Biochimica et Biophysica Acta, 725 (1983) 238–245
Elsevier

BBA 41395

REACTION OF CYTOCHROME *c* IN THE ELECTRON-TRANSPORT CHAIN OF *PARACOCCUS DENITRIFICANS*

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(Received June 8th, 1983)

Key words: Electron transport; Respiratory chain; Cytochrome c; Cytochrome c oxidase; Polarography; (P. denitrificans)

The reaction of the cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) of *Paracoccus denitrificans* cytoplasmic membranes with the endogenous cytochrome *c* of the membranes was studied, as well as its interaction with added exogenous cytochrome *c* from *P. denitrificans* or bovine heart. The polarographic method was employed, using *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine plus ascorbate to reduce the cytochrome *c*. We found that overall electron transport can proceed maximally while the cytochrome *c* remains membrane bound; NADH or succinoxidase activities were not inhibited by the addition of substances which bind the *P. denitrificans* cytochrome *c* strongly. In contrast to our observations with the spectrophotometric method (Smith, L., Davies, H.C. and Nava, M.E. (1976) Biochemistry 15, 5827–5831), in the polarographic assays the membrane-bound oxidase reacts with about equal rapidity with exogenous bovine and *P. denitrificans* cytochromes *c*. The reaction of the oxidase with the endogenous cytochrome *c* proceeds at high rates and preferentially to that with exogenous cytochrome *c*; the reaction with the latter, but not the former is inhibited by positively charged poly(L-lysine). The cytochrome *c* and the oxidase appear to be very closely associated on the membrane.

Introduction

The functioning of the respiratory chain system of *Paracoccus denitrificans* has stimulated interest because of its many similarities to that of the mitochondrial membrane [1–3]. We have also observed some interesting differences between the two systems [4,5]. There appear to be two or three localizations of cytochromes(s) *c* within the bacteria; some is easily removed by washing, while another portion remains firmly membrane bound along with the other cytochromes. The latter can be removed by treatment of the membranes with 60% ethanol (Pinder, P.B., Nava, M.E. and Smith, L., unpublished data).

In contrast to the mitochondrial respiratory chain system, treatment of the isolated *P. denitrificans* membranes with deoxycholate (1 mg/mg protein) does not result in removal of cytochrome *c* or disruption of the electron-transport system, since NADH or succinate oxidase activities are increased rather than inhibited. Thus, we were able to utilize the *P. denitrificans* membrane system to make studies that were not possible with that on the mitochondrial membrane. Here we report observations on the reactions occurring via the endogenous cytochrome *c* and with added exogenous cytochrome *c*.

In vesicles of the *P. denitrificans* cytoplasmic

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

membrane, treated with deoxycholate to expose maximally all reaction sites, the NADH or succinate oxidase activities were not inhibited by the addition of substances which bind strongly to the purified cytochrome *c* of *P. denitrificans*. Overall electron transport can proceed maximally while cytochrome *c* remains membrane bound.

The reactions with the endogenous cytochrome *c* of the membranes proceed at high rates and preferentially to those with added cytochrome *c* of either bovine or *P. denitrificans*, and the reactions with exogenous cytochrome *c* is particularly low when electron transport via endogenous cytochrome *c* is proceeding rapidly. The endogenous cytochrome *c* and the oxidase seem to form a close association on the membrane.

Soluble cytochromes *c* from *P. denitrificans* or bovine heart react with the *P. denitrificans* oxidase with about equal rapidity when assayed polarographically; in contrast, we found previously [5] that the rate *ks* measured spectrophotometrically were higher with the bovine cytochrome. Other differences in the two kinds of assay are apparent. Preliminary presentations of some of these data have been made [4].

