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Electron transport reactions in a cytochrome *c*-deficient mutant of *Paracoccus denitrificans*

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A mutant of *Paracoccus denitrificans* which is deficient in *c*-type cytochromes grows aerobically with generation times similar to those obtained with a wild-type strain. The aa_3-type oxidase is functional in the mutant as judged by spectrophotometric assays of cytochrome *c* oxidation using the membrane particles and cytochrome aa_3 reduction in whole cells. The cytochrome *c* oxidase (aa_3-type) of the *c*-less mutant oxidizes soluble cytochrome *c* at rates equivalent to those obtained with the wild-type. NADH and succinate oxidase activities of the membrane preparations of the mutant and wild-type are also comparable in the absence of detergent treatment. Exogenous soluble cytochrome *c* can be both reduced by NADH- and succinate-linked systems and oxidized by cytochrome aa_3 present in membranes of the mutant strain. Rapid overall electron transport can occur in the *c*-less mutant, suggesting that reactions result from collision of diffusing complexes.

Introduction

The question of the role of *c*-type cytochromes in electron transport reactions is currently under active discussion. Mitochondrial electron transport reactions are dependent on a loosely-bound cytochrome *c*, homologous in structure to the soluble *c*-550 of *Paracoccus* or the c_2 of some photosynthetic bacteria (for review, see Refs. 1, 2). In some bacterial systems only tightly bound *c*-type cytochromes are present. The electron transport system in aerobically-grown *Paracoccus denitrificans* [3,4] is an interesting one for these studies since it has both a soluble, periplasmic *c*-550 of 14.5 kDa, +227–260 mV, p*I* 4.5 [5–7] and a tightly bound cytochrome *c*-552, of 22 kDa, +190–244 mV, p*I* 3.5 [6,8,9]. It contains bc_1 and *c*-552-aa_3 complexes which together form the ubiquinol oxidase [8]. The cytochrome bc_1, NADH- and succinate-ubiquinone reductases, and cytochrome aa_3 complexes have been purified [10–13]. The electron transport components of *Paracoccus* are strikingly similar to those of the mitochondrial system [14].

A number of bacteria which have mutations resulting in deficiency of all *c*-type cytochromes or of soluble cytochrome *c* have been observed to grow aerobically with similar generation times [15–17]. Willison and John [18] measured oxidation and reduction of cytochromes in a *c*-deficient mutant of *Paracoccus*. We have examined some electron transport reactions of a mutant of *P. denitrificans* which can synthesize no *c*-type cytochromes but can make the other electron transport pigments, examining reactions of the whole electron transport chain and of reactions with added soluble cytochrome *c* of *Paracoccus* and of bovine heart. These studies give some new ideas of the functioning of membrane-bound electron transport systems.

Materials and Methods

Growth of Bacteria. Cytochrome *c*-deficient ((HUUG-25 from Henk van Verseveld, [19]) and wild-type (American Type Culture Collection (ATCC) 13543 and National Collection of Industrial Bacteria, Aberdeen, Scotland (NCIB) 8944 (parent of HUUG-25 from Henk van Verseveld)) strains of *P. denitrificans*

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; YEPD, yeast extract/peptone/dextrose; SDS, sodium dodecyl sulfate.

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were grown aerobically in yeast extract/peptone/dextrose (YEPD) (Difco) [4] or succinate [20] media at 30 °C, shaken at 250 rpm to late log phase (16–22 h, 250 Klett units) according to Bolgiano et al. [21]. These cells could be maintained in a functional state when stored at −20 °C for several weeks.

Growth rate measurements. The generation times of the wild-type and cytochrome *c*-deficient strains were determined by measuring growth in glucose- and succinate-based media. Cells were inoculated (1% into 50 ml media) into 250 ml baffled side arm flasks fitted with a cotton and wire top. The turbidity of the inoculated cultures at time of inoculation was measured and subtracted from later values. The cultures were incubated as above and the turbidity was measured hourly during the logarithmic phase using a Klett colorimeter.

Depletion of cytochrome c. Soluble cytochrome *c* was removed from cells by washing them according to the method of Scholes et al. [5]. Rapidly respiring cultures grown aerobically in YEPD media (400 ml in 3-l baffled flasks) were harvested by centrifugation (10000 rpm, 15 min, GSA rotor). 'Unwashed' control cells were resuspended in 50 mM Tris-maleate, 2 mM EDTA, pH 7.0 (10 ml/100 ml original culture volume) and stirred at 4 °C during the procedures with the 'washed' cells. The cells to be washed were suspended in 10 mM potassium phosphate pH 7.0 (15 ml/100 ml original volume) and stirred for 1 h at 4 °C. Following the incubation, the cells were again pelleted and resuspended in 0.5 M KCl, 10 mM potassium phosphate, pH 7.0 (4 ml/100 ml original suspension). After stirring at 4 °C for 1 h, the suspension was again centrifuged and the 'washed' cells were resuspended to 1/10 original volume in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0). The respiration of washed and unwashed cells was measured on the same day.

