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Kinetics of the interaction of the cytochrome *c* oxidase of *Paracoccus denitrificans* with its own and bovine cytochrome *c*

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We have devised a relatively simple method for the purification of cytochrome aa_3 of *Paracoccus denitrificans* with three major subunits similar to those of the larger subunits of the mitochondrial cytochrome oxidase. This preparation has no *c*-type cytochrome. Studies were made of the oxidation of soluble cytochromes *c* from bovine heart and *Paracoccus*. The cytochrome-*c* oxidase activity was stimulated by low concentrations of either cytochrome *c*, providing an explanation for the multiphasic nature of plots of v/S versus v. Kinetics of the oxidation of bovine cytochrome *c* by the *Paracoccus* oxidase resembled those of bovine oxidase with bovine cytochrome *c* in every way; the *Paracoccus* oxidase with bovine cytochrome *c* can serve as an appropriate model for the mitochondrial system. The kinetics of the oxidation of the soluble *Paracoccus* cytochrome *c* by the *Paracoccus* oxidase were different from those seen with bovine cytochrome *c*, but resembled the latter if poly(L-lysine) was added to the assays. The important difference between the two species of cytochrome *c* is the more highly negative hemisphere on the side of the molecule way from the heme crevice in the *Paracoccus* cytochrome. Thus, the data emphasize the importance of all of the charged groups on cytochrome *c* in influencing the binding or electron transfer reactions of this oxidation-reduction system. The data also permit some interesting connotations about the possible evolution from the bacterial to the mitochondrial electron transport system.

Introduction

The bacteria *Paracoccus denitrificans,* grown aerobically, synthesize an electron transport chain with components that are very similar to those of the mitochondrial system, including a soluble cytochrome *c* and a membrane-bound cytochrome oxidase [1,2]. The respiratory chain associated with the *Paracoccus* cytoplasmic membrane differs from the analogous system of the mitochondrial inner membrane by the presence of an additional membrane-bound cytochrome *c* (absorption peak in the reduced form at 552 nm), which is closely associated with the cytochrome aa_3 [3]. A cytochrome c_1 is also present, in a complex with the cytochrome *b*, as in the mitochondrial system [4], as well as a soluble cytochrome *c* localized in the periplasmic space [5] with a peak in the absorption spectrum in the reduced form at 550 nm.

Our previous work shows that the NADH oxidase system of isolated and detergent-treated vesicles of *Paracoccus* cytoplasmic membrane in-

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; SDS, sodium dodecyl sulfate.

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volves only the membrane-bound *c*-cytochromes (c_1 plus *c*-552). During electron transport from NADH, no increase in oxygen uptake is observed on addition of the purified, soluble cytochrome *c*-550 [6]. However, the membrane-bound or the purified oxidase can oxidize the soluble cytochrome *c*-550 and, at an even greater rate, purified bovine heart cytochrome *c*. A known function of the soluble periplasmic cytochrome *c* is that it can be reduced by a methanol dehydrogenase [7] in the periplasmic space [8] and then oxidized by the oxidase.

The purified cytochrome-*c* oxidase of *Paracoccus* has cytochrome aa_3 and copper components like those of the mitochondrial cytochrome oxidase [9] and shows similar behavior in stopped-flow kinetic measurements [10]. The purified oxidase preparation of Ludwig and Schatz [11], has only two subunits, similar to subunits I and II of the mitochondrial system [12]. However, Saraste et al. [13] have found evidence for DNA coding for a protein homologous to subunit III of the mitochondrial oxidase. The additional subunits of the mitochondrial oxidase (as many as ten in bovine heart mitochondrial), synthesized on cytoplasmic ribosomes, are lacking in the *Paracoccus* enzyme. We have devised a new preparation of the *Paracoccus* oxidase, purified using dodecyl maltoside as the only detergent, which has three major subunits of molecular weight similar to those of the mitochondrial oxidase and several minor bands. Most important to these studies is that the preparation has no *c*-type cytochromes.

We have examined the kinetics of oxidation of soluble purified cytochromes *c* of bovine heart and *Paracoccus* by the purified *Paracoccus* cytochrome aa_3. With either of the cytochromes *c*, our data give direct evidence for activation of the oxidase in the presence of cytochrome *c*. These observations offer an explanation for the postulated 'high'- and 'low'-affinity sites on cytochrome *c* for the oxidase. The differences in the reaction of the oxidase with the two species of cytochrome *c* emphasize the importance of charge distribution on the whole cytochrome *c* molecule.

