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Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*

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Spheroplasts from aerobically grown wild-type *Paracoccus denitrificans* cells respire with succinate despite specific inhibition of the cytochrome bc_1 complex by myxothiazol. Coupled to this activity, which involves only *b*-type cytochromes, there is translocation of 1.5–1.9 H^+/e^- across the cytoplasmic membrane. Similar H^+ translocation ratios are observed during oxidation of ubiquinol in spheroplasts from aerobically grown mutants of *Paracoccus* lacking cytochrome *c* oxidase, or deficient in cytochrome *c*, as well as in a strain of *E. coli* from which cytochrome *d* was deleted. These observations show that the cytochrome *o* complex is a proton pump much like cytochrome aa_3 to which it is structurally related.

Cytochrome *o* complex; Cytochrome-*c* oxidase; Electron transfer; Energy coupling; Proton translocation

1. INTRODUCTION

Bacterial respiration is coupled to generation of an electrochemical proton gradient across the cytoplasmic membrane. The gradient may result from scalar protolytic reactions at the inner and outer membrane surface, and/or proton translocation ('pumping') across the membrane [1].

Bacterial respiratory chains are usually branched and several terminal oxidases may be expressed simultaneously. Bacterial cytochrome aa_3 functions analogously to the mitochondrial enzyme [2]. Cytochrome *o* (*bo*) is an alternative oxidase containing *b*-type haem [3–5]. Different 'cytochrome *o*' complexes have been described, which vary at least with respect to their electron donor. The most studied is a ubiquinol:O_2 oxidoreductase of *E. coli*, which contains two *b*-type haems, one of which forms a bimetallic centre with copper, as in cytochrome aa_3 [6,7]. The primary structure of this enzyme shows strong homologies to cytochrome aa_3, particularly with respect to the largest subunit [8]. In cytochrome aa_3 this subunit is believed to bind both haem *a* and the bimetallic centre [9].

The ubiquinol-oxidising cytochrome *o* has generally been described as a scalar generator of an electrochemical proton gradient, releasing 1 H^+ (from oxidation of QH_2) on the outside, and taking up 1 H^+ from the inside of the cytoplasmic membrane, per electron transferred to O_2 [4–6,8,10]. In contrast, we show here that this enzyme functions as a proton pump, probably in much the same way as its structural relative, cytochrome aa_3.

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MX, myxothiazol; UQ-1, ubiquinone-1; QH_2, ubiquinol

2. MATERIALS AND METHODS

Wild-type *Paracoccus denitrificans* S1657 (obtained from Dr H.W. van Verseveld, Vrije Universiteit, Amsterdam) and ATCC 19367 (from Professor B. Ludwig, University of Lubeck) were grown on succinate according to Chang and Morris [11] in a 9 l fermentor (New Scientific Brunswick) at 30°C or room temperature in 3-l Erlenmeyer flasks containing 900 ml medium, with magnetic stirring and efficient aeration. The cytochrome *c*-deficient mutant, HUUG-25, and the mutant NS-3 of *Paracoccus* NCIB 8944, which lacks cytochrome *c* oxidase activity (both gifts from Dr H.W. van Verseveld), were grown in the same way as the wild types. *E. coli* RG 145 (cyd^-) (kindly provided by Dr R. Gennis, University of Illinois, Ur-

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bana) was grown aerobically in the same fermentor as above, at 37°C in minimal salt medium A [12], containing 1% (w/v) succinate, 10 mg/ml vitamin B_1 and 0.15% (w/v) yeast extract (Difco). All cells were harvested in the exponential phase (Klett no.100–200 at 690 nm).

Paracoccus spheroplasts were prepared as described by Lawford [13], using a cell suspension of 1 g wet wt/10–15 ml solution. The cell wall of the NS-3 mutant seemed to differ from that of the wild type, since a longer period of incubation with lysozyme (60 min) was necessary to form spheroplasts. *E. coli* spheroplasts were prepared as in [14], but at 100 mg cells/ml, and for a shorter period of incubation (15 min) with lysozyme (Sigma). The incubation was terminated by addition of 3 mM $MgCl_2$ and centrifugation at 4000 × *g*. In case some lysis occurred, as judged from release of DNA, spheroplasts were treated with DNase I (Sigma) and recentrifuged as above. All spheroplasts were resuspended in 300 mM sucrose, 150 mM KCl, 3 mM $MgCl_2$, 1.5 mM Hepes medium (pH 7.2) at a concentration of 1 g/1–2 ml.

Oxygen consumption was assayed polarographically at 25°C using a Clark oxygen electrode and a Johnson Research Foundation Workshops (PA, USA) amplifier. Spectrophotometry was carried out with a DBS-1 dual-wavelength spectrophotometer (Johnson Res. Found. Workshops), linked to a stirred, thermoelectrically regulated multipurpose cuvette [15].

