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# MINI-REVIEW

# The Reactions of the Oxidase and Reductases of *Paracoccus denitrificans* with Cytochromes *c*

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## Abstract

Electron transport in the *Paracoccus denitrificans* respiratory chain system is considerably more rapid when it includes the membrane-bound cytochrome $c_{552}$ than with either soluble *Paracoccus* $c_{550}$ or bovine cytochrome *c*; a pool function for cytochrome *c* is not necessary. Low concentrations of *Paracoccus* or bovine cytochrome *c* stimulate the oxidase activity. This observation could explain the multiphasic Scatchard plots which are obtained. A negatively charged area on the "back side" of *Paracoccus c* which is not present in mitochondrial *c* could be a control mechanism for *Paracoccus* reactions. *Paracoccus* oxidase and reductase reactions with bovine *c* show the same properties as mammalian systems; and this is true of *Paracoccus* oxidase reactions with its own soluble cytochrome *c* if added polycation masks the negatively charged area. Evidence for different oxidase and reductase reaction sites on cytochrome *c* include: (1) stimulation of the oxidase but not reductase by a polycation; (2) differences in the inhibition of the oxidase and reductases by monoclonal antibodies to *Paracoccus* cytochrome *c*; and (3) reaction of another bacterial cytochrome *c* with *Paracoccus* reductases but not oxidase. Rapid electron transport occurs in cytochrome *c*-less mutants of *Paracoccus*, suggesting that the reactions result from collision of diffusing complexes.


**Key Words:** Electron transport; respiratory chain; cytochrome *c*; cytochrome $c_{552}$; cytochrome $aa_3$; cytochrome *c* oxidase; cytochrome *c* reductase; *Paracoccus denitrificans*.

## The *Paracoccus* Respiratory Chain System

The initial attraction for working with *Paracoccus denitrificans* was the close resemblance of the respiratory chain members on the cytoplasmic

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membranes of these bacteria to those on the mitochondrial membrane (Scholes and Smith, 1968b), which are embedded in similar chemical surroundings (Albracht *et al.*, 1980). The two systems are also responsive to similar inhibitors (Scholes and Smith, 1968b, Berry and Trumpower, 1985). However, there are some interesting differences.

In this review we shall only discuss the electron-transport chains of bacteria which have been grown with strong aeration with succinate or glucose as carbon source in the absence of nitrate (Scholes and Smith, 1968b). Under our growth conditions the bacteria (ATCC 13543) synthesize three cytochromes *c*, two cytochromes *b*, cytochrome $aa_3$, ubiquinone 10, and several dehydrogenases. There is no evidence for a functional cytochrome *o*; after the pigments are reduced anaerobically with substrate there is no combination with CO to produce a typical cytochrome *o*-CO compound (Scholes and Smith, 1968b). In addition, Berry and Trumpower (1985) purified a functional ubiquinol oxidase from the same strain, which gave no evidence of a functional cytochrome *o*. Although claims for an *o*-type oxidase in *P. denitrificans* have appeared often, van Verseveld *et al.* (1983) found no evidence from photolysis experiments for a functional cytochrome *o* in their strain, although there was some evidence for a cytochrome $a_1$. Lawford *et al.* (1976) also found no kinetic evidence for an *o*-type oxidase in strain NCIB 8994. Hubbard *et al.* (1990) found cytochrome $aa_3$ to be the sole oxidase when they used $Mg^{++}$-depleted growth conditions. The nature of the cytochrome *b* which combines with CO, in the dithionite-reduced system seen in cells under some growth conditions (Cox *et al.*, 1978), is not known, but interpretations of experiments are simpler when the bacteria are grown so that there is no evidence of a functional cytochrome *o*.

One difference between the *Paracoccus* and the mitochondrial systems is the synthesis of a strongly membrane-bound *c* cytochrome ($c_{552}$) in addition to cytochrome $c_1$ and a soluble cytochrome *c* ($c_{550}$) in the periplasmic space

**Table I.** Properties of Cytochromes *c*

| | Bovine[a] | *Paracoccus denitrificans* | | |
|---|---|---|---|---|
| Cytochrome | *c* | $c_1$ | $c_{550}$ | $c_{552}$ |
| M.W. (kDa) | 12.4 | 62[b] | 14.5[c] | 22[d] |
| Absorbance, nm (α-band) | 550 | 553 | 550 | 552 |
| Isoelectric pH | 10.5 | 4.2[e] | 4.5[c] | 3.5[f] |

[a]Margoliash and Schejter (1966).
[b]Yang and Trumpower (1986).
[c]Scholes *et al.* (1971).
[d]Berry and Trumpower (1985).
[e]Ludwig *et al.* (1983).
[f]Husain and Davidson (1986) reported this p*I* for a soluble inducible $c_{551}$.

(Scholes and Smith, 1968b) (see Table I). In contrast to the mitochondrial system, the cytochrome $c_{552}$ cannot be removed on treatment of the membranes with detergent or sonication (Davies *et al.*, 1983). The soluble cytochrome $c_{550}$ resides in the periplasmic space and can be removed by washing the intact cells with 0.01 M phosphate buffer, then with the same buffer containing 0.15 M KCl, then with 0.01 M phosphate again (Scholes *et al.*, 1971), a procedure that appears to remove outer membrane material, leaving the cells otherwise unaltered (Hindahl *et al.*, 1981). This procedure has no effect on the overall respiratory rate (Bolgiano *et al.*, 1989).