Methods

P. denitrificans Membrane Fraction. P. denitrificans (ATCC 13543) was cultured aerobically in the medium and under the growth conditions decribed by Scholes and Smith [6]. The cytoplasmic membrane fraction was isolated following osmotic shock of spheroplasts [6]. It was stored in 0.25 M sucrose/0.01 M phosphate, pH 7.4, at −20°C, where the respiratory chain activity was maintained at the original level for several months. Examination of the suspension with a phase contrast microscope revealed a mixture of 'ghosts', as described by Scholes and Smith [6], along with smaller vesicles; the proportion of the two was somewhat variable from preparation to preparation.

Treatment of the vesicle suspension with detergent consisted of addition of 10% detergent to a concentrated suspension of vesicles (at least 13 mg protein/ml), then immediate dilution with cold water to the desired extent. Detergent-treated preparations were utilized for a maximum of 5 h after preparation.

The protein content of the vesicles was assayed by the biuret method [7], with the control described by Scholes and Smith [6].

Reduced minus oxidized difference spectra of the membrane-bound oxidation-reduction pigments were measured with a Cary spectrophotometer [6]. From these the contents of cytochrome aa_3 were calculated, assuming that the $\Delta A_{605-630\ nm}$ was 20.4 mM^{-1}.

Assays. NADH, succinate, formate and lactate oxidase activities were assayed with a Clark-type oxygen electrode [1]. Before measuring succinate oxidase activity, the succinate dehydrogenase was 'activated' by incubating the preparation for 20 min at 26°C with 20 mM succinate. Cytochrome *c* oxidase activity was also measured polarographically in the presence of: (1) 30 mM ascorbate plus added soluble cytochrome *c* or (2) 0.7 mM TMPD plus 30 mM ascorbate in the presence or absence of added cytochrome *c*. TMPD can penetrate the membranes, while ascorbate cannot; thus, TMPD can also react with the cytochrome *c* of the membrane vesicles which is not exposed for reaction on the membrane surface (all of endogenous cytochrome *c*). Corrections were made for any KCN-insensitive O_2 uptake [8]. Cytochromes *c* from both bovine heart and from *P. denitrificans* were employed as added exogenous cytochrome *c*. The former was prepared by the method of Margoliash and Walasek [9] and subjected to isoelectric focusing [10]; the latter was purified following the method of Scholes et al. [11], then also subjected to isoelectric focusing.

We found that variations of buffer type, ionic strength or pH (between 6.4 and 7.8) had only minor effects on the data reported. The buffers used in the different assays are given in the legends to the figures and tables.

The extent of reduction of added cytochrome *c* by the oxidase in the aerobic state in the presence of ascorbate or of ascorbate plus TMPD was measured as described previously [8]

Chemicals. Sodium ascorbate, deoxycholic acid, Hepes and TMPD dihydrochloride were obtained from Sigma Chemical Co. The sodium ascorbate and deoxycholic acid were recrystallized from hot water and from ethanol, respectively, and the TMPD dihydrochloride was brought to pH 6 immediately before use. Poly(L-lysine) hydrobromide

(mol. wt. 19500) was obtained from Miles-Yeda, Ltd., and was converted to the free base and the Br^- removed as described previously [5]. DEAE-Sephadex A-25 was a product of Pharmacia Fine Chemical Co. Tris base was obtained from Schwartz-Mann.

Results

Procedures which disrupt membrane vesicles, such as freeze-thawing or exposure to sonic oscillation or to the detergent Triton-X-100, were found to result in some increase in the NADH or succinate oxidase activities of the untreated suspensions of membrane vesicles [1]. Fig. 1 plots the effect of the addition of the detergent deoxycholate on NADH oxidase or on cytochrome *c* oxidase activities with ascorbate and bovine cytochrome *c* (exogenous cytochrome *c*) or with ascorbate plus TMPD (endogenous cytochrome *c*), measured in 0.05 M Tris-maleate buffer, pH 7.8. The maximal increase in all activities was found at a ratio of 1 mg deoxycholate/mg protein. At this ratio dramatic clarification of the turbid suspension was seen, and no vesicles were apparent when the treated preparations were viewed with the phase contrast microscope. Apparently, rupture of the vesicles resulted in the exposure of reactive sites for NADH and cytochrome *c*. Further increase in the ratio of deoxycholate/mg protein resulted in decreased rates of all activities. Succinate oxidase activity was increased to only a small extent by deoxycholate treatment under optimal conditions, and the lactate and formate oxidase activities of the untreated vesicle were lost (Table I).