Materials and Methods

Cell membrane preparation. Wild-type (ATCC 13543) and cytochrome *c*-deficient (HUUG-25 from Henk van Verseveld) strains of *P. denitrificans* were grown aerobically in yeast extract/peptone/dextrose (Difco) media [14] at 30 °C to late log phase (16–22 h, 250 Klett units) in 400 ml medium in 3-l baffled flasks shaken at 250 rpm or in 7.5 l medium in a 20-l carboy sparged with air at constant pressure. Bacteria were harvested with a Millipore Pellicon durapore cassette system and/or by centrifugation at $2000 \times g$ for 15 min. Cells were frozen as a paste or used immediately. Cell membranes were prepared by lysozyme treatment and osmotic lysis using the method of Scholes and Smith [14] and were frozen at −20 °C. When used in oxidase assays, the membranes were treated immediately prior to assay with an amount of 10% deoxycholate, usually 0.5–1 mg detergent/mg protein, to give a maximal stimulation of activity [15] when assayed with an oxygen electrode using 0.7 mM TMPD and 10 mM ascorbate [16] in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0 at 26 °C). It was noted that including detergent in the assay buffer without prior treatment with detergent resulted in an inhibition of oxidase activity. Also, the preparation was more stable throughout the day when diluted in cold distilled water (4 °C) instead of buffer.

Cytochrome oxidase preparations. Fractions containing membrane-bound cytochrome *c* and cytochrome oxidase (*c*-552-aa_3 complex), described by Berry and Trumpower [3] were obtained from Jeffrey Pennoyer and Xiaohang Yang during their purifications of the succinate-ubiquinone reductase [17] and cytochrome bc_1 [4] complexes from *Paracoccus*. The *c*-552-aa_3 complex eluted at the start of the 250–400 mM NaCl gradient of the 1st DEAE column [17] and could also be collected in fractions at the trailing end of the 200 mM NaCl flow-through of the DE-52 column [4]. These pooled fractions were concentrated with an Amicon PM-10 ultrafiltration cell, diluted with an equal volume of glycerol, and stored at −20 °C.

The cytochrome *c*-552 could be split from cytochrome aa_3 by incubation in a high dodecyl maltoside-containing buffer and chromatography on a DEAE-CL-6B (Sigma) column as follows. The concentrated cytochrome *c*-552-aa_3 fraction was diluted with an equal volume of 50 mM KP_i (pH 8) 60 mg/ml dodecyl maltoside and stirred on ice for 60 min. This sample (20 mg) was loaded

onto a 2.6 × 10 cm DEAE-CL-6B column ($V_t = 25$ ml), which had been pre-equilibrated with 130 mM NaCl, 50 mM KP_i, 0.2 g/l dodecyl maltoside (pH 8) buffer. The column was washed with 4 volumes of the same buffer at a flow rate of 16 ml/h. By monitoring the absorbance of 2 ml fractions at 280 and 422 nm and running difference spectra of the peaks, it was found that the cytochrome *c*-552 came off the column immediately and was followed by residual undissociated *c*-oxidase complex. The cytochrome aa_3 was eluted by a 130–260 mM NaCl gradient (8 volumes). Peak fractions were pooled and stored in 50% glycerol. The concentration of cytochrome aa_3 was determined using $E_{605-630(red)}$ of 11.7 $cm^{-1} \cdot mM^{-1}$ [11].

The cytochrome aa_3 could also be purified from dodecyl maltoside-extracted membranes from the cytochrome *c*-deficient mutant using two successive DEAE columns under conditions similar to those decribed above for the splitting of the cytochrome *c*-552-aa_3 fraction. Membranes were extracted with dodecyl maltoside [4] and incubated with NaCl (130 mM final concentration) for 1 h before loading onto the first column.

Prior to assay, the cytochrome *c*-552-aa_3 and aa_3 preparations were either diluted with cold distilled H_2O or treated with asolectin. In the latter case, using a modification of the direct incorporation procedure of Eytan [18,19], 1–3 μM purified cytochrome aa_3 was incubated at 22°C for 10 min in sonicated asolectin. The asolectin (Associated Concentrates, Long Island, NY) was prepared by homogenizing 50 mg/ml in 12.5 mM potassium-Hepes (pH 7.2). The suspension was sonicated to clarity, and added to the enzyme in a phospholipid-to-protein ratio of around 75 (w/w).

Cytochrome 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 extinction coefficients: $E_{550(red)} = 27.6$ $cm^{-1} \cdot mM^{-1}$ for bovine [20] and $E_{550(red)} = 26.8$ $cm^{-1} \cdot mM^{-1}$ 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) [21]. 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.