The membrane-bound system has a cytochrome $c_1$ with a larger molecular weight than that of the mitochondrial pigment, in addition to the cytochrome $c_{552}$ with a molecular weight of 22,000 (Berry and Trumpower, 1985) (Table I). There are also two *b*-type cytochromes and typical cytochrome $aa_3$. The cytochrome $c_{552}$ appears to be closely associated with the latter (Berry and Trumpower, 1985).

Berry and Trumpower (1985) isolated a "supercomplex" which had high ubiquinol oxidase activity, and which subsequently could be separated into the $bc_1$ and $aa_3$ complexes (Yang and Trumpower, 1986). Clearly the quinol oxidase comprises a functional complex on the membranes. The subunits must have a strong affinity for each other and may fuse under conditions which partially delipidate the components while they are concentrated together on the ion-exchange columnn. Bosma *et al.* (1987a,b) prepared a partially purified ubiquinol oxidase from strain NCIB 8944 with similar methodology and found no other *c*-type cytochromes in the aerobically grown cells and no high-potential cytochrome *b*.

Washed, isolated membrane vesicles, which are easy to prepare by osmotic shock of lysozyme-treated cells (Scholes and Smith, 1968a), have high NADH-and succinic-oxidase activities, and these are not increased either on addition of bovine cytochrome $c_{550}$ or its own soluble cytochrome $c_{550}$ (Kuo *et al.*, 1985) (see Table II). Conditions known to remove or to bind to the soluble *Paracoccus* cytochrome $c_{550}$, such as washing, addition of specific monoclonal antibodies (Kuo *et al.*, 1985), or poly-L-lysine or DEAE cellulose (Davies *et al.*, 1983), do not inhibit the overall electron transport. Berry and Trumpower (1985) found that the rate of quinol oxidase with 5 nM endogenous cytochrome $c_{552}$ equalled the rate of the oxidase with 10 $\mu$M horse cytochrome $c_{550}$. Thus, cytochrome *c* does not seem to require substantial translational motion on the membrane in order to shuttle between the $bc_1$ and $aa_3$ complexes (Berry and Trumpower, 1985).

Waring *et al.* (1980) made covalently attached cytochrome *c* derivatives on cytochrome *c*-depleted mitochondrial membranes and compared the

**Table II.** NADH Oxidase[a]

| | $O_2$ uptake ($\mu$M/s) |
|---|---|
| A. *P. denitrificans* membranes + NADH | 0.47 |
| 1 $\mu$M *P. denitrificans* cytochrome *c* | 0.47 |
| B. *P. denitrificans* membranes + NADH | 0.46 |
| 0.64 $\mu$M antibody | 0.45 |
| 1.28 $\mu$M antibody | 0.44 |
| C. *P. denitrificans* membranes + NADH | 0.47 |
| 1 $\mu$M *P. denitrificans* cytochrome *c* | 0.47 |
| 0.64 $\mu$M antibody | 0.45 |
| 1.28 $\mu$M antibody | 0.41 |
| 1.92 $\mu$M antibody | 0.39 |

[a]Deoxycholate-treated membranes containing 0.43 mg protein and 0.16 $\mu$M *c*-type cytochromes in 2.8 ml of 0.05 M Tris-maleate buffer (pH 7.0) were assayed in the presence of 2.9 mM NADH. Monoclonal antibody to *Paracoccus* cytochrome *c* (F3-29.4) was an IgG pool. The results were expressed as $\mu$M $O_2$ uptake/s. The NADH oxidase is more than 98% inhibited by 2.3 $\mu$g antimycin A/mg protein. (Reproduced from Kuo *et al.*, 1985 with permission.)

respiratory activity with that of the *Paracoccus* system. They also concluded that close contact between the membrane-bound cytochromes leads to rapid electron transport.

Overall the membrane-bound electron transport system of *Paracoccus* has given evidence of the high rates possible when the members are closely associated on the membrane and thus gives insight into the nature of such systems. When the reaction proceeds via the endogenous pigments of the respiratory chain, apparently no sites are exposed for rapid reaction with added soluble cytochrome *c* (Davies *et al.*, 1983). This may be a control mechanism.

To date, no laboratory has reported the purification and characterization of the membrane-bound *Paracoccus* $c_{552}$. It either has a quite different structure from the soluble cytochrome $c_{550}$, since it does not bind the same compounds, or it is largely buried in the membranes.

A "supercomplex" with quinol oxidase activity was isolated from a thermophilic Gram-positive bacterium PS3 (Sone *et al.*, 1987) using ion-exchange chromatography of detergent-treated membranes and by immunoprecipitation with polyclonal antibodies to the purified $caa_3$. However, as with the $bc_1$ and $caa_3$ fractions prepared by Yang and Trumpower (1986), the same supercomplex was not formed when they were recombined. Joliot *et al.* (1989) found evidence in intact photosynthetic bacteria of complexes between soluble cytochrome $c_2$, the reaction center, and cytochrome $bc_1$, seeing rapid electron transport within complexes and slow electron transport between different supercomplexes via diffusing electron carriers.