TABLE I

EFFECT OF DEOXYCHOLATE (1 mg/mg PROTEIN) ON ELECTRON-TRANSPORT ACTIVITIES OF *P. DENITRIFICANS* MEMBRANES

The polarographic assays were run in 50 mM Tris-maleate buffer, pH 7.25, as described in Methods.

	μM O_2 uptake/s per mg protein	
	Untreated membranes	Deoxycholate-treated membranes
NADH oxidase	6.77	13.5
Succinate oxidase	3.45	4.18
Lactate oxidase	1.82	0.25
Formate oxidase	0.77	0

The plots of Fig. 2 show that the cytochrome *c* oxidase of deoxycholate-treated preparations with added exogenous cytochrome *c*, assayed as O_2 uptake in the presence of 30 mM ascorbate, is similar with a wide range of concentrations of bovine cytochrome *c* and *P. denitrificans* cyto-

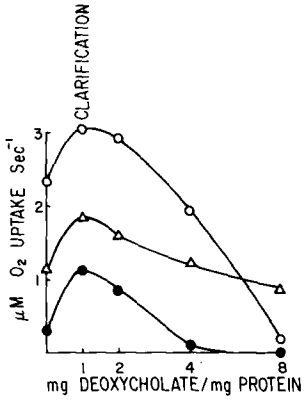

Fig. 1. Effect of detergent treatment on oxidase activities of *P. denitrificans* membranes. All activities were tested in 50 mM Tris-maleate buffer, pH 7.8, with membranes containing 0.14 mg protein. (●) 5 mM NADH was present in the assyas for NADH oxidase. Cytochrome *c* oxidase was assays with 30 μM bovine cytochrome *c* plus 30 mM ascorbate (exogenous cytochrome *c*) (△); or with 30 mM ascorbate plus 1.6 mM TMPD (endogenous cytochrome *c*) (○).

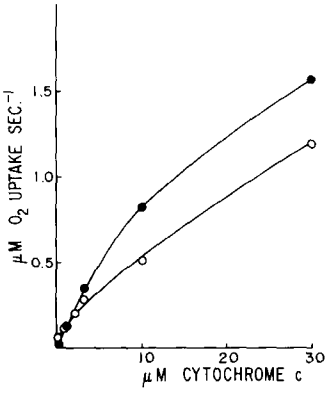

Fig. 2. Oxidation of exogenous bovine (●———●) and *P. denitrificans* (○———○) cytochrome *c* by cytochrome *c* oxidase. Assays were made with deoxycholate-treated membranes containing 0.09 mg protein in 50 mM Tris-acetate buffer, pH 7.0, with 30 mM ascorbate.

chrome *c*. In this respect the membranes are different from those of most bacterial species, which do not oxidize mammalian cytochrome *c* [12]. Because it is more readily available, bovine cytochrome *c* was used in most of the experiments reported here.

As mentioned above, in assaying cytochrome *c* oxidase by measuring O_2 uptake, either ascorbate or a combination of ascorbate plus TMPD can be used to maintain the cytochrome *c* largely in the reduced form during the reaction. Only the TMPD can penetrate membranes [13] and react with any endogenous cytochrome *c* on membrane vesicles oriented so that the cytochrome *c* reactive sites are not exposed. Thus, the assays with ascorbate measure only the reaction of exogenous cytochrome *c* with the exposed cytochrome *c* reactive sites, while TMPD (reduced with ascorbate) can reduce both the endogenous and the added exogenous cytochromes *c*. It is surprising that the rates with TMPD plus ascorbate were increased somewhat by treatment of the vesicles with detergent to expose reaction sites maximally (Fig. 1).