Supernatant fractions from the washed cells were clarified by an additional centrifugation and then concentrated on an Amicon PM-10 membrane. SDS-polyacrylamide gel electrophoresis [22] of this supernatant fraction revealed a single band staining for covalently-bound heme, using tetramethylbenzidine [23], of M_r 14.5 kDa, co-migrating with purified cytochrome *c*-550 of *Paracoccus*. Dithionite reduced minus ferricyanide oxidized difference spectra of the same fraction showed a single α-peak at 550 nm.

Enzyme preparations. Cell membranes were prepared from lysozyme treatment and osmotic lysis using the method of Scholes and Smith [4] and were stored in a frozen state at −20 °C. The membranes were treated just prior to assay with an amount of 10% deoxycholate, usually 0.5–1 mg detergent per mg protein, to give a maximal stimulation of oxidase activity [24] when assayed with an oxygen electrode using TMPD and ascorbate [25]. When treated directly with detergent in this manner, membranes had higher oxidase activity with deoxycholate than dodecyl maltoside; however, when extracted with detergent [10], dodecyl maltoside extracts of membranes yielded higher oxidase rates than membranes extracted with deoxycholate. Purified cytochrome aa_3 was prepared as described in Ref. 21. Briefly, fractions containing membrane-bound cytochrome *c* and cytochrome oxidase (*c*-552-aa_3 complex), described by Berry and Trumpower [8], were obtained from Jeffrey Pennoyer and Xiaohang Yang and were incubated in a high dodecyl maltoside-containing buffer and chromatographed on a DEAE-CL6B (Sigma) column. Prior to assay, the enzyme preparations (stored at −20 °C in 50% glycerol) were either diluted with cold distilled H_2O or treated with asolectin as described [21].

Cytochromes c. Bovine heart cytochrome *c* (Type V) was obtained from Sigma and *Paracoccus* cytochrome *c*-550 was prepared by the methods of Scholes et al. [5]. The concentrations of the cytochrome *c* were determined spectrophotometrically using the appropriate absorption coefficients: $\varepsilon_{550(red)} = 27.6\ cm^{-1} \cdot mM^{-1}$ for bovine [26] and $\varepsilon_{550(red)} = 26.8\ cm^{-1} \cdot mM^{-1}$ for *Paracoccus* [5] cytochromes *c*-550. For use in the spectrophotometric assay, cytochromes *c* were reduced by the addition of a minimal quantity of sodium borohydride (Sigma, 27). The solution was chromatographed on a Sephadex G-25 (Pharmacia) column with 50 mM Tris-maleate, 2 mM EDTA (pH 7.0). Cytochrome *c* preparations were stored at −20 °C.

Spectrophotometric assays. Cytochrome *c* oxidase and reductase were assayed spectrophotometrically at 550–540 nm [28,29] using the turbine-driven, time-sharing, multichannel spectrophotometer built by the Johnson Foundation, University of Pennsylvania, Philadelphia, PA [30]. The activities were expressed as first-order rate constants, k_{obs} [28] and calculated by a least-squares regression method. For the reductase assays the *k* values from the first-order portions of the traces were calculated. Reductase assays were done in the presence of 0.4 mM KCN, and 0.36 mM NADH or 5 mM succinate were used as substrates. Assays were run in 50 mM Tris-maleate buffer, 2 mM EDTA (pH 7.0), 26 °C.

Oxygen electrode assays. NADH, succinate, and cytochrome-*c* oxidase activities were measured using an oxygen electrode (2.6 ml total chamber volume) as described in Ref. 25 at 26 °C in the buffer system used above. The order of addition for the NADH and succinate oxidase assay was as follows: (1) buffer, 50 mM Tris-maleate, 2 mM EDTA (pH 7.0), (2) enzyme, (3) 2 mM NADH or 20 mM succinate, and (4) when added, bovine or *Paracoccus* cytochrome *c* (0.4–0.8 μM). To activate the succinate dehydrogenase prior to the succinate oxidase assay, the membrane preparation was incubated at 26 °C for 20 min in the presence of 20 mM succinate [31]. Cytochrome oxidase was assayed in the presence of 0.7 mM TMPD and 10 mM ascorbate, and

in the case of the wild-type membranes which contain endogenous cytochrome *c*, the rate resulting (endogenous rate) from TMPD and ascorbate is subtracted from the total rate obtained upon the addition of bovine cytochrome *c* (0.2–10 μM) to give the exogenous rate (due to added soluble *c*). Rates are expressed as μM O_2 uptake per s.

