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Monoclonal antibodies to cytochrome *c* from *Paracoccus denitrificans*: effects on electron transport reactions

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The effect of a monoclonal antibody to a soluble cytochrome *c* from *Paracoccus denitrificans* was tested on the membrane-bound electron-transport system of this bacterium. This antibody (F3-10.2) and one previously described (F3-29.4) (Kuo, L.M., Davies, H.C. and Smith, L. (1984) Biochim. Biophys. Acta 766, 472–482) were deduced to bind to the cytochrome *c* in the area including amino acid residue number 23 on a loop on the side of the heme crevice. In contrast to the observations with the previously tested antibody, the present data show the second antibody to block completely the reaction of the cytochrome *c* with cytochrome *c* oxidase but not that with cytochrome *c* reductase. Neither antibody has an appreciable inhibitory effect on the NADH oxidase of the isolated detergent-treated membranes. The two antibodies bind in different ways, giving insight into the interaction of a soluble protein with membrane-bound enzymes. The data indicate that the reaction sites on the cytochrome *c* for the oxidase and reductase moieties of *P. denitrificans* are different. They also argue against the need for a dissociable cytochrome *c* comparable to that which functions on the mitochondrial inner membrane.

Introduction

We have isolated several monoclonal antibodies to a soluble cytochrome *c* from *Paracoccus denitrificans* [1]. We have used these antibodies to investigate a role for *c*-type cytochromes in electron transport in this bacterium. Two of these show considerable specificity for this cytochrome; one binds only to it and to that from *Rhodopseudomonas capsulata* among cytochromes *c* from 29 species tested. The other binds also to that from *Rhodospirillum molischianum*. The two antibodies bind to *P. denitrificans* cytochrome *c* with K_a values of $1.57 \cdot 10^7$ (an IgG_{2a}) and $1.16 \cdot 10^8$ (an IgG_1) [1]. We have found that both bind in the region around amino acid number 23 (equivalent to number 21 in tuna cytochrome *c*), located in a loop on the right-hand side of the heme crevice, looking into the heme crevice as the front of the molecule. Only the cytochromes *c* from *P. denitrificans*, *R. capsulata* and *R. molischianum* have alanine at amino acid residue 23 or in equivalent positions.

The effects of one of the antibodies on the cytochrome *c* oxidase and the NADH cytochrome *c* reductase activities of the respiratory chain system on the cytoplasmic membranes of *P. denitrificans* with its own cytochrome *c* have been

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine.

described [2]. Spectrophotometric assays were employed, where binding of cytochrome *c* to the enzymes is rate limiting. Inhibitory effects were observed both on binding and on electron transport reactions with both oxidase and reductase, and these two effects could be distinguished. Different responses were seen with the oxidase and reductase electron transport reactions.

We have now studied the second antibody for comparison. It differs from the first in that it completely blocks the reaction with the oxidase, measured spectrophotometrically, but does not completely block the reductase reaction, only lowers the rate constant. The effects of both antibodies demonstrate that the reaction sites on the cytochrome *c* for the oxidase and reductase moieties are different. They also show how monoclonal antibodies can bind in different ways to distinguish different aspects of interaction of a protein substrate with its reacting partners.

The two antibodies had little or no effect on the NADH oxidase of the membrane vesicles treated with detergent to expose reaction groups maximally. They also had no effect on the reaction of the oxidase with endogenous cytochrome. Evidently, the electron transport chain of these bacteria functions while all members remain firmly membrane-bound and without the need for a loosely bound dissociable cytochrome *c* comparable to that on mitochondrial inner membranes.

Methods

Preparations

Monoclonal antibodies to P. denitrificans cytochrome c. The growth of the bacteria [3], purification of the cytochrome *c* [4] and the preparation of the antibodies and the purification and properties of antibodies F3-10.2 and F3-29.4 have been described in previous publications [1,2].