Fig. 3 plots the rates of O_2 uptake with the endogenous cytochrome *c* of untreated vesicles with 30 mM ascorbate and increasing concentrations of TMPD. High rates are observed, but rather large concentrations of TMPD are required for maximal rates, as with the membrane vesicles from bovine heart [13]. The rates of reaction with the endogenous cytochrome *c* were always considerably greater than those with added exogenous cytochrome *c*, even with very high concentrations of added cytochrome *c* and were much higher with low concentrations of added cytochrome *c* (Fig. 4).

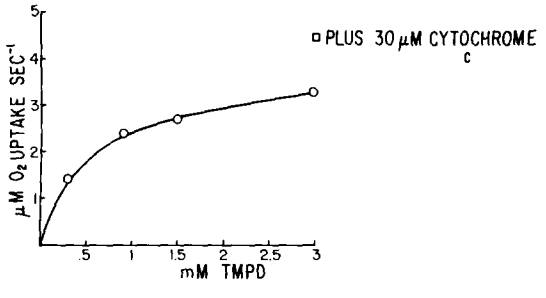

Fig. 3. Effect of the concentration of TMPD on the rate of respiration with endogenous cytochrome *c*. Assays were run in 0.25 M sucrose/0.01 M phosphate, pH 7.0, with membranes containing 0.38 mg protein and 30 mM ascorbate.

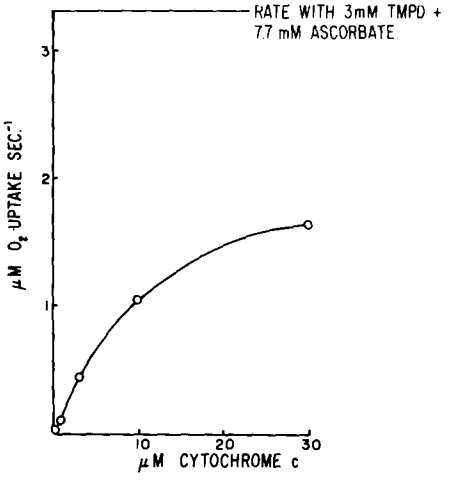

Fig. 4. Reaction of cytochrome *c* oxidase with exogenous and endogenous cytochrome *c*. Assays were run in 0.25 M sucrose/0.025 M Tris-maleate buffer, pH 7.8, with 30 mM ascorbate plus bovine cytochrome *c* or with 7.7 mM ascorbate plus 3 mM TMPD. Each assay contained 0.13 mg membrane protein.

The data of Table II show that the addition of even high concentrations of bovine cytochrome *c* to either untreated or deoxycholate-treated vesicles oxidizing NADH with O_2 gave little or no increase in O_2 uptake rates at either pH 6.4 or 7.8.

The structure of the *P. denitrificans* membrane-bound system, with the endogenous cytochrome *c* firmly attached, shows a strong preference for endogenous cytochrome *c*; sites for reaction with exogenous cytochrome *c* are not available to the same extent.

TABLE II

NADH OXIDASE OF MEMBRANES WITH AND WITHOUT ADDED EXOGENOUS CYTOCHROME *c*

Assays were run with 0.19 mg protein of untreated membranes and 0.073 mg protein of deoxycholate-treated membranes in 50 mM Tris-maleate buffer, pH 6.4 or 7.8, with 1.92 mM NADH and 30 μM bovine cytochrome *c* where indicated. The results are expressed as μM O_2 uptake/s.

pH	Untreated membranes		Deoxycholate-treated membranes	
	No cytochrome *c*	+cytochrome *c*	No cytochrome *c*	+cytochrome *c*
7.8	0.43	0.38	0.96	1.22
6.4	0.42	0.44	0.48	0.65

The measured turnover rates of the cytochrome *c* oxidase were high. With one preparation the rate was 844 s^{-1} in the reaction with endogenous cytochrome *c*, and this was increased to 957 s^{-1} on addition of 30 μM bovine cytochrome *c*.