## Reactions with Exogenous Cytochromes *c*

Although electron transport down the whole chain goes most efficiently via the membrane-bound cytochrome $c_{552}$, the individual oxidase and reductase segments can oxidize or reduce soluble cytochromes *c*, albeit at lower rates. The turnover rate of the oxidase, when reacting with the closely associated cytochrome $c_{552}$ with TMPD[3] plus ascorbate as reductant, can be as high as 1000 per second (Erecinska *et al.*, 1979). The rates are lower in reactions with bovine cytochrome *c* and still lower with added *Paracoccus* cytochrome *c* (see below). The reactions with soluble cytochromes *c* have given some insight into the nature of such interactions.

A 2-subunit (Ludwig and Schatz, 1980) and a 3-subunit (Haltia *et al.*, 1988; Bolgiano *et al.*, 1988) oxidase (cytochrome $aa_3$) have been prepared as well as a pure ubiquinol-cytochrome *c* reductase ($bc_1$ complex) (Yang and Trumpower, 1986). Under most experimental conditions used, the oxidase and reductase react more rapidly with mammalian soluble cytochrome *c* than with purified *Paracoccus* cytochrome $c_{550}$. Studies with the latter have been reported only from our laboratory. Actually the *Paracoccus* oxidase is unusual; most bacterial oxidases do not oxidize mammalian cytochrome *c* (Smith, 1954).

## Oxidase

In spectrophotometric assays, the *Paracoccus* oxidase, on membrane vesicles or as purified cytochrome $aa_3$, oxidizes bovine cytochrome *c* with kinetic characteristics similar to those seen with mammalian oxidase and mammalian cytochrome *c* (Smith and Conrad, 1956). The reaction is first order in ferrocytochrome *c*, and the rate constant decreases with increasing concentrations of total cytochrome *c* (Bolgiano *et al.*, 1988). The purified oxidase reacts in stopped-flow kinetic experiments much as the mitochondrial oxidase does and forms similar intermediates in the reaction with $O_2$ and CO (Reichart and Gibson, 1983, Ludwig and Gibson, 1981). In the reaction with mammalian cytochrome *c* the mammalian and the bacterial oxidases also show similar responses to variations of pH and ionic strength (Bolgiano *et al.*, 1988) and to the inhibitory effects of polycations (Smith *et al.*, 1976, Kennelly *et al.*, 1981). The *Paracoccus* oxidase must have a binding site for mammalian cytochrome *c* equivalent to that of the mammalian oxidase and thus serves as an appropriate model for studies of the mammalian system.

[3]TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.

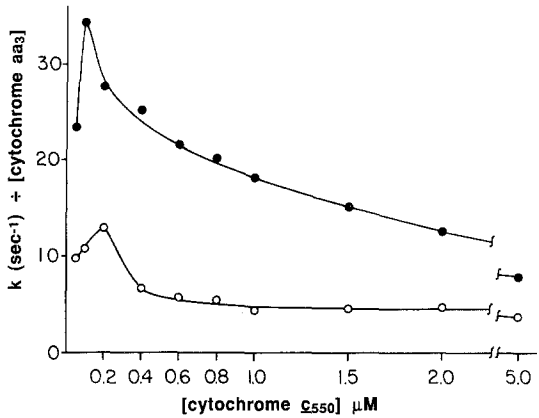


**Fig. 1.** Rate constants of *Paracoccus* cytochrome oxidase on addition of either bovine (●) or *Paracoccus* (○) cytochrome $c_{550}$. Data are expressed as rate $k(s^{-1}) \div$ [cytochrome $aa_3$] $\mu$M. Assays were run at 26°C in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0) with $9.77 \times 10^{-4}$ $\mu$M cytochrome $aa_3$ per assay. (Reproduced from Bolgiano *et al.*, 1988 with permission.)

Exogenous soluble cytochrome *c*, either mammalian or from *Paracoccus*, apparently reacts directly with cytochrome $aa_3$ and not via the bound cytochrome $c_{552}$. Evidence for this is seen in competition experiments with poly-L-lysine (Smith *et al.*, 1976) and by the observation that purified cytochrome $aa_3$ is as active as a preparation containing cytochromes $c_{552}aa_3$ complex (Bolgiano *et al.*, 1988).

In studies with the beef oxidase we had previously found an increase in activity with increasing concentrations of beef cytochrome *c* up to about 0.1 to 0.2 $\mu$M, irrespective of the concentration of oxidase (Smith *et al.*, 1979). This suggested a form of the oxidase influenced by the presence of low concentrations of added cytochrome *c*, a postulate also made by others (Bickar *et al.*, 1985, Kornblatt and Luu, 1986). More recently, as can be seen in Fig. 1, we have obtained direct evidence for this with the *Paracoccus* oxidase; just the addition of the low concentrations of cytochrome *c* (either bovine or *Paracoccus*) increased the oxidase activity (Bolgiano *et al.*, 1988). This stimulatory effect of cytochrome *c* could be an explanation for the postulated "high and low affinity sites" on the oxidase for cytochrome *c* (Ferguson-Miller *et al.*, 1976). The range of cytochrome *c* concentrations giving the increase is that postulated to react at the "high-affinity site." The stimulatory effect seems to be more obvious with the *Paracoccus* oxidase; however we have occasionally also seen it with the bovine oxidase (unpublished). Other explanations for the multiphasic Scatchard plots from kinetic experiments with the beef system, such as multiple binding sites or dimer formation by the oxidase, seem unnecessary.