Binding of antibodies to cytochromes c. Binding of antibodies to cytochromes *c* from numerous different species was assessed by the ELISA method [1]. Cytochromes *c* from *Rhodospirillum molischianum* iso-1, *Rhodospirillum photometricum, Rhodopseudomonas palustris* (Strain 37), *Rhodopseudomonas capsulata* (Strain TJ12), *Rhodopseudomonas globiformis, Rhodopseudomonas viridis, Rhodospirillum salexigens* (Strain WS68) and *Rhodomicrobium vannielii* were a gift from T.E. Meyer. Cytochromes *c* from *Rhodospirillum rubrum* (Strain I, ATC 11170), *Rhodopseudomonas capsulata* (Strain 2.3.1 = ATC 11166), *Rhodopseudomonas sphaeroides* (Strain 2.4.1 ATCC 17023) were a gift from R. Bartsch and *Macacca mulatta* was from M. Reichlin. Bovine cytochromes and cytochromes from horse, pig, chicken, sheep, pigeon, rabbit, rat, tuna, yeast and dog were purchased from Sigma Chemical Company. The bovine and human [5], and that from *Paracoccus denitrificans* [4] were prepared following the methods of Margoliash and Walasek [5] and Scholes et al. [4], respectively, followed by isoelectric focusing [6]. Cytochromes *c* from fly, guanaco, tobacco moth, snapping turtle, *Samia cynthia*, Pacific lamprey, and Pekin duck were gifts of E. Margoliash and had been stored in the frozen state for several years.

Preparation of P. denitrificans membranes. Vesicles of *P. denitrificans* cytoplasmic membranes were prepared by osmotic rupture of spheroplasts [3]; these could be stored at −20°C for several months without loss of activity. To rupture the vesicles [7], sodium deoxycholate was added to a concentrated suspension (at least 14 mg protein/ml) to make the mixture 1 mg deoxycholate per mg protein, which yields maximal reactivity of oxidase and reductase [2]. The suspension was then diluted with cold water to give an appropriate rate and used within 1 day.

Enzymatic assays. Cytochrome *c* oxidase and NADH cytochrome *c* reductase were assayed spectrophotometrically by our established methods [8,9]. The kinetics were followed at 550–540 nm using a turbine-driven, time-sharing, multichannel spectrophotometer built by the Johnson Foundation, School of Medicine, University of Pennsylvania, Philadelphia, PA [10]. In these experiments both oxidase and reductase were first-order in cytochrome *c* throughout, and the activities were expressed as first-order rate constants [8]. All assays were made in 0.05 M Tris maleate buffer (pH 7.0) to eliminate variations due to changes in pH or ionic strength.

Cytochrome *c* oxidase was also assayed polarographically [11] in the presence of 0.7 mM TMPD and 10 mM ascorbate in the same buffer (pH 7.0); NADH oxidase activity was measured polaro-

graphically in Tris-maleate buffer (pH 7.0) with 3 mM NADH.

Results

Localization of the binding site on cytochrome c for antibodies

Binding of the antibodies to cytochromes *c* from the following species, was tested using ELISA methodology [1]; *P. denitrificans, R. capsulata, R. sphaeroides, R. rubrum, R. molischianum* iso-1, *R. palustris* Strain 37, *R. vannielii, R. viridis, R. salexigens* Strain WS 68, *R. globiformis, R. photometricum,* human, bovine (sheep pig), horse, dog, rabbit, rat, chicken, pigeon, tuna, yeast, fly, guanaco, moth, snapping turtle, *Samia cynthia* lamprey and Pekin duck. Antibody F3-10.2 could bind the cytochrome from *P. denitrificans, R. capsulata* and *R. molischianum* but none of those from the other species tested. An examination of the amino acid sequences shows that these three cytochromes all have alanine at residue 23 or equivalent position, in contrast to those from the other species [13]. Antibody F3-29.4 binds only cytochromes from *P. denitrificans* and *R. capsulata.* The next amino acid residue (number 24) for *P.* denitrificans and *R. capsulata* is proline, but for *R. molischianum* is glycine. Thus both antibodies bind to the cytochrome *c* of *P. denitrificans* in different ways in the area around amino acid 23 (equivalent to number 21 in tuna cytochrome *c*), which is on a loop on the right side of the heme crevice looking into the crevice as the 'front' of the molecule [14].