Added exogenous 30 μM cytochrome *c* was found to be largely reduced in the aerobic state in the presence of either 30 mM ascorbate or of 30 mM ascorbate plus 0.7 mM TMPD: 90% with the former and 98% with the latter.

A concentrated membrane preparation was treated with deoxycholate to give maximal exposure of reaction sites, then made 30% saturated with ammonium sulfate and the mixture centrifuged to collect the well packed pellet and the supernatant fluid. Examination of reduced minus oxidized spectra showed that all of the cytochromes were found in the precipitate except for a small amount of what appeared to be a *b*-type cytochrome. The bulk of all of the oxidase reactions was also in the pellet. This observation differs from that seen with membrane fragments from bovine heart mitochodria, where similar treatment removes the cytochrome *c*.

Purified cytochrome *c* from *P. denitrificans* will bind strongly to DEAE-cellulose [11] or DEAE-Sephadex in solutions of relatively low ionic strength and also to poly(L-lysine) [5]. The effect of adding either of these substances to a suspension of membrane preparations oxidizing NADH or succinate is shown in Table III. Some of the measurements were made with vesicles ruptured with deoxycholate treatment to expose reaction sites. Succinate oxidase activity of treated or untreated vesicles was not inhibited when several 3-mg portions of a slurry of DEAE-Sephadex were added to the reaction mixture and, in fact, was slightly stimulated. Succinate oxidase activity was also not inhibited by concentrations of poly(L-lysine) which strongly inhibited the reaction of added exogenous cytochrome *c* with the oxidase. NADH oxidase was not affected by addition of DEAE-Sephadex. Untreated preparations showed no change in activity on addition of poly(L-lysine), but deoxycholate-treated preparations were somewhat inhibited.

Discussion

The respiratory chain system of *P. denitrificans* has been shown to have components similar to those associated with the mitochondrial inner membrane and to have high NADH and succinate oxidase activities [1]. In addition, lactate and for-

TABLE III

EFFECT OF POLY(L-LYSINE) AND DEAE-SEPHADEX ON OXIDASE ACTIVITIES OF *P. DENITRIFICANS* MEMBRANES

(I) Succinate oxidase and cytochrome *c* oxidase assays were run in 50 mM Tris-acetate buffer, pH 7.1, with 0.18 mg of untreated membrane protein or 0.09 mg protein of deoxycholate-treated membranes in each with 19 mM succinate or 30 μM bovine cytochrome *c* plus 30 mM ascorbate. NADH oxidase assays were made with another membrane preparation. Each assay contained 1.6 mg protein of untreated membranes or 0.2 mg protein of deoxycholate-treated membranes in 25 mM Hepes, pH 7.15, with 2 mM NADH. The concentration of poly(L-lysine) was 0.38 μM except with succinate and cytochrome *c* oxidase of untreated membranes, where it was 0.58 μM. The results are expressed as μM O_2 uptake/s. (II) NADH oxidase was run with 0.2 mg protein of deoxycholate-treated membranes in 25 mM Hepes, pH 7.15, with 2 mM NADH. Succinate oxidase was assayed with a different membrane preparation; each assay contained 0.18 mg protein in 50 mM Tris-acetate buffer, pH 7.1. DEAE-Sephadex was added as a slurry in the same buffer (3 mg dry weight per addition). The results are expressed as μM O_2 uptake/s.