Respiration of intact cells. The respiration of cell suspensions was also measured using the oxygen electrode. Cells were incubated at 26 °C and vortexed for 45 s prior to adding to the 50 mM Tris-maleate, 2 mM EDTA (pH 7.0) in the chamber. The time for the stirred suspension to become anaerobic (t_{an}) was measured and expressed as minutes.

Absorption spectrum measurements. The dithionite-reduced minus oxidized and CO + dithionite-reduced minus dithionite reduced difference spectra of cells, membranes and preparations were obtained by scanning from 650 to 400 nm with the Hitachi dual wavelength spectrophotometer. Membranes and purified preparations were diluted in 50 mM Tris-maleate, 0.1% dodecyl maltoside, 2 mM EDTA (pH 6.9). Whole cell suspensions were diluted 1 : 1 with glycerol. For the CO spectra, samples were bubbled with 20–40 bubbles of CO and immediately covered with aluminum foil and placed on ice just prior to recording spectra. Calculations of cytochrome concentrations were made using the absorption coefficients for wavelength pairs of cytochromes *b* and *c* according to Williams [32,33]. The concentration of cytochrome aa_3 was determined using $\varepsilon_{605-630(red)}$ of 11.7 $cm^{-1} \cdot mM^{-1}$ [11]. Reduction of cytochromes *a* and *c* was measured on anaerobiosis using mutant and wild-type cells or membrane vesicles in the presence of NADH or succinate using the Hitachi 557 recording spectrophotometer in the dual wavelength mode using conditions described in the figure legend.

Analytical procedures. Protein was determined by the BCA (Pierce) [34] or modified Lowry [35] procedures.

Materials. Dodecyl maltoside from Boehringer Mannheim; TMPD from Kodak; electrophoresis chemicals from Biorad; and all other reagents from Sigma.

Results

Growth of bacteria

The cytochrome *c*-deficient strain used in these studies was shown to lack all *c*-type cytochromes [19]. Cytochrome *c*-deficient strains of *Paracoccus*, independently isolated by other investigators [18,36], also appear to contain no cytochrome *c* as evidenced by low-temperature difference spectra of cells and membranes. In spite of the cytochrome *c* deficiency, generation times determined from growth of the wild-type and mutant examined here in succinate- or glucose-based media under aerobic conditions (Table I) are about the same.

TABLE I

Growth rates for wild-types and cytochrome c-deficient strains of Paracoccus denitrificans

Cultures were inoculated (1%) from a log culture and were grown at 30 °C, 250 rpm. YEPD, yeast extract/peptone/dextrose [4]. Succinate medium was made according to the recipe of Chang and Morris [20]. n.d., not determined.

Strain	Generation time	
	YEPD medium	Succinate medium
Wild-type (NCIB 8944)	108 min	114 min
(ATCC 13543)	n.d.	94 min
Mutant (HUUG-25)	141 min	91 min

Purified cytochrome oxidase

We have purified the cytochrome aa_3-type oxidases from both wild-type (ATCC 13543) and mutant strains of *Paracoccus* and have found them to contain the same 3-major subunit composition [21], although they show slightly different peaks in reduced minus oxidized difference spectra (Fig. 1). The absorption peaks of the wild-type oxidase in the reduced form are at 607, 580 and 446 nm in agreement with Ludwig and Schatz [11]. The isolated oxidase from the mutant, however, has slightly different aa_3 peaks (605 and 445 nm) and evidence for a small amount of a *b*-type (560 nm) cytochrome. The mutant preparation also has an additional CO-binding component (peak at 418 in CO-reduced minus reduced spectra) compared to wild-type in Fig. 1. The cytochrome-*c* oxidase activity of this preparation, tested polarographically with 0.4 μM bovine cytochrome *c*, was totally inhibited by 50 μM KCN.