As mentioned above, in our laboratory we have also studied the reaction of the *Paracoccus* oxidase with its own soluble cytochrome $c_{550}$. X-ray crystallographic studies showed that *Paracoccus* cytochrome *c* has the same "cytochrome fold" characteristic of mammalian *c*-cytochromes and the sequence of amino acids surrounding the heme crevice is almost identical (Timkovich and Dickerson, 1976). The *Paracoccus* cytochrome is larger than the bovine pigment and there is a vast difference in the amino acid side chains on the surface. In the *Paracoccus* cytochrome the ratio of dicarboxylic acids to diamino acids is twice that of bovine, leading to very different isoelectric points. Both cytochromes have a group of lysines around the heme crevice, but that of *Paracoccus* is more of a dipole than is the bovine cytochrome, having a highly negative hemisphere on the side of the molecule away from the heme crevice (Timkovich and Dickerson, 1976). The properties of its reaction with the *Paracoccus* oxidase are very different from those with bovine cytochrome *c*, described above (Smith *et al.*, 1976). The reaction rates are lower, and the great sensitivity of the reaction with bovine cytochrome *c* to ionic strength and the large decrease in rate constant with increasing

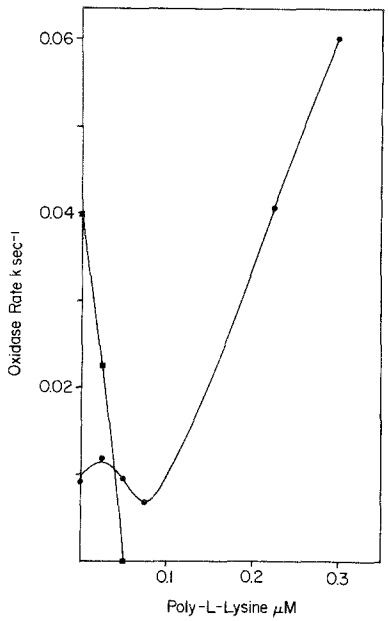


**Fig. 2.** Effect of poly-L-lysine on the oxidation of bovine and *P. denitrificans* cytochromes *c* by *P. denitrificans* oxidase. The assays were run in 0.05 M Tris-maleate, pH 6.0, with either 4.3 $\mu$M (■) bovine or *P. denitrificans* (●) cytochrome *c* and *P. denitrificans* membranes containing 0.0025 mg of protein. Poly-L-lysine was added to the buffered cytochrome *c* and the reactions were initiated by addition of the membranes containing the oxidase. (Reproduced from Smith *et al.*, 1976 with permission.)

concentrations of cytochrome *c* are not apparent in the reaction with the *Paracoccus* pigment (Bolgiano *et al.*, 1988). Plots of *v*/*S* versus *v* according to Eadie–Hofstee with *Paracoccus* oxidase and bovine cytochrome *c* give multiphasic plots similar to those published so often with the mammalian system. With *Paracoccus* cytochrome *c* the plot is more nearly monophasic. Thus it is the nature of the cytochrome *c* and not that of the oxidase which influences this type of interaction.

Because of the negatively charged back hemisphere, *Paracoccus* cytochrome *c* can actually bind polycations such as poly-L-lysine (Smith *et al.*, 1976) or a copolymer of lysine and homoarginine (Kennelly *et al.*, 1981). Whereas these polymers competitively inhibit the reaction of bovine cytochrome *c* with the *Paracoccus* oxidase (Smith *et al.*, 1976), as with the bovine oxidase (Davies *et al.*, 1964), similar concentrations will greatly stimulate the activity of the oxidase with *Paracoccus* cytochrome *c* (Smith *et al.*, 1976) (see Fig. 2). Also on addition of poly-L-lysine the properties of the reaction (such as the effects of changes of pH and ionic strength) are altered to resemble those in the reaction with bovine cytochrome *c* (Bolgiano *et al.*, 1988) (see Fig. 3). The bovine cytochrome oxidase oxidizes the *Paracoccus* cytochrome