	Untreated membranes		Deoxycholate-treated membranes	
(I) Effect of poly(L-lysine) (PL):	No PL	+PL	No PL	+PL
succinate oxidase	0.20	0.35	0.11	0.20
NADH oxidase	0.41	0.40	0.80	0.44
cytochrome oxidase with exogenous bovine cytochrome *c*	0.64	0.16	0.83	0.38
(II) Effect of DEAE-Sephadex (DS)	No DS	+DS	No DS	+DS
succinate oxidase	0.28	0.35		
NADH oxidase			0.84	0.82

mate can be oxidized. However, the association of the oxidation-reduction pigments with the lipoprotein membranes differs in some respects in the bacterial as compared with the mitochondrial system. There are at least two different localizations of cytochrome *c* in *P. denitrificans* (Ref. 11 and Pinder, P.B., Nava, M.E. and Smith, L., unpublished data). Part of the cytochrome is easily extractable, but part is tightly membrane bound. Here we show that treatment of the isolated membranes with deoxycholate, then suspension in buffer, increases the NADH oxidase activity, in contrast to the the loss of activity on similar treatment of heart submitochondrial particles. Also, the precipitate obtained on addition of ammonium sulfate to the deoxycholate-treated membranes contained all of the cytochrome *c* which was associated with the membranes, as well as nearly all of the other cytochromes. This procedure results in loss of the cytochrome *c* from fragments of inner mitochondrial membrane. Using different conditions, Erecinska et al. [14] found that treatment of *P. denitrificans* membranes with cholate and ammonium sulfate resulted in retention of some cytochrome *c* along with rapid respiration rates.

The reaction of the *P. denitrificans* oxidase with the endogenous membrane-bound cytochrome *c* of the vesicles is very rapid. The turnover rate approaches 1000 s^{-1} when assays are run in 50 mM Tris-maleate buffer, pH 7.0, at 26°C. Similar values were reported by Erecinska et al. [14] for intact cells.

The experiments reported here give evidence that electron transport can proceed in such systems with the cytochrome *c* remaining tightly membrane bound, refuting postulations that it must come off and on from the reductase to the oxidase segment during electron transport. When the membrane fraction treated with deoxycholate to disrupt the vesicles was oxidizing NADH or succinate, addition of DEAE-Sephadex did not inhibit O_2 uptake. DEAE-Sephadex binds purified cytochrome *c* of *P. denitrificans* strongly under the conditions used [11] and should bind all such cytochromes *c* with acid isoelectric points, even if the endogenous cytochrome *c* has some different properties from that easily extracted from intact bacteria. Thus, it should have picked up any cytochrome *c* which dissociated from the membranes during electron transport, resulting in inhibited respiration. Poly(L-lysine), which also binds the purified cytochrome *c* [5], did not inhibit the succinate oxidase activity of untreated or deoxycholate-treated membranes or the NADH oxidase activity of untreated membranes. A different kind of evidence implies that the cytochrome *c* of mitochondrial membranes also functions while membrane bound, in spite of its ease of removal from the membranes. When modified bovine cytochrome *c* was covalently bound to cytochrome *c*-depleted rat liver mitochondria, significant ability to respire was retained [15]; tight binding of the cytochrome *c* did not block electron-transport activity. However, the extent of formation of the derivative was uncertain.

Two questions that emerge from the present observations on the *P. denitrificans* system are:

(1) With what does added exogenous cytochrome *c* interact?

(2) How do the reactions measured here with the polarographic assays compare with those previously seen using the spectrophotometric method?

The soluble exogenous cytochrome *c* could react directly with the oxidase or with the endogenous cytochrome *c* of the membranes, since cytochrome *c* can undergo intermolecular electron exchange. Our data imply that the soluble cytochrome *c* reacts directly with the oxidase. Evidence for this lies in the competitive inhibition by poly(L-lysine) of the reaction of the oxidase with exogenous bovine or *P. denitrificans* cytochromes *c*. Also, both bovine and *P. denitrificans* cytochromes *c* react with the oxidase with about equal rapidity in the polarographic assays, although the isoelectric points are very different. The two cytochromes do have a common binding site for the *P. denitrificans* oxidase [5].