The oxidant pulse method [16] was used to determine proton translocation, as described [17]. The reaction medium was composed of 100 mM KCl, 100 mM KSCN and 0.5 mM Hepes (pH 7.4). The pH of anaerobic spheroplast suspensions was kept within the range pH 6.5–7.5 by additions of acid or alkali.

FCCP and ubiquinone-1 were generous gifts from Dr P.G. Heytler and Hoffman-La Roche, respectively.

3. RESULTS

Spheroplasts from wild-type *Paracoccus* cells translocate 2.6–2.9 H^+/e^- linked to succinate oxidation by O_2 (fig.1A), confirming the earlier report by Lawford [13]. With ferricyanide as oxidant, accepting electrons at the cytochrome *c* level, the ratio fell by approx. 1 unit (fig.1B). This is similar to data obtained using isolated mitochondria [18], and confirms the report that cytochrome *c* oxidase of *Paracoccus* cells translocates protons with a comparable efficiency to that found for mitochondria [19], although lower efficiencies have been reported for reconstituted *Paracoccus* enzyme [20].

Oxygen consumption with succinate as substrate was reduced to 40–50% by myxothiazol (MX), a specific inhibitor of the cytochrome bc_1 complex, in the present wild-type *Paracoccus* spheroplasts (cf. [21]). Parsonage et al. [21] suggested that the remaining MX-insensitive activity takes place via an alternative oxidase, cytochrome *o*. In agreement, we found (not shown) that this activity was characterised by a high apparent K_m value for O_2 (3–4 μM), whilst the apparent K_m was below 1 μM in the absence of MX. It is interesting that in the absence of MX, the electron flux appears mainly to be channeled through a pathway involving the cytochrome bc_1 and aa_3 complexes.

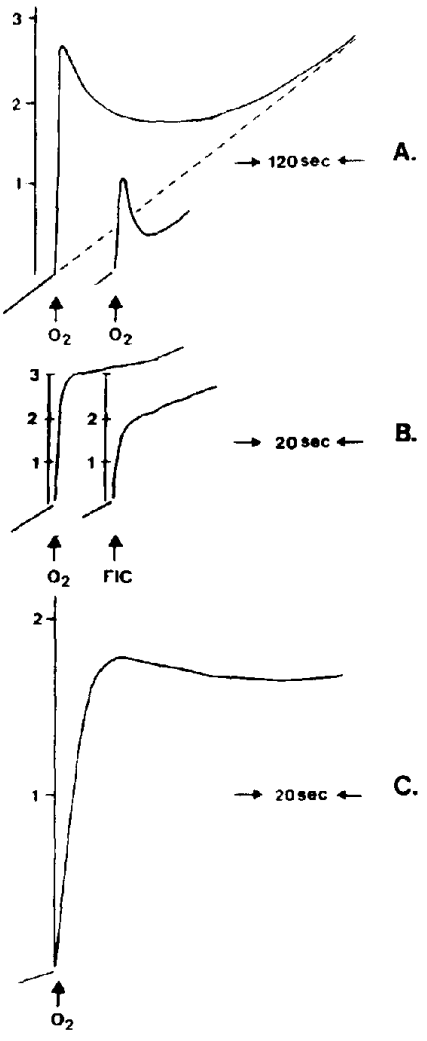

Fig.1. Proton translocation in wild-type *P. denitrificans*. 1.7 ml reaction medium (section 2), supplemented with 18 μM rotenone and 1.5 mM potassium succinate, was made anaerobic with a stream of Ar. ATCC 19367 spheroplasts (1.5 mg protein) were added and the system incubated anaerobically for 15 min. The ordinates indicate the H^+/e^- ratio [17]. (A) Injections of 0.645 nmol O_2 were made in the absence (left) and presence (right) of 0.7 μM FCCP. (B) As (A) without FCCP (left), and with 2.5 nmol anaerobic potassium ferricyanide (FIC) in place of O_2 (right). (C) 8.8 μM myxothiazol was added prior to injection of 2.58 nmol O_2.

Further support for the involvement of cytochrome *o* in MX-insensitive respiration was provided by the spectral responses of cytochromes upon anaerobiosis. Fig.2 shows that *b*-type cytochromes become reduced early, whilst reduction of cytochromes *c* and *a* lags behind. This indicates that the cytochrome *o* involved is of the quinol-oxidising cytochrome *b*-type (see section 4).