Effect of antibody F3-10.2 on cytochrome c oxidase and NADH cytochrome c reductase of P. denitrificans membranes, measured spectrophotometrically

(1) Oxidase. Addition of antibody to the assay mixture rendered some *P. denitrificans* cytochrome *c* non-oxidizable by the oxidase (Figure 1). There was a very slow change in absorbance after the first order part of the reaction was completed, which we assume resulted from a slow dissociation of the cytochrome *c* from the antibody. This was not seen in the experiments with antibody F3-29.4 [2], which has a larger association constant with the cytochrome [1]. The low rate was subtracted from the entire tracing, then the first-order rate

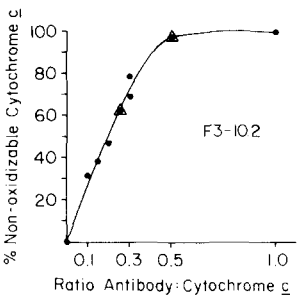

Fig. 1. Production of non-oxidizable cytochrome *c* on addition of antibody F3-10.2. Assays were run spectrophotometrically as described in Methods with *P. denitrificans* membranes containing 0.042 mg protein together with 0.48 μM *P. denitrificans* cytochrome *c* for all points except those at ratios of antibody to cytochrome *c* of 0.25 and 0.5, where purified *P. denitrificans* cytochrome aa_3 of E. Berry was used (0.48 mg protein per assay) instead of the *P. denitrificans* membrane preparation.

constant and the amount of non-oxidizable cytochrome *c* were calculated. Measurements with increasing ratios of antibody to cytochrome *c* (Fig. 2, Table 1 showed that near 100% inhibition was reached with 0.5 mol antibody per mol cytochrome *c* (one antibody site per cytochrome *c*; the antibodies are divalent). Any cytochrome *c* not bound to antibody was completely active, i.e., had an unchanged rate constant (Table I). The data show that this antibody binds strongly to cyto-

TABLE I

EFFECT OF ANTIBODY F3-10.2 ON THE REACTIONS OF *P. DENITRIFICANS* CYTOCHROME *c* WITH *P. DENITRIFICANS* CYTOCHROME *c* OXIDASE

The spectrophotometric assays were performed as described in the legend to Fig. 2.

Antibody added/ mol cytochrome *c*	Oxidase	
	non-oxidizable cytochrome *c*	rate contant of oxidizable cytochrome *c* (% of control)
(mol)	(%)	(%)
0.1	31	109
0.15	38	100
0.2	47	100
0.3	69, 79	98
0.5	98	– [a]
1.0	100	– [a]

[a] Completely inhibited.

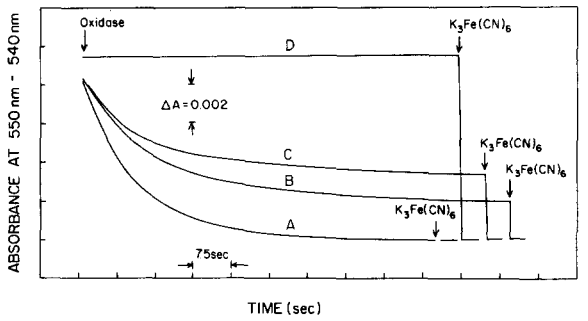

Fig. 2. Recording of the change in absorbance at 550 minus 540 nm as 0.48 μM *P. denitrificans* cytochrome *c* is oxidized by *P. denitrificans* cytochrome *c* oxidase in the presence of F3-10.2 antibody. The different experiments were run with the same concentration of cytochrome *c* in 0.05 M Tris maleate buffer (pH 7.0) and with the same concentration of oxidase containing 0.042 mg protein in 2.8 ml, but with varying concentrations of antibody. At the arrow marked oxidase, 10 μl of diluted *P. denitrificans* detergent-treated membrane fragments were added. At the arrow marked $K_3Fe(CN)_6$, 2 μl of a 10-fold dilution of saturated solution were added. Curve A, no antibody added; curve B, 0.1 mol antibody per mol cytochrome *c*; curve C, 0.2 mol antibody per mol cytochrome *c*; curve D, 1.0 mol antibody per mol cytochrome *c*.

chrome *c* to mask completely the site at which it binds to, and reacts with, the oxidase.

(2) Reductase. The effects of this antibody on the reductase reaction were different from those seen with the oxidase (Fig. 3). No non-reducible cytochrome *c* was formed, but the rate was seen to decrease at all ratios of antibody to cytochrome *c* tested. Thus, the binding/reaction site on cytochrome *c* for the reductase is different from that for the oxidase. The latter is not completely covered by the antibody so that the cytochrome *c* can still bind and react, but at a lower rate.

The effects of antibody F3-10.2 are similar to those seen previously [15] with a single site antibody to human cytochrome *c* isolated from rabbit serum.