Differences are apparent when data obtained with the polarographic and spectrophotometric methods are compared. In contrast to the similarity of the rates with bovine and *P. denitrificans* cytochrome *c* measured polarographically, in the spectrophotometric assays with 4.3 μM cytochrome *c* the rate constants for the reaction with *P. denitrificans* cytochrome were only 40% of those with the bovine cytochrome [5]. Also, poly(L-lysine) gave different effects on the activities with the two

assay methods. Low concentrations of the positively charged polymer bind to a negatively charged site on *P. denitrificans* cytochrome *c* and stimulate its reaction with the oxidase measured spectrophotometrically, but no stimulation is observed with bovine cytochrome *c* [5]. No stimulation is observed with either cytochrome *c* when the assays are run polarographically. The importance of the negatively charged group is only evident in the spectrophotometric assays, where the cytochrome *c* must be released from the oxidase before another molecule can react. In the polarographic method there is continuous turnover of the cytochrome *c* while bound to the oxidase. We have emphasized that the two types of assays measure different aspects of the reaction of cytochrome *c* with the oxidase [8,16].

High enough concentration of poly(L-lysine) can inhibit the reaction of the *P. denitrificans* oxidase with exogenous bovine or *P. denitrificans* cytochrome *c* [5] (Table III); however, the reaction with endogenous cytochrome *c* is not inhibited by comparable concentrations of poly-L-lysine) (Table III). The positively charged poly(L-lysine) must compete with a positively charged site of lysines on the exogenous cytochrome *c* for binding to the oxidase [17]. Thus this positively charged site on the endogenous cytochrome *c* must be closely associated with the oxidase on the membrane and not available for binding to poly(L-lysine), even for brief intervals.

The turnover rate with the endogenous pigment (measured with TMPD plus ascorbate), even in deoxycholate-treated vesicles, is much higher than that with exogenous cytochrome *c* (measured with ascorbate plus cytochrome *c*). Also, the addition of even high concentrations of cytochrome *c* to the system oxidizing NADH does not increase the rate of O_2 uptake, even though both oxidase and reductase segments react with added cytochrome *c* [5]. Clearly, the pathway via endogenous cytochrome *c* proceeds preferentially. Perhaps sites are not available to the exogenous cytochrome *c* during rapid electron transport due to a change in conformation. The relatively tight binding of the cytochrome *c* in the *P. denitrificans* system should allow studies yielding further insight into the interactions of these membrane-bound oxidation-reduction pigments.

The precise effect of detergents on the *P. denitrificans* vesicles is not clear. We previously showed that treatment with Triton X-100 increased the activity of succinate oxidase under some conditions, but not that of NADH oxidase [1]. We now find that deoxycholate treatment increased NADH oxidase activity, but has much less effect on the succinate oxidase system. We have also obtained evidence that there are two pathways for the oxidation of succinate in *P. denitrificans* [4]. The maximal stimulation of NADH oxidase and cytochrome *c* oxidase activities on treatment with deoxycholate was seen at a ratio of deoxycholate to membrane protein where clarification of the turbid suspension occurred, presumably where the vesicles were ruptured and formed mixed micelles with the detergent. Thus there was increased exposure of reaction sites that had been unavailable. What is surprising is that deoxycholate treatment also increased the maximal rate of O_2 uptake with TMPD plus ascorbate and to the same extent as with ascorbate plus cytochrome *c*. Since TMPD can penetrate the membranes, detergent treatment should not have been necessary for maximal activity. Some of the problems of assaying cytochrome oxidase using TMPD as a reductant have been apparent in studies with membrane vesicles [8,18], so the explanation may partly lie here.

Acknowledgements

We appreciate the valuable assistance of George McLain in the preparation of the purified cytochromes *c*. This research was supported by the National Institutes of Health through Grant GM-06270 and General Research Support Grant RR-05392 to L.S. and by Grant HL28272 and Biochemical Research Support Grant RR-07083 to H.C.D.

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