Fig.1C shows that the cytochrome *o* activity is coupled to translocation of >1 H^+/e^- in *Paracoccus* spheroplasts. The observed H^+/e^- ratio was dependent on the magnitude of the O_2 pulse, rising to maximal values near 2 (fig.3). In contrast, the H^+/e^- ratio for cytochrome bc_1 plus aa_3 activity behaved in the same way as in mitochondria (fig.3), being apparently lowered at larger O_2 pulses. This is due mainly to the enhanced velocity of H^+ backflux across the membrane that is difficult to account for quantitatively.

The odd O_2 dependence of the observed H^+/e^- ratio for the cytochrome *o* system is probably

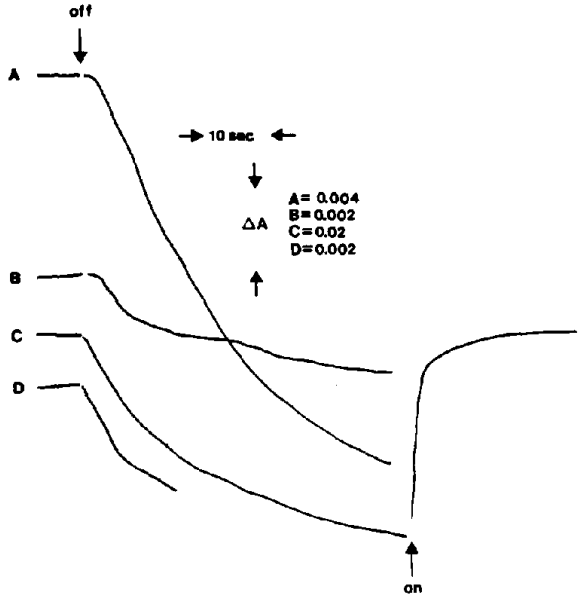

Fig.2. Reduction of cytochromes at anaerobiosis. S1657 spheroplasts (3 mg protein) were suspended in 2 ml deaerated 200 mM sucrose, 20 mM KCl, 20 mM Tris-Hepes medium (pH 7.2), supplemented with 1.5 mM succinate and 10 μM myxothiazol. Temperature, 25°C. Stirring of the cuvette contents introduced oxygen from a narrow port. Turning the stirrer off caused anaerobiosis and cytochrome reduction (downward deflections). (A) 550–540 nm (cytochromes *c*), (B) 605–630 nm (cytochrome *a*), (C) 430–410 nm (cytochromes *b*), (D) 562–575 nm (cytochromes *b*).

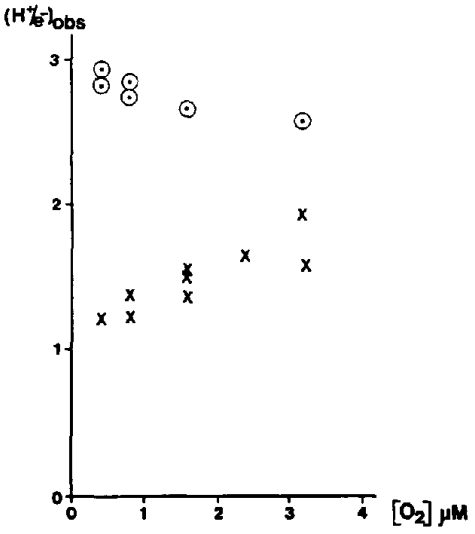

Fig.3. $[O_2]$ dependence of observed H^+/e^- ratio. See legend to fig.1. (Circles) Without myxothiazol; (crosses) with 6–9 μM myxothiazol.

related to the high K_m for O_2. The oxidant pulse method depends on a reasonably high affinity of the measured system for the oxidant [16]. At lower values of the O_2 affinity, the turning point of H^+ ejection is no longer a good estimate for the point of anaerobiosis, but reflects a point where the velocity of oxygen consumption (and linked proton translocation) is decreased due to lowered $[O_2]$, to an extent such that it equals proton backflux. Consequently, division of total proton ejection with total *added* O_2 underestimates the true H^+/e^- ratio. The extent of this error will decrease for larger O_2 pulses. It also follows that H^+ translocation, as measured via the O_2-pulse technique, will be especially vulnerable to high membrane permeability to protons due to the high K_m for O_2 (see section 4).

Oxygen consumption with ubiquinol-1 as substrate is faster than with succinate. Most of the proton-translocation experiments using mutants were therefore performed with UQ-1, which was kept reduced with dithiothreitol. In strain HUUG-25 of *Paracoccus* (which we find does contain some cytochrome *c*, not shown), we confirmed the absence of MX-sensitive respiration [21]. Proton translocation was similar to that in the MX-supplemented wild type (table 1).

