of antimycin A. It is not clear from the data of fig.1 how many *c*-type cytochromes are present in *P. denitrificans*, and in particular the original evidence for cytochromes c_1 in *P. denitrificans* [14] must be considered suspect, and indeed difficult to demonstrate conclusively except possibly by thermodynamic characterization, since its expected spectral properties would be similar to those of one of the *b*-type cytochromes.

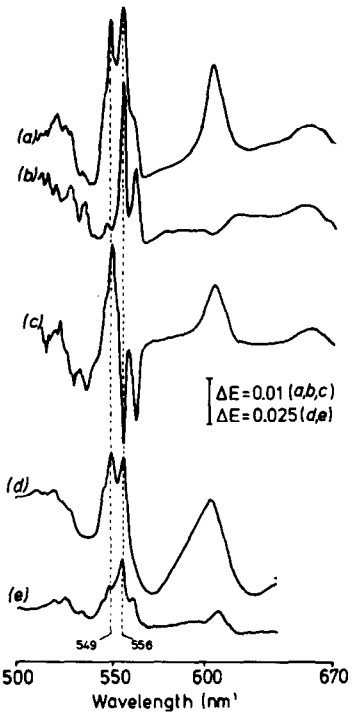

Fig.1. Low temperature difference spectra of particles from *P. denitrificans*. Difference spectra were recorded at 77°K after the following additions to the test and reference cuvettes: (a) NADH (1 mM) reduced minus H_2O_2 (2 mM) oxidized; (b) NADH (1 mM) + antimycin A (20 μg) reduced minus H_2O_2 (2 mM) oxidized; (c) NADH (1 mM) reduced minus NADH (1 mM) + antimycin A (20 μg) + H_2O_2 (2 mM) reduced; (d) L-ascorbate (0.7 mM) + TMPD (70 μM) reduced minus H_2O_2 (2 mm) oxidized; and (e) NADH (1 mM) reduced minus L-ascorbate (0.7 mM) + TMPD (70 μM) reduced. Protein concentrations were 10.0 mg protein/ml (curves a, b, c) or 8.1 mg protein/ml (curves d, e).

3.2. *Kinetic analysis of cytochrome redox changes*

Previous kinetic studies have been performed with *P. denitrificans* cells grown anaerobically in the presence of nitrate [15]. The data of fig.2 compare the kinetics of oxidation and re-reduction of membrane bound cytochromes in particles from aerobically grown *P. denitrificans.* Measurements at 605–625 nm (fig.2ii) showed that cytochromes $a + a_3$ were fully oxidized during the flow ($t_{1/2} < 3$ ms) and are kinetically competent to serve as a terminal oxidase.

Of the total extent of oxidation of cytochrome *c* resulting from mixing anaerobic particles with oxygen (fig.2iii), approx. 50% occurred during the flow and the remainder within 30 ms of the cessation of flow. There are two extreme interpretations of this kinetic behaviour. The first is that there was a single pool of cytochrome *c* that was oxidized with a half-time of about 3 ms. This would account for the 50% oxidation during the flow and although the oxidation of the remaining 50% should proceed with an identical half time the observed rate would be slowed by the 5 ms time constant in the measuring circuit. The other interpretation is that there are two distinct pools of cytochrome *c*, one mainly oxidized and the other mainly unoxidized during the flow. The former interpretation is supported by the monophasic kinetics of re-reduction of cytochrome *c* when the pulse of oxygen was exhausted (fig.2vi).

Two phases of cytochrome *b* oxidation were seen in measurements at 562–575 nm, the first occurred rapidly after the flow stopped ($t_{1/2} < 10$ ms) followed by a much slower oxidation ($t_{1/2} < 100$ ms), suggesting two kinetically distinguishable pools of cytochrome *b* (fig.2iv). This was confirmed by following the re-reduction kinetics after the exhaustion of oxygen (fig.2vii and viii). Of the two *b*-type cytochromes detected by low-temperature difference spectroscopy (fig.1) and from a comparison of the relative amounts of the slow and fast reacting cytochrome *b* components measured at different wavelength pairs (compare fig.3vii and fig.3viii), the *b*-type cytochrome with maximum absorption at 556 nm can be identified with the faster component, and the *b*-type cytochrome showing maximum absorption at 563 nm with the slower component. Significantly no *b*-type cytochrome was oxidized during the flow (the overshoot shown at the start of trace iv in fig.2 is probably due to slower flow at the onset of flow and can be disregarded) unlike previous studies with *Haemophilus parainfluenzae* [16] and *E. coli* [8], and so there is no kinetic evidence for cytochrome *o*, a *b*-type cytochrome with oxidase activity, in *P. denitrificans.*