NADH and succinate oxidase activities

Membrane preparations from both wild-type and mutant cells possessed NADH and succinate oxidase activities, measured polarographically (Table II). As found previously [31,37], O_2 uptake with NADH or succinate in the wild-type was not increased on addition of soluble bovine cytochrome *c*. The same was true for *Paracoccus* cytochrome *c*-550 (data not shown). Similarly the NADH oxidase of the mutant membranes was not stimulated by soluble cytochrome *c*; however, the succinoxidase was. The effect of detergent treatment on wild-type and mutant membranes was very different (Table II), both the NADH and succinate oxidase of wild-type being increased by this treatment, while that of the mutant was decreased. The activities of the two oxidases in untreated membranes was rather similar in mutant and wild-type, when expressed per milligram of protein, but detergent-treated wild-type membranes showed considerably higher NADH oxidase. The lower succinoxidase may be a reflection of the lability of the succinate dehydrogenase during the preparation of the

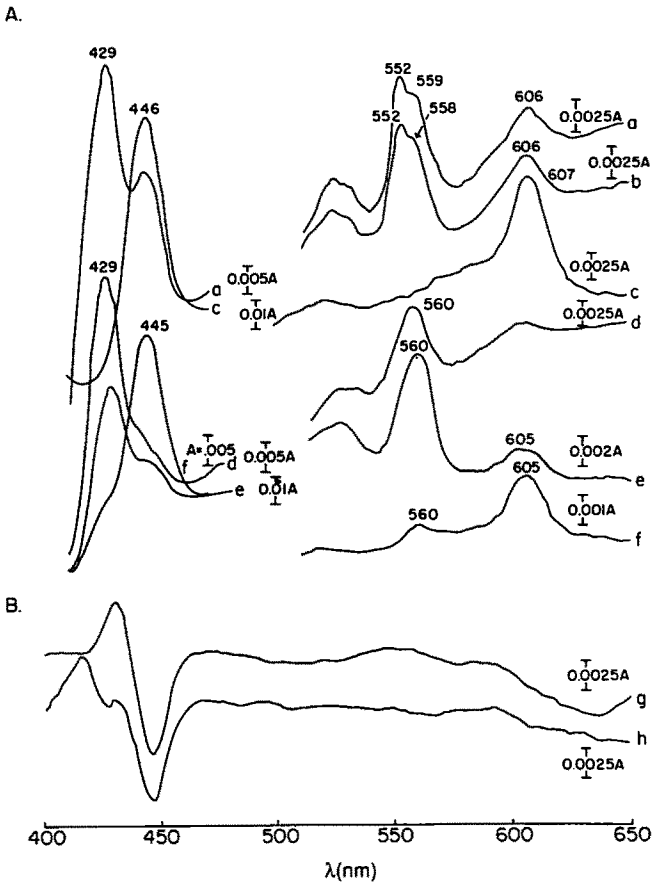

Fig. 1. Difference spectra of cells (a, d), membranes (b, c) and purified cytochrome oxidase (c, f, g, h) from wild-type (a–c, g) and cytochrome *c*-deficient (d–f, h) strains of *Paracoccus*. (A) Dithionite-reduced minus ferricyanide-oxidized spectra and (B) CO + dithionite-reduced minus dithionite-reduced difference spectra were recorded by scanning from 650 to 400 nM at room temperature.

membrane suspensions as seen in bovine heart mitochondria [38].

Evidence that the soluble *c*-550 is not involved when cells are respiring (in this case from endogenous substrate) is seen from the results of Table III. In experiments in which 25–30% of the cytochrome absorbing at 550 nm (which thus includes an absorption contribution from cytochrome *c*-552) was washed out of the cells (14.5 kDa co-migrating with purified cytochrome *c*-550 with absorption peak at 550 nm), it was found that respiration, as measured with an oxygen electrode, was unaffected (Table III). This procedure appears to remove outer membrane material from *Paracoccus*, while leaving cells otherwise unaltered [39].

TABLE III

Effect of depletion of cytochrome c-550 on Paracoccus (NCIB 8944) respiration

The respiration of washed and unwashed wild-type *Paracoccus* (NCIB 8944) were assayed using an oxygen electrode at 25 °C (total volume, 2.6 ml). The washed cells had lost 25–30% of the cytochrome *c*-550 when compared to unwashed cells. The estimate of the cytochrome *c* washed out is conservative due to the overlapping absorbances of the two other *c*-type cytochromes. The respiration is expressed as the total time (min) for the suspension to become anaerobic (T_{an}). The absorbances (A) of the cells (7.5 × diluted) were measured by a Klett colorimeter (green filter).

	Experiment 1		Experiment 2	
	$1/T_{an}$ (min)	A (Klett units)	$1/T_{an}$ (min)	A (Klett units)
Unwashed cells	0.114	238	0.185 [a]	228
Washed cells	0.100	233	0.161 [a]	205
% Respiration retained in *c*-depleted cells	88		87 [a]	

[a] Cells were diluted 1.75× into the 50 mM Tris-maleate, 2 mM EDTA (pH 7.0) assay buffer.