Oxidase assays. Cytochrome *c* oxidase was assayed spectrophotometrically at 550–540 nm [22,23] using the turbine-driven, time-sharing, multichannel spectrophotometer built by the Johnson Foundation, University of Pennsylvania, Philadelphia, PA [24]. The activities were expressed as first-order rate constants, k_{obs} [22] and calculated by a least squares regression method. Unless otherwise indicated, assays were run in 50 mM Tris-maleate buffer, 2 mM EDTA (pH 7.0, 25°C). Buffer pH was adjusted by titrating with 3 M maleic acid (H_2Mal) or concentrated NaOH. Buffers varying in ionic strength were all made with 2 mM EDTA (pH 7.0). The post-assay pH values were 7.00 ± 0.05. Calculations of ionic strength were made using the following p*K* values: Tris, 8.1; maleic acid (pK_1), 1.92; maleic acid (pK_2), 6.23; 2 mM EDTA contributed 0.0082 M to the final value [25].

The conditions for the polarographic assays were as described above. Rates are expressed as μM O_2 uptake/s per μM cytochrome aa_3; to obtain equivalent spectrophotometric rates (μM cytochrome *c* oxidized/s per μM cytochrome aa_3), rates of oxygen uptake were multiplied by 4 [23].

Difference spectra. Dithionite-reduced minus oxidized spectra of membranes and preparations were obtained by scanning from 400 to 650 nm with a dual wavelength spectrophotometer. Calculations of cytochrome concentrations were made using the extinction coefficients for wavelength pairs according to Williams [26,27].

Analytical procedures. Protein was determined by the BCA (Pierce) [28] or modified Lowry [29] procedures. Polyacrylamide gel electrophoresis was carried out using 12.5% or 11–17% gradient gels, in the presence of sodium dodecyl sulfate (SDS), according to the Laemmli procedure [30]. Gels were stained for protein with Coomassie blue R-250 [31].

Materials. 20 kDa poly(L-lysine) was purchased from Yeda, dodecyl maltoside was from Boehringer Mannheim, TMPD was from Kodak, chemicals for electrophoresis were from Biorad and all other reagents were from Sigma.

Results

Purification of the oxidase

Following the methods of Yang and Trumpower [4] and Pennoyer and Trumpower [17], a fraction of *Paracoccus* membranes containing cytochrome aa_3 and a *c*-type cytochrome was obtained. Berry and Trumpower [3] showed that this complex was split from the ubiquinol-cytochrome-*c* oxidase 'supercomplex' studied by them. By incubating this fraction in a high concentration of dodecyl maltoside, the cytochrome *c*-552 could be removed from the cytochrome aa_3. An SDS-polyacrylamide gel of a heavily stained preparation of the purified cytochrome aa_3 (Fig. 1) shows three major bands of molecular mass 44, 28 and 23.5 kDa and several minor bands. The dithionite-reduced minus oxidized difference spectrum (Fig. 2) is typical of cytochrome aa_3 with absorption peaks at 607 and 445 nm. There is no evidence of a *c*-type cytochrome in the preparation.

The method of preparation is relatively simple. It uses dodecyl maltoside as the only detergent and can be completed in 2 days. The enzyme is stable when stored at −20 °C in 50% glycerol.

Activity of the oxidase

Effect of pH and ionic strength. In agreement with numerous studies, the amount of oxidase in these assays was directly proportional to the first-order rate constant at a fixed concentration of cytochrome *c*. Spectrophotometric assays in 50 mM Tris-maleate buffers showed a decrease of rate *k* with increasing pH (Fig. 3) with both bovine and *Paracoccus* cytochromes *c*. At any pH value, the activities with bovine cytochrome *c* exceeded those with the *Paracoccus* cytochrome. Increasing ionic strength at pH 7 with 1 μM cytochrome *c* had little effect with *Paracoccus* cytochrome *c*, but a large effect with bovine cytochrome *c*, which showed peak activity at an ionic strength of 0.06 M (Fig. 4). When using Tris-cacodylate, which is a 'non-binding' buffer [32] we

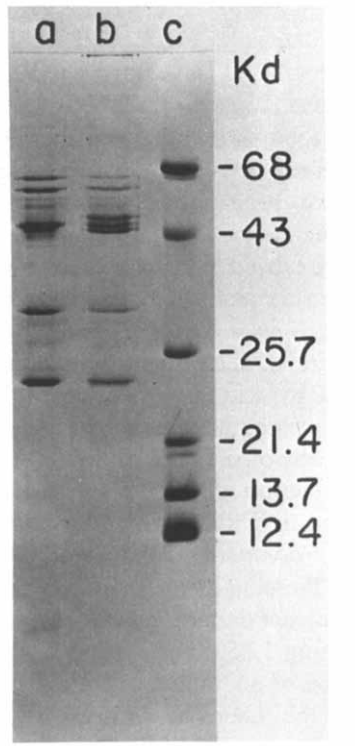

Fig. 1. Sodium dodecyl sulfate-gel electrophoresis of purified cytochrome aa_3 from wild-type (lane a) and cytochrome *c*-deficient (lane b) *Paracoccus*. Three major bands of 44, 28 and 23.5 kDa are apparent in each preparation. Molecular weight standards (lane c) are of the indicated kDa. Approx. 25 μg of the oxidase preparation was applied to each lane of the 11–17% gel. The identity of the minor polypeptides is unknown.