3.3. *Stoicheiometry of respiration driven proton translocation*

Scholes and Mitchell [17] have shown previously that protons are ejected through the plasma membrane

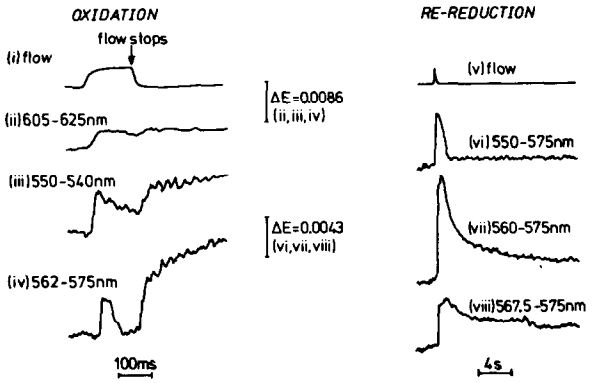

Fig.2. Kinetics of oxidation and reduction of membrane-bound cytochromes in aerobically-grown *P. denitrificans.* Stopped-flow dual-wavelength spectrophotometric measurements were made with an apparatus having a 17:1 mixing ratio for the relative volumes of delivery from the major and minor syringes, and a dead-time of 3 ms. The optical path of the observation chamber was 10 mm long. Information was recorded with a transient recorder, and, in parallel a pen recorder. For each experiment the major syringe contained particles (6.0 mg protein/ml, traces ii, iii, iv: 11.7 mg protein/ml, traces vi, vii, viii) suspended in 50 mM TES (pH 7.0), 50 mM KCl with 2 mM succinate-KOH (pH 7.0) to produce anaerobiosis and the minor syringe contained air saturated 50 mM TES (pH 7.0) 50 mM KCl, giving an oxygen concentration on mixing of 25 ng-atoms/ml. Spectrophotometric sensitivies and the time scales are shown by labelled bars, the temperature was 24°C, 0.75 ml of mixed reactants were used for each trace, and in each case an upward deflection of the trace indicates an oxidation of a particular cytochrome. The start and stop of flow are shown by the flow velocity traces (i) and (v) in which an upward deflection indicates increased flow velocity. Key to fig: (i) flow velocity trace for traces (ii), (iii), (iv); (ii) rapid kinetics of cytochrome $a + a_3$ oxidation; (iii) rapid kinetics of cytochrome *c* oxidation; (iv) rapid kinetics of cytochrome *b* oxidation; (v) flow velocity trace for traces (vi), (vii), (viii); (vi) kinetics of re-reduction of cytochrome *c* following oxidation; (vii) and (viii) kinetics of re-reduction of cytochrome *b* following oxidation at two different wavelength pairs. The measuring time constants were 5 ms (traces ii–iv) or 50 ms (traces vi–viii).