Effects of antibodies F3-10.2 and F3-29.4 on electron transport from NADH to O_2 and cytochrome c to O_2, measured polarographically

Table IIA records the effect of addition of purified soluble *P. denitrificans* cytochrome *c* on the NADH oxidase of *P. denitrificans* membranes treated with detergent to expose reaction sites maximally; the rate of O_2 uptake remained unchanged. Also, the two antibodies to the soluble cytochrome *c* had only small inhibitory effects on the NADH oxidase activity (Table IIB, IIC il-

TABLE II

NADH OXIDASE

Deoxycholate-treated membranes containing 0.43 mg protein and 0.16 μM *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. Antibody F3-29.4 was an IgG pool. The results were expressed as μM O_2 uptake/s. The NADH oxidase is more than 98% inhibited by 2.3 μg antimycin A/mg protein.

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

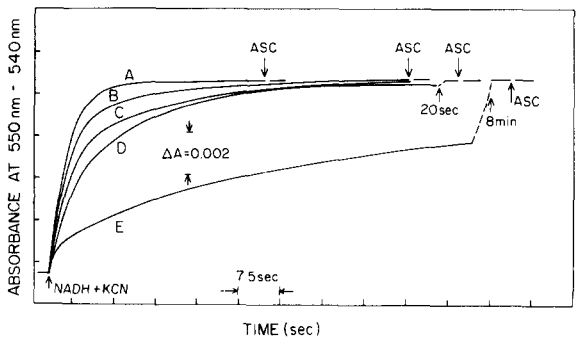

Fig. 3. Recording of change in absorbance at 550 minus 540 nm as 0.48 μM *P. denitrificans* is reduced by the reductase segments of *P. denitrificans* membrane fragments in the presence of F3-10.2 antibody. 10 μl of the diluted *P. denitrificans* membrane fragments containing 0.042 mg of protein were added to the ferrocytochrome *c* in 0.05 M Tris-maleate buffer (pH 7.0). NADH and KCN were added to the reaction mixtures to give final concentrations of 0.36 mM NADH and 0.4 mM KCN. At the arrow marked Asc., approx. 1 mg of solid sodium ascorbate was added. Curve A, no antibody; curve B, 0.1 mol antibody per mol cytochrome *c*; curve C, 0.2 mol antibody per mol cytochrome *c*; curve D, 0.3 mol antibody per mol cytochrome *c*; curve E, 1.0 mol antibody per mol cytochrome *c*.

TABLE III

EFFECT OF ANTIBODY F3-10.2 ON THE OXIDATION OF CYTOCHROME *c* BY *P. DENITRIFICANS* CYTOCHROME *c* OXIDASE

O_2 uptake was measured polarographically in 0.05 M Tris maleate buffer with 2 mM EDTA (pH 7.0) at 26°C, in the presence of 10 mM ascorbate + 0.75 mM TMPD with sequential addition of the substances indicated. Purified *P. denitrificans* cytochrome aa_3 containing 0.234 μM cytochrome $c + c_1$ and 0.183 μM aa_3 was a gift from Dr. E.A. Berry.

	O_2 uptake (μM/s)
A TMPD + ascorbate	0.04
+ *P. denitrificans* cytochrome aa_3 (1.2 mg)	0.21
+ *P. denitrificans* cytochrome *c* (0.96 μM)	0.33
+ antibody (0.246 μM)	0.24
+ antibody (0.49 μM)	0.17
B Antibody (0.442 μM) + TMPD + ascorbate	0.04
+ *P. denitrificans* cytochrome aa_3 (1.2 mg)	0.20
+ *P. denitrificans* cytochrome *c* (0.96 μM)	0.20
+ antibody (0.565 μM)	0.18

lustrates this with antibody F3-29.4) either with or without added cytochrome *c*.

Cytochrome *c* oxidase activity was measured polarographically with TMPD plus ascorbate, since TMPD can penetrate membranes and thus reduce any endogenous cytochrome *c* (or c_1) in addition to any exogenous cytochrome *c* added. Neither antibody F3-10.2 nor F3-29.4 inhibited the reaction of the oxidase with endogenous cytochrome *c* of the detergent-treated membranes, even with a large excess of antibody (data not shown).