H^+ translocation with the cytochrome *d*-

Table 1

Proton translocation coupled to oxidation of ubiquinol-1

	H^+/e^-
P. denitrificans	
HUUG-25	1.4–1.9
NS-3	1.5–1.9
E. coli	
cyd^-	1.7–1.9

Basic experimental procedure was as described in section 2 and the legend to fig.1. The medium was supplemented with 2.5–4 mM dithiothreitol and 30–125 μM ubiquinone-1, replacing succinate. O_2 pulses ranged between 1.29 and 5.16 nmol. In the *E. coli* experiments the medium was further supplemented with 100 mM sucrose, 3 mM $MgCl_2$, and 1.6 μg/ml valinomycin. The range of observed H^+/e^- ratios is listed for each source of spheroplasts

depleted strain of *E. coli* was observed to proceed in a rapid and a slow phase in the usual KSCN-supplemented medium (not shown). The slow phase was most likely due to insufficient electrical charge compensation, because the response was converted to a single fast phase (cf. fig.1) on addition of valinomycin. No endogenous respiration was observed, which leads one to ascertain that oxidation of ubiquinol through the cytochrome *o* system is being studied exclusively.

The NS-3 strain of *Paracoccus*, which lacks cytochrome aa_3 as judged from spectroscopy, behaved similarly to the *E. coli* spheroplasts, but exhibited some rotenone-insensitive endogenous respiration.

Table 1 shows that the H^+/e^- ratios observed with spheroplasts from these organisms were comparable to those of wild-type *Paracoccus* supplemented with MX. Taken together, the results indicate that the *b*-type ubiquinol-oxidising cytochrome *o* catalyses translocation of up to 2 H^+/e^- across the cytoplasmic membrane.

4. DISCUSSION

Although the cytochrome *o* complex is the most widespread of the bacterial cytochrome oxidases [4], its role in energy conservation has remained uncertain (see section 1). The present data demonstrate that the ubiquinol-oxidising *b*-type cytochrome *o* functions as a proton pump in both *P. denitrificans* and *E. coli* cells. This function is closely analogous to that of cytochrome aa_3 [19,20,22,23], except that the latter exhibits no scalar proton release, since cytochrome *c* is the electron donor rather than ubiquinol.

A previous study of cytochrome *o* in *E. coli* cells did not report proton pumping [24]. However, the H^+/e^- ratio for succinate oxidation was significantly above unity, without correction for proton backflux. In addition, cytochrome *d* might have been present, competing with cytochrome *o* [6]. Studies of *E. coli* membrane vesicles [25], and of purified cytochrome *o* reconstituted into proteoliposomes [10,25], also indicated only scalar protolytic properties. The reason for this might primarily be high membrane permeability for H^+, which depresses observed H^+ translocation especially for a 'high-K_m' enzyme (see section 3). Adverse changes in the protein during isolation are also possible. It may be noted that the first studies of reconstituted *Paracoccus* cytochrome aa_3 also failed to reveal proton translocation (Sigel, E. et al., quoted in [26]), although this was subsequently observed in both intact cells [19] and proteoliposomes [20]. In view of the present results, it is obvious that the functioning of cytochrome *o* in reconstituted proteoliposomes should be further tested.

Since a ubiquinol-oxidising cytochrome *o* has not so far been isolated from *Paracoccus* it is important to establish whether this enzyme is indeed involved in the present work. The measured activity appeared to involve *b*-type cytochromes only. It showed an elevated K_m for O_2, similar to that reported for the *E. coli* enzyme [5], and the data were analogous for *Paracoccus* mutants that oxidise ubiquinol through a pathway not involving the cytochrome bc_1 or aa_3 complexes. In our hands, the NS-3 mutant is, in fact, entirely devoid of *c*-type cytochromes (cf. [27]). EPR studies of this mutant showed a low-spin haem signal practically identical to that of *E. coli* cytochrome *o* (Haltia, T., unpublished). Taken together, this evidence strongly supports our conclusion.

Based on growth-yield studies [28,29] it has been proposed that oxidation of ubiquinol through the cytochrome *o* pathway of *Paracoccus* is associated with translocation of two electrical charges per e^-, which agrees with the present observations. However, in the past it was assumed that this pathway involves the cytochrome bc_1 complex,

which was taken to explain the high charge translocation ratio (see, e.g. [19,28]). Yet, Willison and Haddock [29] and Parsonage et al. [21] showed that the bc_1 complex is not involved in this pathway. To account for the growth-yield data, they suggested that cytochrome *o* might be proton-pumping, as we have demonstrated here.

The structural analogies between cytochromes *o* and aa_3 (see section 1) can now be complemented by an analogous energy-conserving function. This opens up interesting comparisons between the two enzymes, which may help in narrowing down the possibilities of proton-translocating mechanisms.

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