Oxidation and reduction of soluble cytochrome c

Membrane vesicles and purified oxidase and reductase of wild-type *P. denitrificans* can oxidize and reduce both its own soluble cytochrome *c* and that of

TABLE II

Effect of bovine cytochrome c on NADH, succinate and cytochrome-c oxidase activities of Paracoccus membranes: comparison of wild-type and cytochrome c-deficient strains

The assays were done polarographically in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0). The following amounts of substrate were used for the appropriate assays: 1 mM NADH, 20 mM sodium succinate, 0.7 mM TMPD and 9.3 mM ascorbate, and 0.4 μM bovine cytochrome *c*. Prior to succinate oxidase assays, the membrane preparation was incubated at 26 °C. for 20 min in the presence of 20 mM succinate. Membranes were incubated with an amount of deoxycholate that gave maximal stimulation (0.5 or 1 mg detergent/mg protein) and immediately diluted with cold H_2O.

Prepn.	Deoxycholate	O_2 uptake (μm O_2/s per mg)					
		NADH oxidase		Succinate oxidase		Cytochrome oxidase	
		−c	+c	−c	+c	−c	+c
Mutant	−	0.217	0.217	0.068	0.163	0	0.177
	+	0.011	0.076	0.059	0.059	0	0.669
Wild-type	−	0.149	0.149	0.048	0.048	2.89	3.33
	+	1.780	1.780	0.056	0.056	2.89	3.56

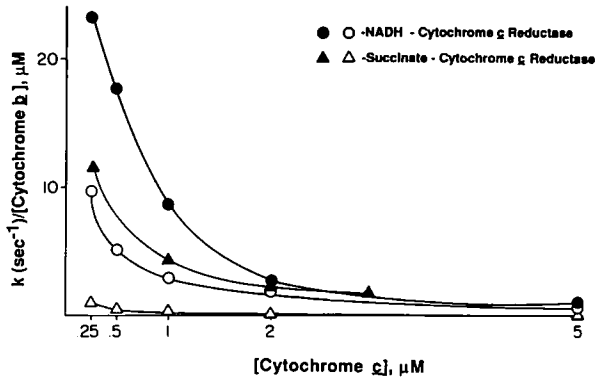

Fig. 2. Comparison of NADH (●, ○) and succinate (▲, △) cytochrome-c reductase activities using detergent-treated membrane preparations from wild-type (●, ▲) and cytochrome *c*-deficient (○, △) *Paracoccus*. First-order rate constants ($k(s^{-1})$/[cytochrome *b*] (μM)) were calculated from spectrophotometric data. The buffer used was 50 mM Tris-maleate, 2 mM EDTA (pH 7.0). Assays were performed at 26 °C using 0.36 mM NADH or 5 mM succinate and bovine cytochrome *c* in the presence of 0.4 mM KCN.

bovine heart mitochondria [10,11,27]. The spectrophotometric assays of Figs. 2 and 3 show that mutant membranes have the same type of activities despite the lack