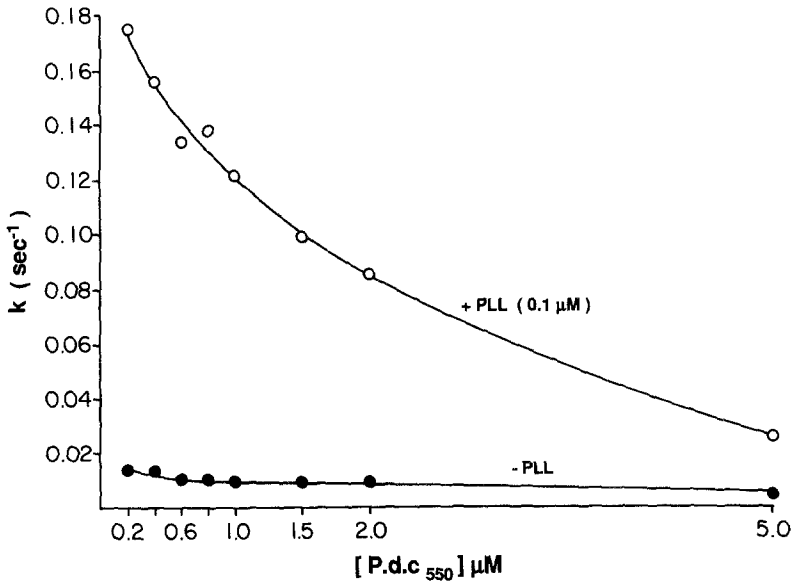


**Fig. 3.** Effect of poly-L-lysine on rate constants with *Paracoccus* cytochrome $c_{550}$. The oxidation of *Paracoccus* cytochrome $c_{550}$ was assayed with (○) or without (●) the addition of 0.1 μM poly-L-lysine (PLL), 20 kDa at pH 6.0 in 50 mM Tris-maleate, 2 mM EDTA. The *Paracoccus* cytochrome oxidase fraction used in the assay contained $4.88 \times 10^{-4}$ μM cytochrome $aa_3$. The assays were run by adding poly-L-lysine to the buffered cytochrome *c* before the addition of the oxidase fraction. (Reproduced from Boligano *et al.*, 1988 with permission.)

*c* at a low rate, but this is also somewhat increased on addition of the proper concentration of poly-L-lysine (Smith *et al.*, 1976). Apparently the polycations bind more strongly to *Paracoccus* cytochrome *c* and this masks the negative charges and makes the complex a more acceptable substrate for the oxidase, emphasizing the importance of charged groups in the reaction of cytochrome *c* and the oxidase (Davies *et al.*, 1964, Minnaert, 1965). Electrostatic interactions can orient oxidants and reductants during an electron transfer encounter. The observations with *Paracoccus* oxidase and cytochrome *c* give evidence of the effects of charged groups around the whole molecule in determining the stability of the complexes formed or in favoring electron-transfer reactions. Koppenol and Margoliash (1982) have discussed the effect of the dipole moment in orienting molecules like cytochrome *c*.

## Reductases

The reaction of the NADH- and succinate cytochrome *c* reductases of the membrane vesicles (succinate or NADH dehydrogenase plus cytochrome $bc_1$) with mammalian cytochrome *c* has the same properties as the reductases of bovine heart mitochondrial fragments with bovine cytochrome *c* (Smith *et al.*, 1974). The reaction is zero order in ferrocytochrome *c* at high concentrations of cytochrome *c*, then becomes first order at lower concentrations, when the reaction with cytochrome *c* becomes rate-limiting. We have measured the first-order rate constants.

Berry and Trumpower (1985) found a turnover rate of 700 per second for the uniquinol cytochrome *c* reductase of the membranes on addition of horse cytochrome *c*. Their purified ubiquinol cytochrome *c* reductase ($bc_1$ complex) was activated by phospholipid and inhibited by substances that inhibit the mitochondrial cytochrome $bc_1$ complex.

We found the *Paracoccus* reductases to be active with cytochromes *c* from beef, *Paracoccus*, and *Rhodospirillum rubrum*, in contrast with the oxidase, which does not oxidize *R. rubrum* cytochrome at all and oxidizes *Paracoccus* cytochrome only slowly (Smith *et al.*, 1976).

The extra loops and the tail on the *Paracoccus* cytochrome *c* constitute large perturbations on the surface of the molecule, making these regions unlikely as reaction sites for the oxidase and reductases. The remaining possibilities are the top of the molecule and the heme crevice, which are the most structurally invariant surface features among the different species of cytochrome *c* (Timkovich and Dickerson, 1976). There is evidence in data from the mammalian systems that the reaction sites are in the area at the top of the heme crevice.

In contrast to the stimulatory effect on the oxidase, poly-L-lysine does not stimulate the reductases (unpublished data). Thus the binding of the poly-L-lysine does not affect its reaction with the reductases. This observation plus that of the reaction of *R. rubrum* cytochrome *c* with the reductases but *not* the oxidase gives good evidence that cytochrome *c* reacts differently with the oxidase and reductases.

Additional evidence that the reaction sites on cytochrome *c* for the oxidase and reductases are different came from our studies with monoclonal antibodies to cytochromes *c*. In spectrophotometric assays, monoclonal antibodies to *Paracoccus* cytochrome *c* could affect both the binding of the cytochrome *c* (evidenced as decreased rates) as well as the electron-transfer reactions (giving complete inhibition) and could distinguish between the two. The antibodies showed different effects on the blocking of the reductase reaction, compared to the oxidase (Kuo *et al.*, 1984); one antibody completely blocked the reaction with the oxidase, but not the reductase (Kuo *et al.*, 1985). Thus the reaction sites for reaction of cytochrome *c* with the oxidase are different from those with the reductase. This suggestion has also come from work with cytochrome *c* from other species (Smith *et al.*, 1976, Kuo *et al.*, 1986).