A partially purified preparation of *P. denitrificans* oxidase (kindly furnished by E. Berry) showed the presence of cytochrome aa_3 and some *c*-type cytochrome. This preparation gave some O_2 uptake with TMPD and ascorbate in the absence of added soluble cytochrome *c* (endogenous activity), but the O_2 uptake was increased on addition of exogenous cytochrome *c* (Table IIIA). The reaction with the endogenous cytochrome *c* was not inhibited in the presence of an excess of antibody (Table IIIB). But the reaction with the added exogenous cytochrome *c* was blocked (Table IIIA and IIIB), and in the presence of the soluble cytochrome, the rate with equivalent antibody site was less than that seen with the endogenous cytochrome *c* only. The reaction with added cytochrome *c* was completely blocked if equivalent antibody site was added before addition of the cytochrome *c* (Table IIIB).

Thus the reaction with exogenous cytochrome *c* is inhibited by the antibody, whether assayed spectrophotometrically or polarographically, implying that the oxidase-cytochrome *c* complex formed in the polarographic assays rapidly forms and dissociates.

Discussion

Antibodies F3-10.2 and F3-29.4 bind to similar areas on the surface of *P. denitrificans* cytochrome *c*. F3-10.2 binds only to cytochrome *c* from *P. denitrificans* and to cytochromes *c* from *R. capsulata* and *R. molischianum* among a large number of cytochromes from the different species tested. The amino acid sequences of parts of the chains of these three are [13]:

	20	21	22	23	24	25	26
P. denitrificans	Met	Ile	Gln	Ala	Pro	Asp	Gly
R. capsulata	Ser	Ile	Ile	Ala	Pro	Asp	Gly
R. molischianum	Ser	Ile	Asp	Ala	Gly	–	–

These three cytochromes are the only ones having alanine at position 23, and this is the only region in which these three cytochromes have the same residue, in contrast to the other cytochromes *c* tested. Antibody F3-10.2 binds to *P. denitrificans* cytochrome *c* so that it is not possible to align the heme group with that of the oxidase so that electron transfer can proceed; thus, this localizes the site on the cytochrome *c* for reaction with the oxidase. The area is on a loop on the right side of the heme crevice looking into the crevice as the 'front' of the molecule. Any cytochrome *c* in excess of antibody sites reacts with the oxidase with an unchanged rate constant (Table I). This would be consistent with the tight binding of this antibody to the cytochrome *c*, $K_A = 1.6 \cdot 10^7$ [1].

The sequences of the cytochromes from *P. denitrificans* and *R. capsulata* are identical for amino acids 23–26, while that for *R. molischianum* is very different beyond amino acid 23. This makes it look as if the area involved in the binding of antibody F3-10.2 includes amino acid 23 and some of those preceding it or, possibly, nearby in three dimensions.

The second antibody (F3-29.4) binds only to cytochromes from *P. denitrificans* and *R. capsulata*. Since these two cytochromes are identical between amino acids 23 and 26, this may well be the area of binding. We reported previously [2] that antibody 29.4 can completely inhibit both the oxidase and reductase reactions, although at different stoichiometries. Also, the reaction of free, unbound cytochrome *c* with the oxidase and reductase proceeds at decreased rates, and thus some limited area for binding remains. As suggested above, the site at which binding completely inhibits electron transfer to the oxidase may include amino acid 23 and preceding amino acids, which would not be covered in the binding of antibody F3-29.4 to the cytochrome *c*. Because of the way it binds to cytochrome *c*, antibody F3-29.4 can distinguish between binding and electron transfer reactions. Jemmerson and Margoliash [16] have also reported multiple rabbit antibodies to horse cytochrome *c* which recognize 'single sites'.

Antibody F3-10.2 completely blocks the reaction between cytochrome *c* and the oxidase, but does not block the reaction with the reductase. It does produce a decrease in the rate of the reductase reaction, most likely because the incompletely covered site is distorted or less accessible and thus binds and reacts at a lower rate. The effects of this antibody are similar to the effects of an antibody to human cytochrome *c* seen previously [15].