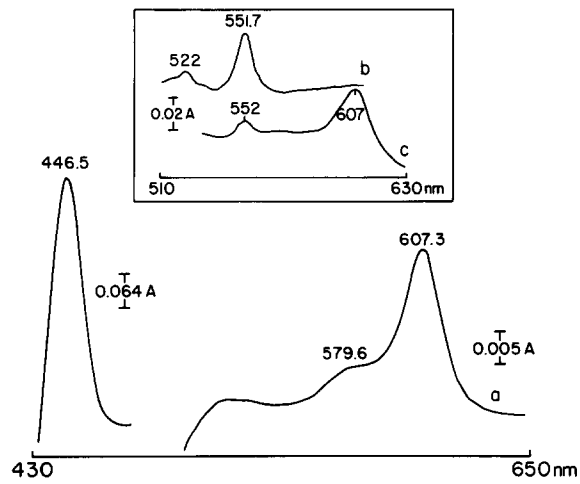

Fig. 2. Difference spectra of *Paracoccus* cytochromes *c*-552-aa_3, *c*-552, and aa_3. Traces a–c are dithionite-reduced minus air-oxidized spectrum as follows: trace a, purified aa_3 from wild-type *Paracoccus*; trace b, cytochrome *c*-552 from the flow-through of the DEAE-column as described in Materials and Methods; trace c, cytochrome *c*-552-aa_3 used as starting material. The absorbance scale for traces b and c is as indicated by the bar in a.

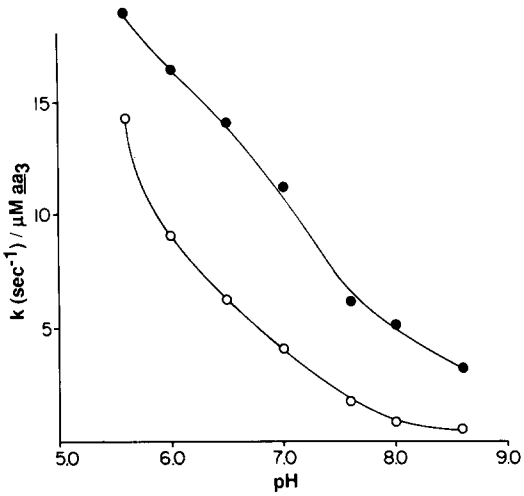

Fig. 3. Effect of pH on rate constants. Rate constants of the oxidase reaction upon addition of 1 μM bovine (●) or *Paracoccus* (○) cytochromes *c*-550 are expressed as k (s^{-1})÷ [cytochrome aa_3]. Assays were performed in 50 mM Tris-maleate, 2 mM EDTA buffer (26 °C). The cytochrome *c*-552-aa_3 preparation used contained the following amounts of cytochrome aa_3: $7.81 \cdot 10^{-3}$ μM for the *Paracoccus* assay, and $3.91 \cdot 10^{-3}$ μM for the bovine assay.

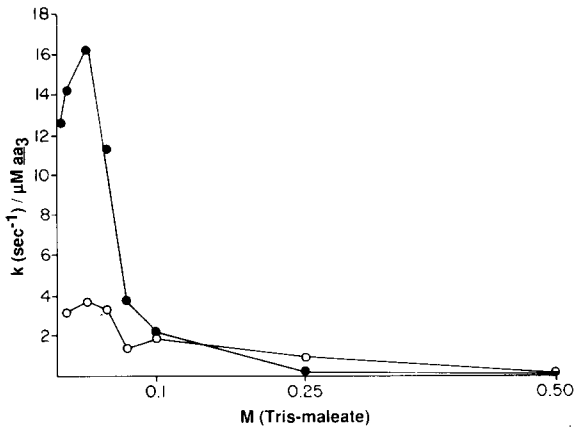

Fig. 4. Effect of ionic strength on rate constants. Rate constants of the oxidase reaction upon addition of 1 μM bovine (●) or *Paracoccus* (○) cytochrome *c*-550 are expressed as k (s^{-1})÷[cytochrome aa_3]. Assays were performed at pH 7.0, 26 °C. In Tris-maleate, 2 mM EDTA buffer. The preparation used contained the following concentration of cytochrome aa_3: $3.91 \cdot 10^{-3}$ μM (*Paracoccus* assay) and $1.95 \cdot 10^{-3}$ μM aa_3 (bovine asay).

obtained the same results. The inhibitory effect at higher ionic strengths with bovine cytochrome *c* is similar to that seen in the interaction of bovine oxidase with bovine cytochrome *c* [33].