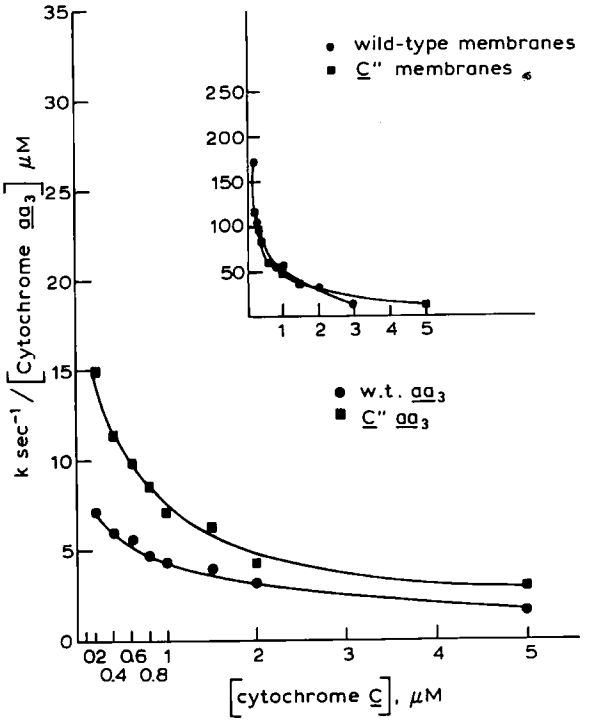

Fig. 3. First-order rate constants for the oxidation of bovine cytochrome *c* using *Paracoccus* oxidase from purified preparations or membrane particles (inset) of cytochrome *c*-deficient (■) and wild-type (●) strains measured spectrophotometrically. Data are expressed as rate k (s^{-1})/[cytochrome aa_3] (μM). Assays were run at 26 °C. in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0). Membrane fragments were treated with deoxycholate (0.5 mg/mg protein for the mutant and 1 mg/mg for the wild-type). The following amounts of aa_3 (μM) were used in the assays: $3.0 \cdot 10^{-3}$ (wild-type, purified); $1.79 \cdot 10^{-3}$ (mutant, purified); $5.83 \cdot 10^{-5}$ (wild-type, membranes); and, $4.25 \cdot 10^{-4}$ (mutant, membranes).

of cytochromes *c*-552 and c_1. Plots of rate k vs. concentration of bovine cytochrome *c* show a decrease of rate k with increasing cytochrome *c*, as seen with wild-type *P. denitrificans* [21] and bovine [28,29] enzymes. The rates with the reductases of the mutant are lower than those with wild-type, when expressed in terms of cytochrome *b*. The oxidase activity with bovine cytochrome *c* of the wild-type and mutant membrane fragments (assayed spectrophotometrically) are similar when expressed per cytochrome aa_3 (Fig. 3). The oxidase activities of purified cytochrome aa_3 are also similar, although approx. 60% of the activity is lost during the purification procedure when measured with the addition of asolectin [21].

As seen in Table II, the oxidase activity of wild-type membranes with TMPD plus ascorbate is high with the endogenous cytochrome *c* and is only a little higher on addition of soluble exogenous bovine cytochrome *c*. The increase due to the reaction with exogenous cytochrome *c* is about equal to the rate of the oxidase of the mutant membrane with added cytochrome *c* (Fig. 4).

As with the wild-type membranes [27], the activity of the *Paracoccus* oxidase in purified or membrane preparations with its own cytochrome *c*-550 was much lower than that with bovine cytochrome *c* (Fig. 4).

Overall electron transport in intact cells and membranes

We also measured the reduction of cytochrome aa_3 upon the exhaustion of O_2 due to endogenous respira-

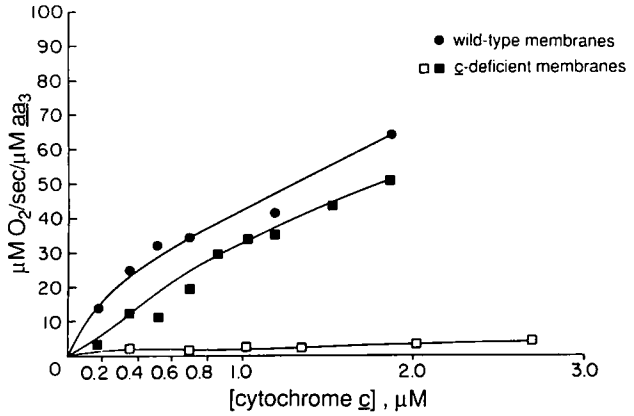

Fig. 4. Comparison of cytochrome oxidase activity from wild-type (●) and cytochrome *c*-deficient (■, □) mutant strains of *Paracoccus* measured polarographically. Data are expressed as rates in the form of oxygen uptake (μM O_2/s per μM aa_3) resulting from the addition of soluble cytochrome *c*-550 of bovine heart (■, ●) or *Paracoccus* (□). When assaying wild-type membranes, the oxidase rate from addition of soluble *c*, as plotted, was obtained by subtracting the rate due to the presence of endogenous cytochrome *c* (97.4 μM O_2/s per μM aa_3) from the total (endogenous + exogenous) rate. The oxidase rate of wild-type membranes with *Paracoccus* cytochrome *c*-550 was negligible. Assays were run in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0), 26 °C with 0.75 mM TMPD and 10 mM ascorbate. The concentration of aa_3 (μM) in the preparations assayed were: $4.37 \cdot 10^{-3}$ (wild-type membranes) and $5.31 \cdot 10^{-3}$ (mutant membranes). The ratio of cytochrome *a*: *c* in the wild-type membranes was approximately 1 : 1.

tion in whole cells, and in the presence of succinate in cytochrome *c*-deficient membranes (Fig. 5). The reduction of cytochrome aa_3 is rapid and complete (no further increase with dithionite). A somewhat slower rate of cytochrome aa_3 reduction in another cytochrome *c*-less (N7) mutant has been reported previously by Willison and John using NADH and succinate as substrates for membrane particles [18].

