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Purification and properties of succinate-ubiquinone oxidoreductase complex from *Paracoccus denitrificans*

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Highly active succinate-ubiquinone reductase has been purified from cytoplasmic membranes of aerobically grown *Paracoccus denitrificans*. The purified enzyme has a specific activity of 100 units per mg protein, and a turnover number of 305 s^{-1}. Succinate-ubiquinone reductase activity of the purified enzyme is inhibited by 3'-methylcarboxin and thenoyltrifluoroacetone. Four subunits, with apparent molecular masses of 64.9, 28.9, 13.4 and 12.5 kDa, were observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme contains 5.62 nmol covalently bound flavin and 3.79 nmol cytochrome *b* per mg protein. The 64.9 kDa subunit was shown to be a flavoprotein by its fluorescence. Polyclonal antibodies raised against this protein cross-reacted with the flavoprotein subunit of bovine heart mitochondrial succinate-ubiquinone reductase. The 28.9 kDa subunit is likely analogous to the bovine heart iron protein, and the cytochrome *b* heme is probably associated with one or both of the low-molecular-weight polypeptides. The cytochrome *b* is not reducible with succinate but is reoxidized with fumarate after prereduction with dithionite. Iron-sulfur clusters S-1 and S-3 of the *Paracoccus* oxidoreductase exhibit EPR spectra very similar to their mitochondrial counterparts. *Paracoccus* succinate-ubiquinone reductase complex is thus similar to the bovine heart mitochondrial enzyme with respect to prosthetic groups, enzymatic activity, inhibitor sensitivities, and polypeptide subunit composition.

Introduction

Understanding the mechanisms of electron transfer and energy transduction in the respiratory chains of bacteria and mitochondria has been facilitated by the purification of the oligomeric lipoproteins complexes which participate in these reactions. Succinate dehydrogenase is one of the major entry points into the electron-transport chain in both mitochondria and numerous prokaryotes. The dehydrogenase (SDH) consists of two peripheral subunits (FP and IP), which are associated with two membranous polypeptides to form succinate-ubiquinone oxidoreductase complex, alternatively referred to as complex II [1]. The two subunit dehydrogenase can be removed from membranes with high pH or chaotropic agents, whereas detergents must be utilized to isolate succinate-ubiquinone reductase complex.

Abbreviations: DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; DFP, diisopropylfluorophosphate; DM, dodecyl maltoside; FP, flavoprotein; I_{50}, inhibitor concentration necessary for 50% inhibition; IP, iron protein; Q_2, 2,3-dimethoxy-5-methyl-6-*n*-decaprenyl-1,4-benzoquinone; SDH, succinate dehydrogenase; SCR, succinate-cytochrome *c* reductase; SQR, succinate-ubiquinone reductase.

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When isolated in a water-soluble form the dehydrogenase contains a 70 kDa polypeptide (FP subunit) carrying covalently bound 8-alpha-*N*(3)-histidyl-FAD, and a 27 kDa polypeptide (IP subunit) with one binuclear (S-1), one tetranuclear (S-2), and one trinuclear (S-3) iron-sulfur cluster [2–4]. The two-subunit, soluble succinate dehydrogenase can transfer electrons to artifical acceptors such as phenazine methosulfate, but cannot transfer electrons to ubiquinone, which is thought to be the physiological electron acceptor. When the soluble dehydrogenase is reconstituted to membranes from which it has been extracted, succinate-ubiquinone oxidoreductase activity is restored.

There are three schools of thought concerning the membranous factor(s) that confer succinate-ubiquinone reductase activity to soluble succinate dehydrogenase preparations. One view is that a cytochrome *b* which is unique to complex II and contains two polypeptides of 15.5 and 13.5 kDa is required for this activity [5]. Alternatively, it has been proposed that a 15 kDa ubiquinone binding protein (Q_p) is responsible, and that this 'Q protein' is distinct from the *b* cytochrome [6–10]. A third view is that the simplest unit capable of confering succinate-ubiquinone reductase activity to soluble succinate dehydrogenase contains two polypeptides of 13.5 and 7 kDa (C II_3 and C II_4, respectively), in addition to the 70 and 27 kDa components of the dehydrogenase [11].