Effect of cytochrome c concentration. Fig. 5 plots the activity of a partially purified cytochrome aa_3 preparation as a function of the concentration of cytochrome *c* at pH 7 in 50 mM Tris-maleate. With both bovine and *Paracoccus* cytochromes *c* as substrate a sharp peak in rate constant is seen at concentrations around 0.1 to 0.2 μM (with a ratio of cytochrome *c* to aa_3 of around 30). The peak in activity is sometimes seen as a plateau or levelling off. Past the activation, there is little or no change of rate constant with an increase in the *Paracoccus* cytochrome *c* concentration. With bovine cytochrome *c*, the rate constant decreases in the manner previously observed in the reaction of bovine oxidase with bovine cytochrome *c* [22]. Except at even higher concentrations of cytochrome *c* (above 5 μM), the rate constants with bovine cytochrome *c* were greater than those with *Paracoccus* cytochrome *c*. The same pattern was seen with all types of *Paracoccus* oxidase preparation, including membrane vesicles from wild-type and a cytochrome *c*-deficient mutant (Bolgiano, B., unpublished results) as well as the partially purified cytochrome *c*-552 aa_3 complex and purified cytochrome aa_3.

Plots of this type of datum according to Eadie-

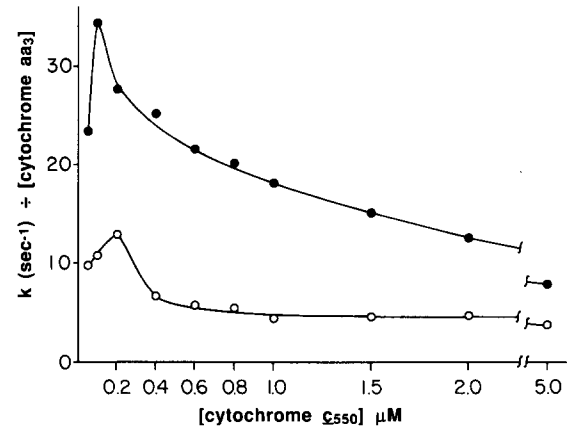

Fig. 5. Rate constants of *Paracoccus* cytochrome *c*-552-aa_3 complex on addition of either bovine (●) or *Paracoccus* (○) cytochromes *c*-555. Data are expressed as rate k (s^{-1})÷ [cytochrome aa_3] μM. Assays were run at 26 °C in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0) with $9.77 \cdot 10^{-4}$ μM cytochrome aa_3 per assay.

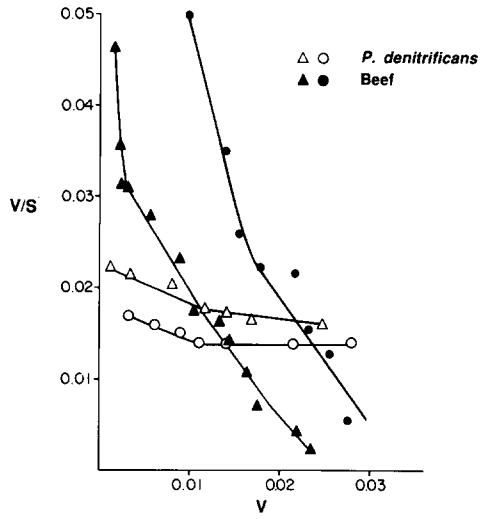

Fig. 6. Oxidase activity with membrane fragments from a cytochrome *c*-deficient mutant (●, ○) and cytochrome aa_3 (▲, △). Assays were run spectrophotometrically with activities expressed as $v(k \cdot [\text{cytochrome } c])$, and plotted in the form of Eadie-Hofstee (v/S vs. v). Membrane fragments were treated with deoxycholate (0.5 mg/mg membrane protein); the purified cytochrome aa_3 was incubated with asolectin (75 mg/mg protein). The following amounts were used in the assays: 0.02 (bovine) and 0.050 (*Paracoccus*) mg of cytochrome *c*-deficient membranes; and, $8.95 \cdot 10^{-4}$ (bovine) and $3.58 \cdot 10^{-3}$ (*Paracoccus*) μM cytochrome aa_3 of the purified oxidase. Assays were run in 50 mM Tris-maleate, 2 mM EDTA (pH 7.0, 26 °C) with either bovine (●, ▲) or *Paracoccus* (○, △) soluble cytochrome *c*-550.

Hofstee (v/S vs. v) from experiments with mitochondrial enzymes are bi- or multi-phasic [34,16]; this has been seen either as: (1) evidence for multiple binding sites on the oxidase for cytochrome *c* [34]; or (2) an indication of dimer formation by the oxidase [35]. When the results described above for the *Paracoccus* oxidase are plotted in this way (Fig. 6), those with bovine cytochrome *c* give a multiphasic plot with steep slopes, as expected, but the data with the *Paracoccus* cytochrome gives a nearly monophasic plot with a gradual inclination and with the slope approaching zero because the v/S remains constant beyond 0.4 μM. Thus, the data suggest that it is the nature of the cytochrome *c* rather than the state of the oxidase that influences the type of interaction.