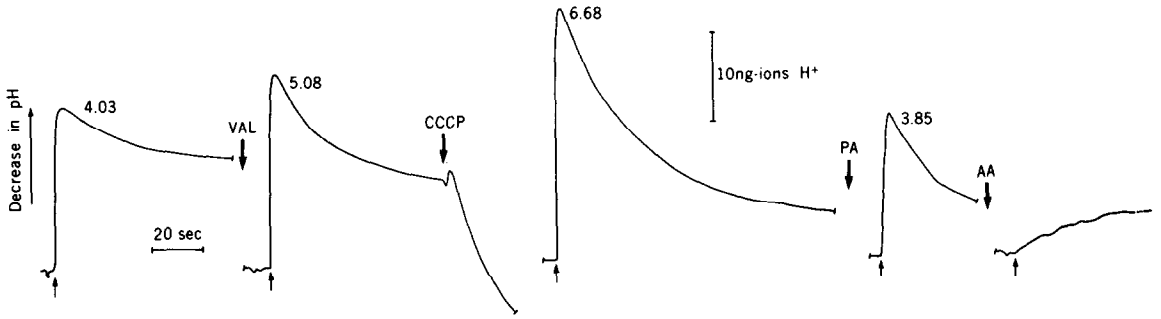

Fig.3. Respiration-driven proton translocation in intact cells of *C. denitrificans*. Cells (1.64 mg protein), grown with glycerol as carbon source were suspended in 1.0 ml of 0.3 M sucrose–150 mM KCl–1.5 mM glycyl/glycine buffer, pH 7 at 25°C, containing 5 mM glycerol. The incubation chamber was sealed and respiration proceeded until the oxygen content of the cell suspension was exhausted, during which time the pH fell to 6.7. After a further incubation period of 20 min, the anaerobic cell suspension was pulsed with oxygen by injecting 10 μl of air-saturated 150 mM KCl (4.7 ng-atoms of oxygen) with a microsyringe. The moments of injection are indicated in the figure. Further additions of valinomycin (VAL, 2 μg); CCCP (2×10^{-10} moles); piericidin A (PA, 6×10^{-9} moles) and antimycin A (AA, 1 μg) are indicated by the thick arrows. After each addition, an equilibration period of approx. 10 min. was allowed before pulsing with oxygen. The corrected $\rightarrow H^+/O$ ratio (see text) is given for each oxygen-pulse. The response of the pH electrode to changes of H^+ concentration in the bulk-phase was determined by injecting 4 μl of N_2-saturated 5 mM HCl in 150 mM KCl into the suspension.

of *P. denitrificans* during respiratory pulses and limiting $\rightarrow H^+/O$ quotients of 8 were obtained with cells oxidizing endogenous substrates. To investigate the stoicheiometry of proton translocation associated with the respiration of known added substrates we used a starvation technique previously developed for similar studies with *E. coli* [6]. A rapid acidification of the bulk-phase of the incubation medium was observed when a small amount of oxygen was rapidly introduced into a stirred anaerobic suspension of cells, that had been grown aerobically with glycerol as carbon source, starved and subsequently treated, as shown in fig.3. In the absence of added substrate (glycerol), an oxygen pulse failed to elicit a proton pulse with starved anaerobic cells. The plasma membrane of *P. denitrificans* is known to have a low proton conductance [18] and we observed a slow and partial decay of the proton pulse. In a KCl-containing incubation medium, the extent of proton ejection for a given amount of oxygen consumed (the $\rightarrow H^+/O$ ratio) was markedly increased in the presence of both the K^+-conducting ionophore valinomycin (2 μg/ml) and the proton-conducting agent, CCCP (0.2 μM). In the absence of a moving counter ion (in this case K^+), the opposing membrane potential that developed during the short burst of respiration tended to limit the extent of respiration-driven proton translocation [17]. The addition of a small amount of CCCP (0.2 μM final conc.) shortly after an oxygen pulse resulted in a collapse of the pH change (fig.3). In the presence of both valinomycin and CCCP, the pH change decayed exponentially with a half-time of 30 s. However, with increased membrane proton-permeability, the observed peak of acidification was under-estimated by an error that arose from the flow of protons back across the membrane during the non-instantaneous burst of respiration. We have made a correction for the slight under-estimation of the observed $\rightarrow H^+/2e^-$ ratio [17,19] by making semi-logarithmic plots of the decay-phase and extrapolating them back to the moment of oxygen addition. The corrected $\rightarrow H^+/O$ ratio associated with glycerol oxidation was 6.68 while after the addition of piericidin A, a site specific inhibitor of NADH dehydrogenase [EC 1.6.99.3] at this concentration, the $\rightarrow H^+/O$ ratio was 3.85. Further inhibition of respiration through the addition of antimycin A resulted in the virtual abolition of proton translocation associated with glycerol oxidation (fig.3).

By using cells grown with D,L-malate as carbon source, harvested in the late exponential phase of growth, we were able to extend these studies to include $\rightarrow H^+/O$ ratios associated with the oxidation of succinate and L-malate; the results are summarized

Table 1
Observed → H^+/O ratios and oxidase rates for D,L-malate grown *P. denitrificans* respiring different substrates

Substrate added	→ H^+/O	Oxidase activity (ng atom O/min/mg protein)
None (endogenous)	4.04 ± 0.29 (10)	40
Succinate (1 mM)	3.25 ± 0.16 (10)	172
Succinate (1 mM) + Piericidin A (0.24 μM)	3.67 ± 0.15 (3)	169
L-malate (1 mM)	4.61 ± 1.11 (8)	93
L-malate (1 mM) + Piericidin A (0.24 μM)	0.67 ± 0.35 (4)	< 5