## Physiological Role of Soluble Periplasmic Cytochrome $c_{550}$

As discussed above, when respiration catalyzed by the electron transport chain on membrane vesicles proceeds from NADH or succinate via the endogenous cytochrome $c_{552}$, there is no reaction with added soluble cytochrome $c_{550}$. Also spectrophotometric recordings at 550 and 552 nm with suspensions of intact bacteria showed that the cytochrome $c_{552}$ was rapidly reduced on anaerobiosis, but the reduction of the cytochrome $c_{550}$ began only after the membrane-bound cytochrome was reduced (Bolgiano *et al.*, 1989). Thus, other functions must be served by the soluble pigment. The level of the soluble periplasmic cytochrome *c* varies widely with growth conditions and is low in cells grown with high aeration with succinate as substrate (Scholes and Smith, 1968b). It increases markedly, along with additional cytochromes *c*, in cells grown aerobically with methanol (Davidson and Kumar, 1989) or anaerobically with nitrate (Scholes and Smith, 1968b, Sapshead and Wimpenny, 1972, Bosma *et al.*, 1987a, b). It has been shown to be reduced by methanol dehydrogenase in the periplasm; then the reduced cytochrome can react directly with the oxidase without involving the rest of the respiratory chain (van Verseveld *et al.*, 1981, Alefounder and Ferguson, 1981). It also reacts with the soluble nitrite reductase found in the periplasmic space of cells grown with nitrate (Alefounder and Ferguson, 1980). As discussed in the

section on the oxidase, after the initial stimulation of the oxidase at low concentrations of cytochrome *c*, the rate of the oxidase reaction is controlled in a nearly linear fashion by the concentration of cytochrome *c*. Thus the rate of reaction with the soluble cytochrome is increased by increased synthesis of the pigment.

When the negatively charged back hemisphere of the soluble cytochrome $c_{550}$ is masked with a polycation, the rate of reaction with the oxidase is increased markedly. It appears as if the negative charge can be attracted to a positively charged region on the oxidase, so that the cytochrome is not aligned properly for rapid electron transport. Perhaps something is synthesized during the reaction with methanol dehydrogenase, for example, which combines with the negatively charged site and thus promotes the reaction with the oxidase. Combination with polycations does not increase the rate of reduction by the reductase, which is all right, since the reduced soluble cytochrome *c* reacts directly with the oxidase. This could supply a control mechanism for reactions with the periplasmic cytochrome *c*. The mitochondrial oxidase system lacks the bound cytochrome $c_{552}$, and its reactions with its soluble cytochrome *c* without the negative patch are different, in that the reaction can be influenced by environmental factors, such as ionic strength or the concentration of the cytochrome *c*, giving possible sources of control lacking in the *Paracoccus* system.

Other bacterial species also synthesize both membrane-bound and periplasmic cytochromes *c*. Examples are *Methylophilus methylotrophus* and a thermophilic Gram-positive bacterium PS3. A complex of cytochrome $caa_3$ can be isolated from *Thermus thermophilus*, which can then be dissociated in detergent (Lorence *et al.*, 1981). *Alcaligenes eutrophus* has a membrane-bound electron-transport system containing two or three cytochromes *c*, which has NADH oxidase activity; there is also soluble cytochrome *c* in the periplasm (Probst and Schlegel, 1976).

Most interesting, some species of photosynthetic bacteria synthesize a membrane-bound cytochrome *c* in the reaction center and also a soluble cytochrome *c* (Matsuura *et al.*, 1988). It is thought that *in vivo* the soluble cytochrome *c* reacts with the bound cytochrome, but that it can also react directly with the oxidized bacteriochlorophyll. Thus the latter could function if the bound cytochrome *c* were lost by mutation. Such a mutation could lead to the change from the *Paracoccus* system to one like that in mitochondria, and postulates have been made that an organism like *Paracoccus* was the source of mitochondria (John and Whatley, 1975). Beginning with the *Paracoccus* system, only the soluble cytochrome *c* need change and parallel changes in the oxidase and reductases would not be necessary to arrive at active mitochondrial-type oxidase and reductase systems.

## Studies with Cytochrome *c*-Deficient Mutants: Collision of Complexes?

A mutant of *Paracoccus* deficient in soluble cytochrome $c_{550}$ was prepared by van Spanning *et al.* (1990); it also synthesized decreased levels of cytochrome $aa_3$. The maximum specific growth rate of the mutant was the same as that of the wild type with succinate or choline as substrates, but the growth yield was decreased by 14% and the lag phase increased 4-fold. The authors conclude that electron transport in the mutant proceeded entirely via an "alternate pathway" not involving cytochrome $c_{550}$. However, the data also fit with the conclusion that the soluble cytochrome is not necessary for the main energy-yielding pathway via cytochrome $c_{552}$, in accordance with our evidence that respiration remains unchanged after removal of the periplasmic cytochrome *c* from wild-type cells (Bolgiano *et al.*, 1989).