Effect of poly(L-lysine). We have previously shown that poly(L-lysine) competitively inhibited

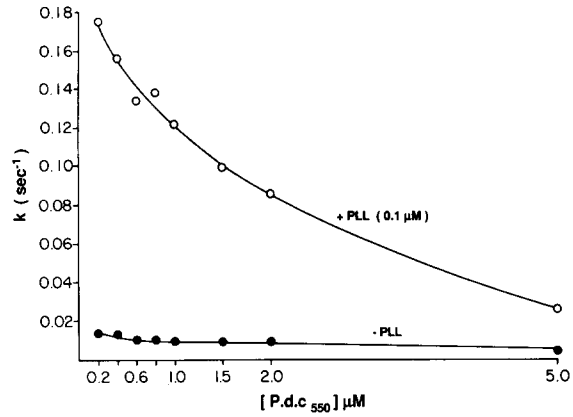

Fig. 7. Effect of Poly(L-lysine) on rate constants with *Paracoccus* cytochrome *c*-550. The oxidation of *Paracoccus* cytochrome *c*-550 was assayed with (○) or without (●) the addition of 0.1 μM poly(L-lysine) (PLL), 20 kDa at pH 6.0 in 50 mM Tris-maleate, 2 mM EDTA. The *Paracoccus* cytochrome *c*-552-aa_3 fraction used in the assay contained $4.88 \cdot 10^{-4}$ 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.

Paracoccus oxidase when bovine cytochrome *c* was substrate [21], in accordance with similar data obtained with the bovine oxidase-bovine cytochrome *c* system [33]. Surprisingly, similar concentrations of poly(L-lysine) gave significant stimulation of the *Paracoccus* oxidase oxidizing *Paracoccus* cytochrome *c* [21]. In the presence of these stimulating concentrations of poly(L-lysine), the effect of the concentration of cytochrome *c* on the oxidase rate constants changed; the resulting plot resembles that obtained with bovine cytochrome *c* (Fig. 7). The same change was seen in the effect of ionic strength. In the presence of poly(L-lysine) the reaction of the *Paracoccus* oxidase with *Paracoccus* cytochrome *c* became sensitive to changes in ionic strength, with a peak activity at around 50 mM Tris-maleate.

Effect of asolectin. Treatment of the purified *Paracoccus* cytochrome aa_3 with asolectin, as a source of phospholipids, increased the rate constant with bovine cytochrome *c* 4-fold, but had only a small effect on the reaction with *Paracoccus* cytochrome *c* (Fig. 8). The turnover numbers obtained (μM cytochrome *c* per s^{-1} per μM cytochrome aa_3) using 5 μM cytochrome *c* were 48.6 s^{-1} with bovine cytochrome *c* and 18.7 s^{-1}

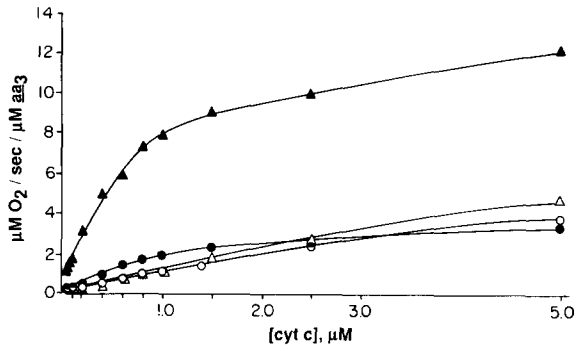

Fig. 8. Effect of asolectin on cytochrome oxidase activity with bovine (▲, ●) or *Paracoccus* (△, ○) cytochrome *c*-550. Purified cytochrome aa_3 was treated with asolectin as described in Materials and Methods to a final concentration of 75 mg/mg protein. Asolectin treated (▲, △) or untreated (●, ○). Assays were carried out spectrophotometrically in 50 mM Tris-maleate, 2 mM EDTA, (pH 7.0, 26 °C), and activities are expressed as velocities in the form of O_2 uptake (μM O_2/s) ÷ [cytochrome aa_3] μM. In the experiments, $8.95 \cdot 10^{-3}$ μM cytochrome aa_3 was used, except for the assay with bovine cytochrome *c* with the asolectin preparation, in which $3.58 \cdot 10^{-3}$ μM was used.

with *Paracoccus* cytochrome *c* when measured spectrophotometrically, and 548 s^{-1} with bovine cytochrome *c* and 170 s^{-1} with *Paracoccus* cytochrome *c* measured polarographically.