Cells were grown in batch culture as indicated in Materials and methods with D,L-malate (0.5% w/v) as carbon source. Cells were harvested in the late exponential/early stationary phase of growth and starved of endogenous substrates by shaking in growth medium (containing no added carbon source) for 2 h at 30°C. Measurements of → H^+/O ratios and oxidase activities were performed as described in Materials and methods and the legend to fig.3. Values for → H^+/O ratios were obtained in the presence of valinomycin (4 μg/ml) and are presented as means ± S.D. with the number of observations in parentheses.

in table 1. The oxidation of succinate yielded an → H^+/O rate of between 3 and 4 whereas the oxidation of L-malate produced a value for the → H^+/O ratio close to 5 with a large standard deviation. Significantly the respiration and associated → H^+/O ratio of L-malate, but not succinate, was inhibited by piericidin A.

Because of the large variation in the observed → H^+/O ratio observed for different batches of cells oxidizing L-malate, we harvested cells at various stages in the growth cycle, subjected the cells to an identical starvation procedure, and compared the observed → H^+/O ratios for cells oxidizing both unknown endogenous substrates and added L-malate. The data are summarized in table 2 and clearly indicate that, although the rate of L-malate oxidation did not change appreciably during the growth cycle, the observed → H^+/O ratio gradually decreased from 8 to a value closer to 4.

We interpret these data to show: (a) the ubiquinol to cytochrome oxidase segment of the respiratory chain resembles that of mammalian mitochondria [20], is so arranged as to allow the translocation of 4 H^+

Table 2
Variation in the → H^+/O ratio associated with L-malate oxidation by batch grown *P. denitrificans* harvested at different points in the growth cycle

Harvest E_{420} (hours after inoculation)	Endogenous substrate(s)		L-malate (1 mM)	
	→ H^+/O	Oxidase activity (ng atom O/min/mg protein)	→ H^+/O	Oxidase activity (ng atom O/min/mg protein)
0.24 (3)	5.83 ± 0.39 (4)	62	7.89 ± 0.13 (6)	132
0.55 (4.5)	5.96 ± 0.79 (6)	41	7.80 ± 0.32 (7)	121
1.25 (6.5)	4.83 ± 0.82 (7)	51	7.07 ± 0.49 (8)	136
1.45 (11)	4.53 ± 0.08 (5)	42	6.03 ± 0.33 (7)	121
1.60 (24)	2.94 ± 0.19 (5)	73	3.51 ± 0.32 (6)	146

Cells from a 12 h stationary phase culture of *P. denitrificans* were added to a mineral salts medium containing D,L-malate (0.5% w/v) as carbon source to give an initial E_{420} of 0.07, and incubated at 30°C in shake flasks. Bacterial growth was measured turbidimetrically at 420 nm and at various times after inoculation cells were harvested, starved, and the → H^+/O ratios and oxidase activities determined as indicated in the legend to table 1. After a lag phase (1.5 h), growth was exponential for a further 4.5 h.

per O consumed, and is synthesized throughout the growth cycle of aerobically grown cells; (b) in cells harvested from the early exponential phase of growth the oxidation of L-malate proceeds via the reduction of both $NADP^+$ and NAD^+ and involves both a proton translocating transhydrogenase [EC 1.6.1.1] and a proton translocating NADH dehydrogenase activity [17]; and (c) in cells harvested from the stationary phase of growth, L-malate oxidation proceeds either primarily through an additional flavoprotein-linked dehydrogenase, which communicates directly with the ubiquinone pool and does not involve $NAD(P)^+$ reduction, or alternatively both the transhydrogenase and NADH dehydrogenase are modified in these cells such that neither activity results in net proton translocation. The second alternative appears more likely, since L-malate oxidation is still sensitive to inhibition by piericidin A in stationary phase cells as shown in table 2. The data also suggest, that sensitivity towards piericidin A and the occurrence of proton translocation associated with the NADH dehydrogenase are distinct and separate properties of this region of the respiratory chain. This is a point of some controversy in analogous studies with mitochondria derived from the yeast *Torulopsis utilis* where it has been claimed that these phenomena are either independent [7,21] or functionally related [22,23] properties of the NADH dehydrogenase. Interestingly, it is reported [22,23] that in *T. utilis* the presence of phosphorylation associated with the NADH dehydrogenase and piericidin A sensitivity of respiration are a property of mitochondria from cells harvested in the stationary phase of growth rather than of mitochondria from exponentially growing cells. Clearly further work with *P. denitrificans* will be of importance in resolving these controversies.

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