The reduction of separate pools of *c*-type cytochromes in wild-type cells could be measured by following absorbance at 550 or 552 nm (traces a and b). Both populations go rapidly reduced (up to 90–95% of their total reduction) without the addition of substrate. Adding NADH or succinate increases the absorbance so that there is no effect with dithionite. The cytochromes measured by the trace at 552 begin to go reduced upon anaerobiosis (measured independently by O_2 uptake of the suspension), at the same time that the cytochrome *a* does, while the pool at 550, which would be predominantly the soluble *c*-550, begins to go reduced later, when the aa_3 reduction is almost complete. This provides additional evidence that the reaction of *c*-550 with the oxidase can be distinguished from that of *c*-552 with the oxidase. Lawford et al. [40] using stopped-flow techniques to study steady-state kinetics of *Paracoccus* suspensions found evidence of two pools of cytochrome *c*: one which appeared to be non-reactive and another which was rapidly reduced at anaerobiosis.

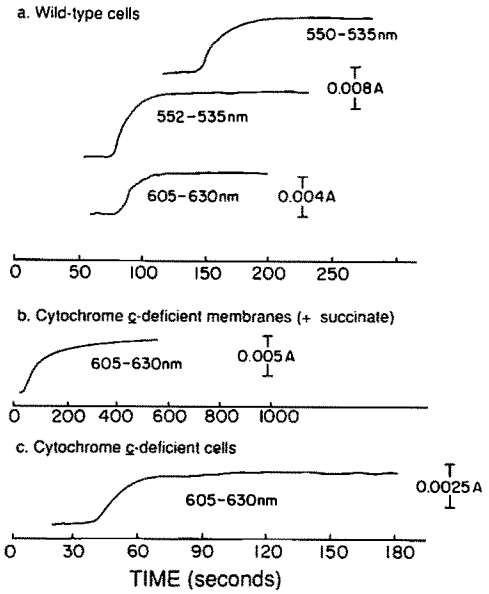

Fig. 5. Reduction of cytochromes *a* (605–630 nm) and *c* (550 or 552–535 nm) from wild-type cells (a) and cytochrome *c*-deficient (c) cells and cytochrome *c*-deficient membranes (b) of *Paracoccus* on anaerobiosis. 1 ml suspension of cells (50% glycerol, 25 mM Tris-maleate (pH 7.0)) or membrane fragments (25 mM sucrose, 10 mM potassium phosphate (pH 7.6)) was aerated by shaking the cuvette and the absorbance change was monitored as the cytochromes became reduced during anaerobiosis with an Hitachi 557 spectrophotometer. For the cytochrome *c*-deficient sample, 20 mM sodium succinate was added prior to aerating the cuvette.

Discussion

Since the aerobic growth rate of the cytochrome *c*-deficient mutant of *Paracoccus* with succinate or glucose is the same as that of the wild-type (Table I), the energy-yielding reactions of the mutant are not rate-limiting. The NADH and succinate oxidase activities of membrane fragments of wild-type and mutant organisms are also similar in the absence of detergent treatment.

There is some suggestion that the mutant utilizes an alternative oxidase, such as cytochrome *o* which may be directly reduced by ubiquinone, or cytochrome *b* according to Willison and John [18] and Parsonage et al. [41]. There is no evidence that cytochrome *o* participates in respiration in wild-type strains grown aerobically [3,18] and no direct tests have been made with mutant cells. Although Willison and John [18] observed much slower reduction from substrate of cytochrome aa_3 on anaerobiosis of mutant membranes, our data using whole cells rather than membranes show similar rates of reduction in mutant and wild-type cells. Also they found decreased NADH oxidase in mutant membranes, while in the absence of detergent we did not. In our experiments respiration of NADH was inhibited by low concentrations of KCN typical for the usual oxidase.

We have shown that the cytochrome aa_3 can be fully functional in the cytochrome *c*-less mutant. Thus our data suggest that electron transport can proceed by collision and reaction of large complexes within the membrane and can do so at rates large enough to fulfill the energy requirements of the cells. It is not clear with what the cytochrome aa_3 reacts; it could be with a cytochrome *b*, with the iron-sulfur protein or with ubiquinone, whichever can form a reactive combination with the cytochrome aa_3. Similar suggestions have been made by others from data with other species [15,42]. Hochli et al. [43] and Zhu and Beattie [44] have cited evidence that complexes such as the bc_1 and aa_3 diffuse independently of one another in the plane of the membrane, while Hochman et al. [45] suggest a dynamic aggregation of colliding complexes in rat liver mitochondria.