Discussion

Our method for isolating and purifying the *Paracoccus* oxidase is relatively simple and yields an enzyme with an absorption spectrum typical of cytochrome aa_3 with no evidence of a *c*-type cytochrome. The activity compared favorably with preparations obtained by more tedious methods, when the effects of pH, ionic strength and cytochrome *c* concentrations are taken into consideration. Assays previously reported with a purified preparation were made only with mitochondrial cytochrome *c* and not with its own cytochrome *c*-550.

The purified oxidase shows three major bands in SDS-polyacrylamide gel electrophoresis. In this respect, it differs from the preparation described by Ludwig and Schatz [11], which gives evidence of two subunits resembling subunits I and II of the mitochondrial oxidase. Recently, however, Saraste et al. [13] found a DNA sequence in *Paracoccus* coding for protein homologous to subunit III of the mitochondrial enzyme that is similar in molecular size. Perhaps we have retained the third subunit with our method of preparation.

We found the oxidase activity of the purified cytochrome aa_3 preparation with soluble cytochrome *c* to be similar to that of preparations which also contained the membrane-bound cytochrome *c*-552. The oxidase of a cytochrome *c*-deficient mutant also had similar properties (Bolgiano, B., unpublished results). This is all good evidence that the soluble periplasmic cytochrome *c*-550 of *Paracoccus* reacts directly with the cytochrome aa_3 independently of the membrane-bound cytochrome *c*-552, in agreement with our other results [21]. As discussed below, the soluble cytochrome *c*-550 appears not to react with the cytochrome aa_3 when the aa_3 is interacting with the membrane-bound cytochrome *c*-552 [3,6].

The reaction of the *Paracoccus* oxidase with bovine cytochrome *c* resembles that of the bovine oxidase with bovine cytochrome *c* in every way. Similar responses were observed in both systems: (1) variations of pH or ionic strength [33]; (2) variations of concentration of cytochrome *c* [22]; (3) addition of phospholipids [36]; and (4) addition of poly(L-lysine) [33]. The *Paracoccus* oxidase must have a cytochrome *c* binding site equivalent to that of the bovine enzyme and can act as an appropriate model for electron transfer reactions of the mitochondrial enzyme with mitochondrial cytochrome *c*.

X-ray crystallographic studies of the two species of cytochrome *c* showed that both possess the characteristic 'cytochrome fold', and the sequence of amino acids surrounding the heme crevice is almost identical [37]. Thus, the method of operation must be similar. However, the *Paracoccus* cytochrome *c* is larger than bovine cytochrome *c*, and there is a vast difference in amino acid side chains on the surface of the molecules, giving the large difference in isoelectric pH (bovine 10.5 vs. *Paracoccus* 4.5). Both cytochromes have a group of lysines around the top of the heme crevice, where the binding/reaction site for the oxidase appears to be localized in the mitochondrial system [38,39]. *Paracoccus* cytochrome *c* is more of a dipole than bovine cytochrome *c* is, having a

highly negative hemisphere on the side of the molecule away from the heme crevice [40], which could account for some of the differences in its reactivity.

The interaction of the *Paracoccus* oxidase with its own soluble cytochrome *c*-550 has very different properties from those seen in its reaction with bovine cytochrome *c*. The rates of oxidation of *Paracoccus* cytochrome are lower than those with bovine cytochrome *c*. Also the great sensitivity of the reaction with bovine cytochrome *c* to ionic strength and the decrease in rate constant with increasing concentration of cytochrome *c* are not apparent in the reactions with the *Paracoccus* pigment. However, the addition of poly(L-lysine) changes the properties to those seen in the reaction with bovine cytochrome *c*. The bovine oxidase oxidizes *Paracoccus* cytochrome *c* at a very low rate; but this rate is also increased somewhat on addition of poly(L-lysine) [21].

The highly negatively charged 'back' hemisphere is the most obvious difference between bovine and *Paracoccus* cytochromes. The effect of the positively charged poly(L-lysine) (which binds to the *Paracoccus* cytochrome [21]) on the response of the reaction to variations in ionic strength and cytochrome *c* concentration points to the importance of this charge in the interaction of the oxidase with cytochrome *c*. Electrostratic interactions are important in orienting oxidants and reductants during an electron transfer encounter, and the ionic strength dependence of electron transfer between proteins has often been related to the surface charge environment in the vicinity of the interacting prosthetic groups. However, the present data give evidence of the role of interactions among the charged groups around both proteins in determining the electrostatic stability of the complexes or in favoring electron transfer reactions, as shown by others [41,42]. The calculations of Koppenol and Margoliash [43] demonstrated a role for the dipole moment in orienting molecules like cytochrome *c* for the formation of productive electron transfer complexes.