The two antibodies to *P. denitrificans* cytochrome *c* affect the reaction of the cytochrome with the oxidase and reductase differently. The data seem to indicate that the sites on cytochrome *c* at which it binds to and reacts with the oxidase and reductase are not identical, although they may be close or even overlapping. While we have not excluded the interpretation that steric hindrance by the antibody is responsible for the differential inhibition, it is difficult to envisage a combination of antibody with cytochrome *c* where the oxidase is completely blocked while the reductase remains reactive unless the binding sites on cytochrome *c* are different. The observations of several groups [16–20] using derivatization of side-chains on cytochrome *c* and antibody studies lead to the conclusion that the sites on cytochrome *c* for reaction with the oxidase and reductase are the same. However, examination of the data shows that there are some differences in the effects on oxidase and reductase, and probably the types of experiment used would not have revealed the differences seen in these studies of antibody effects. In addition, the antibody studies using the spectrophotometric assay distinguish between effects on binding and effects on electron transfer, in contrast with conclusions using polarographic assays.

The studies by others cited above, with horse cytochrome *c*, localized the reaction sites for oxidase and reductase in an area including the top three-quarters of the heme crevice and an adjoining area on the top left of the molecule. The present studies on *P. denitrificans* cytochrome *c* place these sites on a loop on the right-hand side of the heme crevice including alanine number 23. The horse equivalent, number 21, is not included in the area postulated for horse cytochrome *c*. Since the *P. denitrificans* oxidase and reductase react equally well with cytochrome *c* from bovine, equine (unpublished data) and *P. denitrificans* [21] sources, the results are puzzling. They question the concept of highly specified binding areas. This may be only a reflection of our ignorance of the exact mechanism of electron transport.

Although the soluble cytochrome *c* of *P. denitrificans* can be rapidly oxidized and reduced by the *P. denitrificans* membrane-bound system, addition of the soluble cytochrome *c* to the membranes oxidizing NADH does not increase the rate of O_2 uptake. With one preparation of *P. denitrificans* membranes the cytochrome oxidase rate with 0.48 μM exogenous cytochrome *c* measured spectrophotometrically was 0.042 μM/s calculated from the rate constant, and faster rates of the oxidase were possible. The equivalent O_2 uptake should have been detectable as an increase over the NADH oxidase activity. It appears that the reaction with the soluble cytochrome *c* is inhibited during electron transport through the membrane-bound system. This may be a built-in control mechanism.

Neither of the two antibodies to the soluble cytochrome *c* had appreciable effects on the NADH oxidase activity or on the oxidation of endogenous cytochrome by the oxidase. These data plus our previous observations [22] and the recent work of Berry and Trumpower [23] suggesting an

additional membrane-bound *c*-type cytochrome focus on the interesting question of the role of the soluble *c*-550 in *P. denitrificans*. It is clear that electron transport can proceed without the soluble cytochrome *c*. However, it reacts very rapidly with the oxidase and reductase segments of the electron transport chain, thus must have some functions such as that in the metabolism of methanol [23] or nitrite [24] or others not yet revealed. What is important is to compare this kind of chain with that of mitochondria, where the cytochrome *c* is loosely membrane-bound and dissociates easily with a loss of electron-transport activity.

Whatever is the function of the soluble cytochrome *c* of *P. denitrificans*, it can react very rapidly with the oxidase and reductase segments of the electron transport system, and it has been shown to react directly with the oxidase [22]. Thus, the reactions are good models for the interaction of a soluble cytochrome with membrane-bound enzymes, and the two monoclonal antibodies are revealing subtle differences in binding and in electron-transfer reactions with the oxidase and reductase.

A perusal of the data available leads to the idea that actually there are few bacterial electron transport systems which function like that of mitochondria, with a very loosely bound cytochrome *c* which can be reversibly removed by alteration of the salt concentration. Possibly only those bacteria which have a soluble cytochrome *c* in the periplasmic space (*P. denitrificans* and, for example, some photosynthetic ones) can function with a soluble cytochrome reacting with the membrane-bound ones. Viable cytochrome *c*-deficient mutants have been isolated from bacteria such as *Azotobacter vinelandii* [26] and *P. denitrificans* [23], and bacteria such as *Escherichia coli* synthesize a *c*-type cytochrome only when grown under anaerobic conditions [27].

If, in fact, a bacterium such as *P. denitrificans* was the source of mitochondria [28], it could have supplied the loosely bound *c*-cytochrome which reacts rapidly with oxidase and reductase and which can now function in interchain or intermembrane electron transport. The present arrangement in mitochondria with only the dissociable *c*-cytochrome may have evolved to supply some as yet unrecognized function.

An intriguing aspect of the accumulated data is the difference revealed in reaction sites on the cytochromes *c* from *P. denitrificans* and bovine heart, while they both interact with the respective oxidases and reductases.

Acknowledgments

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