Willison and John (1979) investigated a mutant of *Paracoccus* lacking all *c*-type cytochromes. They found that in membrane vesicles cytochrome $aa_3$ was reduced more slowly than in wild-type cells and that NADH and succinoxidase activities were lower.

We compared a cytochrome *c*-deficient mutant obtained from van Verseveld *et al.* (1981) with its parent wild type (see Fig. 4) and found that they grew aerobically with the same generation time (Bolgiano *et al.*, 1989). Also the NADH and succinate oxidase activities of membrane vesicles of mutant and wild type were comparable. Although soluble cytochrome *c* could be oxidized and reduced, adding it did not increase the NADH oxidase activity. Most significant, in both membrane fragments and in intact cells, cytochrome $aa_3$ was completely reduced when anaerobiosis was reached, and the rate was only slightly lower in the mutant (see Fig. 5), in contrast with the observations of Willison and John (1979). Thus rapid electron transport from the dehydrogenases goes through to the oxidase without the presence of cytochromes $c_1$, $c_{552}$, or $c_{550}$, and the respiration is inhibited by low concentrations of cyanide. The simplest way to explain these observations is that electron transport reactions result from collision of diffusing complexes. Similar suggestions have been made by others from data with different bacterial species. A mutant of a photosynthetic bacterium lacking periplasmic cytochrome $c_2$ could grow aerobically or photosynthetically, although growth was slower in dim light (Daldal *et al.*, 1986). The authors suggest that the oxidized reaction center is reduced by electron transport from the bound cytochrome $c_1$, since it is still antimycin A-sensitive. Another mutant deficient in both cytochrome $c_2$ and the "alternate oxidase" could also grow photosynthetically and aerobically in the dark (Daldal, 1988), again suggesting interaction with the cytochrome $bc_1$ complex. Hochli *et al.* (1985) and Zhu and Beattie (1988) have cited evidence that the $bc_1$ and $aa_3$ complexes diffuse independently in the plane of the mitochondrial membrane,

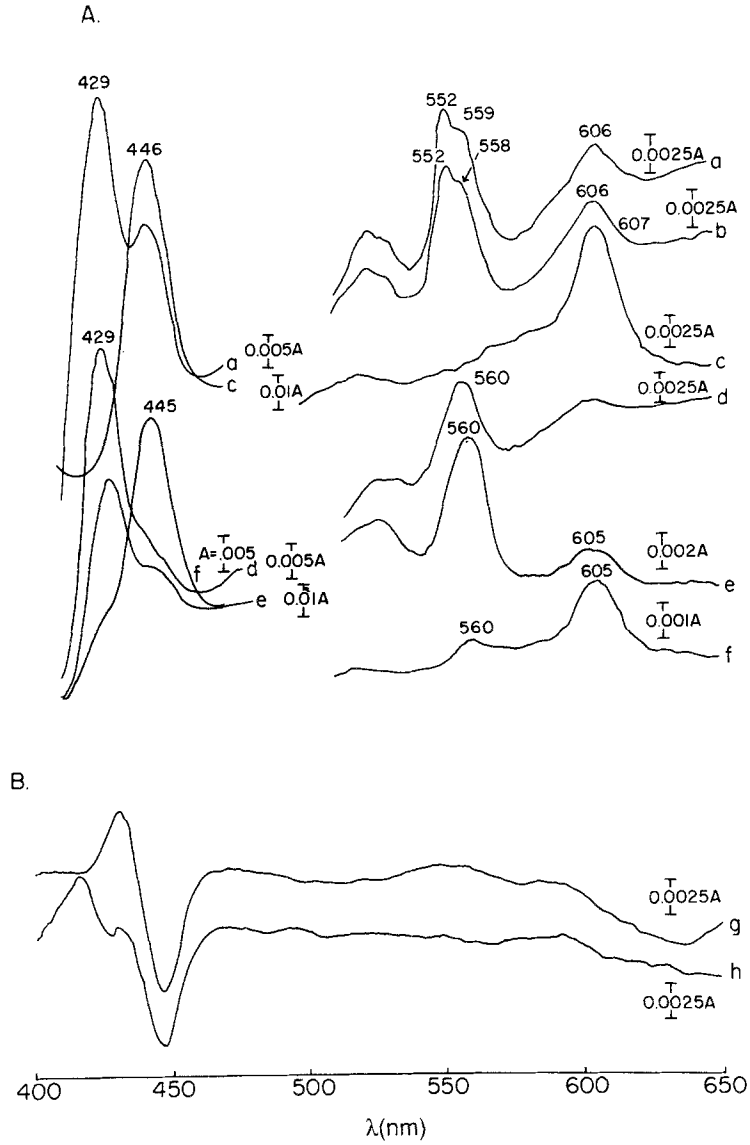


**Fig. 4.** Difference spectra of cells (a, d), membranes (b, e), and purified cytochrome oxidase (c, f, g, h) from wild-type (a, b, c, g) and cytochrome *c*-deficient (d, e, f, h) strains of *Paracoccus*. (A) Dithionite-reduced minus ferricyanide-oxidized spectra and (B) CO + dithionite-reduced minus dithionite-reduced difference spectra were recorded by scanning from 650 to 400 nm at room temperature. (Reproduced from Bolgiano *et al.*, 1989 with permission.)