We previously reported [44] that with the bovine heart system the oxidase always showed increasing activity, however assayed, as the cytochrome *c* concentration was increased up to about 0.1–0.2 μM, irrespective of the concentration of cytochrome aa_3 in the assays. Our observations were suggestive of a conformational form influenced by the concentration of cytochrome *c*. The observations with the *Paracoccus* oxidase give direct evidence for an increase in oxidase activity in the presence of around 0.1–0.2 μM cytochrome *c* (Fig. 5), since at a given cytochrome *c* concentration the amount of oxidase has been shown to be linearly related to the first-order rate constant, k (s^{-1}). We have also, occasionally, seen a similar stimulatory effect in the reaction of bovine oxidase with bovine cytochrome *c* (Davies, H.C., Smith, L. and Nava, M.E., unpublished results). Suggestions of this type have been made by others. Bickar et al. [45] for example, reported that the cytochrome *c* or some other metal porphyrins influenced electron transport between cytochromes *a* and a_3, and Kornblatt [46] found such an effect even with porphyrin cytochrome *c*. The nature of the cytochrome *c* does not appear to be critical to this significant effect.

The stimulatory effect of cytochrome *c* could be the explanation for the 'high affinity' site postulated from plots of v/S versus v, since in this range of cytochrome *c* concentration the v/S would be increased (Figs. 5 and 6). Apparently, the stimulatory effect on the oxidase is more easily seen in the reaction with *Paracoccus* cytochrome *c* due to the absence of the decrease in activity on increasing concentration of cytochrome *c* observed with the mitochondrial system. This agrees with recent data suggesting that the same domain on the surface of cytochrome *c* is involved in both so-called 'high'- and 'low'-affinity reactions of cytochrome *c* with the oxidase [47]. Because of the stimulating effect of cytochrome *c* on the oxidase, it seems unnecessary to postulate an effect of cytochrome *c* bound at a noncatalytic site in equilibrium with that at the catalytic site as an explanation for the unusual oxidase kinetics [48]. Another suggestion that the multiphasic nature of the plots is evidence for the dimeric state of the oxidase does not fit with the present data where the nature of the plots depends upon the cytochrome *c*, rather than upon the oxidase. The decreased rate constant with increasing concentration of cytochrome *c* above that which gives the stimulatory effect, seen with the mitochondrial system, has been most simply explained [49–51] as

derived from the equality of the binding constant of the ferrous form with the dissociation of the ferric form.

What do these results suggest regarding the physiological role of *Paracoccus*-soluble cytochrome *c*-550? Only the membrane-bound cytochrome *c*-552 is involved in electron transport in the NADH oxidase system [6] and this reaction is not increased on addition of soluble cytochrome *c*. Possibly, the soluble cytochrome *c* can react with the oxidase only when it is not reacting with the membrane-bound cytochrome, that is, when the reaction site is exposed externally to the periplasm. The soluble cytochrome *c* participates in additional kinds of reactions with the oxidase, such as that initiated by a soluble methanol dehydrogenase [7], also located in the periplasmic space [8] and, under anaerobic conditions, with a soluble nitrite reductase [52]. As has been shown here, the rate of its reaction with the oxidase is controlled in a linear fashion by the concentration of the cytochrome *c*, and it has been shown that these bacteria can increase the synthesis of the cytochrome under some conditions [53–55]. The mitochondrial system differs in that the reaction of the oxidase with its cytochrome *c* can also be controlled by environmental factors such as ioinic strength and cytochrome *c* concentration, giving possible controls lacking in the *Paracoccus* system. The soluble *Paracoccus* cytochrome *c* does not have the mitochondrial function of reacting alternately with the membrane-bound reductases and oxidase of electron transport chains, and thus does not require all the functional properties of the mitochondrial pigment.

The similarities of the respiratory chain pigments of *Paracoccus* to those of the mitochondrial system [1,56] lead to the suggestion that an ancestor of *Paracoccus* was the original source of mitochondria [57]. Evolution would have involved changes in both the oxidase and the cytochrome *c*. Unlike the oxidases of many other bacteria [58], that of *Paracoccus* was found to be able to oxidize mitochondrial cytochrome *c* rapidly [59,60]. Thus, during evolution, the membrane-bound cytochrome *c* was lost and the oxidase could function efficiently with the soluble cytochrome *c*. Then changes in the cytochrome *c* may have introduced the possibility for controlling the system by affecting its binding under various conditions. Regulation of the binding of cytochrome *c* to the oxidase has been suggested [61] to be the function of the smaller, nuclear-encoded subunits of the oxidase which are lacking in prokaryotic systems. A parallel evolution for the oxidase and cytochrome *c* was not necessary and, while the cytochrome oxidase has involved into a very complex bigenomic enzyme, the cytochrome *c* has become more electrostatically and sterically simpler, to facilitate its role in electron transport.

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