In wild-type cells and membranes electron transport to the oxidase via the membrane-bound cytochrome *c*-552 is much greater than that which is possible in the reactions with added soluble cytochrome *c* under any conditions measured polarographically (Fig. 4, Table III). The cytochrome *c*-552 on the membrane can apparently form a highly reactive complex with the cytochrome aa_3. This could be the explanation for our previous [37] and present (Table II) observations that when rapid turnover proceeds via NADH or succinate, addition of soluble cytochrome *c* results in no increase

in O_2 uptake and only small increases when TMPD plus ascorbate are used as substrate. During rapid turnover with endogenous cytochrome *c* no reaction sites may be available for reaction with exogenous cytochrome *c*. This suggests that the cytochrome aa_3 of the mutant must also form a reactive complex with some redox component, since, soluble cytochrome *c* does not stimulate NADH or succinate oxidase of the mutant to a great extent either.

One dramatic difference between the wild-type and mutant membranes is the effect of detergent treatment. Whereas the NADH and succinate oxidases of the wild-type were stimulated by detergent, presumably by reaction sites becoming exposed, similar treatment resulted in inhibition of the NADH oxidase of the mutant. Thus, in the absence of the membrane-bound c_1 and *c*-552, the structure of the membrane changes so that it can no longer form the reactive micelles with detergent. Some experiments with cytochrome *c*-deficient mutants have shown overall electron transport not to be inhibited by chemicals which inhibit electron transport in wild-type cells in the cytochrome bc_1 segment (Refs. 18, 41, and Bolgiano, Smith and Davies, unpublished data). This might be expected if abnormal interactions are taking place. Also the different membrane structure could affect accessibility of the inhibitors.

In spite of the lack of cytochrome *c*-552 in the mutant, the oxidase of the mutant can oxidize soluble cytochrome *c* at rates comparable to that of the wild-type. This is conclusive evidence that soluble cytochrome *c* can react directly with cytochrome aa_3, as we postulated previously from indirect evidence [21]. Mammalian and *Paracoccus* cytochromes *c* can bind to subunit II of *Paracoccus* oxidase [46], which is also the subunit involved in cytochrome *c* binding in the mitochondrial system [47]. The purified oxidase complex of the mutant shows the same reactivity as that purified from the wild-type and both oxidases have the same major bands on SDS-gel electrophoresis [21]. Although the absorption spectra of the two are slightly different, this might result from the difference in membrane strucutre. The present data show that the rates of oxidation of soluble bovine cytochrome *c* by mutant and wild-type oxidases and the properties of the kinetics are similar. With both mutant and wild-type oxidase, cytochrome *c* from *Paracoccus* is oxidized less rapidly than that from bovine heart. We have established previously that the differences in reactivity with the two species of cytochrome *c* rests in the charge distribution around the molecules [21] as shown by changes seen on addition of charged polycations.

What is surprising is that soluble cytochrome *c* is reduced via NADH in the mutant system at rates that compare with those seen in wild-type, in spite of the absence of cytochrome c_1. The somewhat lower rates with succinate, as with the succinoxidase, may be a reflection of the lability of the succinate dehydrogenase during the preparation of membrane suspensions, as seen with bovine mitochondria [38]. The decrease in first order rate constant with increase in the concentration of cytochrome *c* resembles that seen with the wild-type reductase and with the mitochondrial reductase [29]. As yet it is not clear with which redox pigment of the mutant reductase the soluble cytochrome *c* interacts, or whether it is the same as that which can react with the membrane-bound oxidase. It has been shown with bovine heart and *Paracoccus* membrane particles that the reductase reacts with different species of cytochrome *c* with less specificity than does the oxidase [27,48].

Additional data presented here reinforce the conclusion that the *Paracoccus* soluble cytochrome *c*-550 of the periplasmic space does not participate in overall electron transport from substrates such as NADH and succinate. Removing some of the periplasmic cytochrome *c* by washing intact cells did not decrease the rate of O_2 uptake with endogenous substrates (Table III). Also we could show spectrophotometrically that the membrane-bound cytochrome *c*-552 is reduced more rapidly on anaerobiosis than is the soluble cytochrome *c*-550 (Fig. 5). The function of the soluble cytochrome *c* as shown by others, resides in reactions such as that from periplasmic [49,50] methanol- and methylamine-dehydrogenases in methanol- and methylamine-grown cells [6,7] or with the nitrite and nitrous-oxide reductases in nitrate-grown bacteria [51,52].

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