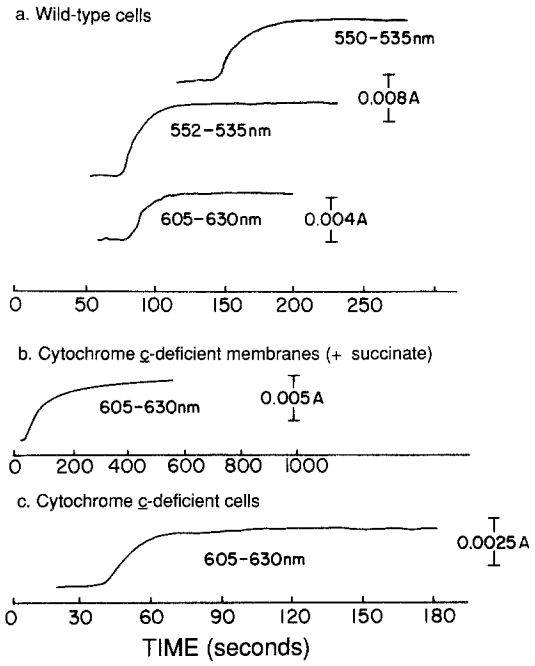


**Fig. 5.** Reduction of cytochromes *a* (605–630 nm) and *c* (550 or 552–535 nm) from wild-type cells (a) and cytochrome *c*-deficient (c) cells and cytochrome *c*-deficient membranes (b) of *Paracoccus* on anaerobiosis. A 1-ml suspension of cells [50% glycerol, 25 mM Tris-maleate (pH 7.0)] or membrane 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 a Hitachi 557 spectrophotometer. For the cytochrome *c*-deficient sample, 20 mM sodium succinate was added prior to aerating the cuvette. (Reproduced from Bolgiano *et al.*, 1989 with permission.)

while Hochman *et al.* (1985) suggest a dynamic aggregation of colliding complexes in rat liver mitochondria. At any rate, it appears that interaction is possible between any cytochromes in the membrane with appropriate redox potentials that can collide with the reactive groups in effective alignment.

In spite of the lack of cytochromes $c_1$ and $c_{552}$, the oxidase and reductases of the *Paracoccus* mutant oxidized and reduced both mammalian and *Paracoccus* soluble cytochromes *c* with characteristics similar to those seen in the wild type.

A dramatic difference between the membranes of the mutant and wild type *Paracoccus* is the response to detergent treatment. Whereas the NADH and succinate oxidases of the wild type are stimulated by detergent treatment, similar treatment results in inhibition of NADH oxidase of the mutant. In the absence of intact cytochromes $c_1$ and $c_{552}$ the membrane structure is different, so that it can no longer form the reactive micelles with detergent.

## Summary

In-depth studies of the reactions of *Paracoccus* oxidase and reductases with membrane-bound and with soluble cytochromes *c* from *Paracoccus* and beef have given some insights into the nature of such electron transport interactions:

1. No pool function is necessary for cytochrome *c*. Electron transport is more rapid via solidly membrane-bound cytochromes.

2. The presence of low concentrations of cytochrome *c* (either *Paracoccus* or bovine) stimulates the activity of cytochrome oxidase. This can explain the observed multiphasic plots of $v/S$ vs. $v$; other postulates are unnecessary.

3. The distribution of charges around the entire cytochrome *c* molecule is important in its reaction with the oxidase. The negatively charged area on the "back side" of *Paracoccus* cytochrome *c* inhibits its reaction with the oxidase. This negatively charged site is not prominent in the structure of mitochondrial cytochrome *c*; it could offer a control mechanism for *Paracoccus* reactions with the oxidase by inhibiting the reaction unless blocked by a polycation.

4. The reactions of *Paracoccus* oxidase and reductases with mammalian cytochrome *c* show the same properties as those of the mammalian systems with mammalian cytochrome *c*. The same is true of the reaction of *Paracoccus* oxidase with soluble *Paracoccus* cytochrome *c* if a polycation is added to mask the negatively charged area. The *Paracoccus* pigments have reaction sites comparable to those of the mammalian systems and can serve as appropriate models.

5. The reaction sites on soluble *Paracoccus* cytochrome *c* for the oxidase and the reductases are different, as shown by: (a) stimulation of the oxidase, but not the reductase, by poly-L-lysine; (b) different inhibition of the oxidase and reductases by monoclonal antibodies to the cytochrome *c*; and (c) reaction of *R. rubrum* cytochrome *c* with *Paracoccus* reductases, but not with the oxidase.

6. The soluble periplasmic cytochrome is reduced on anaerobiosis after the membrane-bound cytochrome $c_{552}$. The soluble cytochrome *c* has other functions than that of a member of the energy-yielding respiratory chain system.

7. Studies of mutants of *Paracoccus* deficient in all cytochromes *c* suggest collision of large complexes within the membrane. Apparently some component(s) of the $bc_1$ complex can reduce the cytochrome $aa_3$. Any pigments with appropriate redox potentials can interact if properly oriented.

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