Cytochrome *b* is present in all succinate-ubiquinone reductase preparations thus far isolated, but the role of this cytochrome is not clear. Some investigators feel that this *b* cytochrome is an artifact of isolation derived from complex III [8], while others feel that it is an intrinsic and necessary component for succinate-ubiquinone reductase activity [5]. Cytochrome *b* of complex III from bovine heart has an apparent molecular weight of 30–40 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis [12], while the cytochrome *b* isolated with complex II by Hatefi and coworkers contains two polypeptides of 13 and 15 kDa [5].

Succinate-ubiquinone reductase complexes isolated from *Neurospora crassa* and *Bacillus subtilis* also contain a cytochrome *b* [13–16]. A 5-aminolevulinate requiring mutant of *B. subtilis*, when deprived of this heme precursor, showed increased levels of non-membrane bound succinate dehydrogenase in the cytoplasm, implying that cytochrome *b* may have a role in binding of succinate dehydrogenase to the membrane in this organism [16].

In another report it was shown that immunoprecipitated succinate-ubiquinone reductase had a high cytochrome *b* to FAD ratio, and the cytochrome was reducible by succinate. The flavoprotein, IP subunit, and cytochrome *b* were estimated to be equimolar in the isolated enzyme complex [15,17].

Hatefi and Galante [5] showed that, although the *b* cytochrome of bovine heart complex II is only partially and slowly reduced by succinate, the cytochrome can be reoxidized with fumarate or ubiquinone after reduction with dithionite. In addition, the reoxidation by fumarate is blocked by mersalyl, a known inhibitor of succinate dehydrogenase [5]. This suggests that the cytochrome is an integral part of the enzyme complex, or at least can participate in electron transfer through the physiological pathway.

Yu and Yu implied that cytochrome *b* of succinate dehydrogenase is an artifact of isolated [8]. Their most highly purified Q_p preparations contained very little cytochrome *b*, but could reconstitute succinate-ubiquinone reductase activity with soluble succinate dehydrogenase. However, their most active Q_p preparation contained higher levels of the cytochrome [8]. More recent results from their laboratory suggest that the cytochrome may play at least a structural role in this enzyme complex [9], as noted in the studies with *B. subtilis* [16].

A distinct succinate-ubiquinone reductase cytochrome *b* has been demonstrated by a genetic approach in *Neurospora crassa*: in this organism it was shown that, whereas the gene for the cytochrome *b* of the cytochrome bc_1 complex is mitochondrial, the gene for the *b* cytochrome of succinate-ubiquinone reductase is nuclear [14].

In *Bacillus subtilis*, the genes for the FP and IP subunits, and for cytochrome *b*-558 are organized in an operon and transcribed in the sequence sdhA (cytochrome *b*-558), sdhC (IP) [18]. Recently, the sdhA gene was cloned and sequenced [19]. The hydropathy profile showed that the pro-

tein was very hydrophobic and probably spans the membrane five times. There was little sequence homology with the gene for the cytochrome *b* of the mitochondrial cytochrome bc_1 complex [19,20]. Surprisingly, there was also very little homology of the *b* cytochrome of *Bacillus subtilis* with either of the *Escherichia coli* hydrophobic peptides encoded by the genes sdhC and sdhD [19,21].

In order to define the polypeptide components required for succinate-ubiquinone oxidoreductase activity better and to gain a better understanding of the possible role of cytochrome *b* in ubiquinone reductase activity, we have purified succinate-ubiquinone oxidoreductase from membranes of aerobically grown *Paracoccus denitrificans*. *P. denitrificans* was chosen for this study because of its similarity to mitochondria in terms of redox components and inhibitor sensitivities [22], and because the cytochrome containing complexes of the respiratory chain of this bacterium contain many fewer polypeptides than their mitochondrial counterparts. Cytochrome oxidase isolated from *P. denitrificans* contains only two polypeptides [23], whereas the mitochondrial complex contains 11 or more [24]. The cytochrome bc_1 complex from *P. denitrificans* contains only three subunits [25], whereas the mitochondrial counterpart purified by essentially the same procedure contains nine or more [26]. It was thus of special interest to ascertain whether succinate-ubiquinone reductase from this bacterium might be structurally simpler than that isolated from eukaryotes.

The synthetic nonionic detergent dodecyl maltoside has proven to be effective for dispersing energy-transducing membranes [25–31], and has been utilized for the isolation or stabilization of cytochrome oxidase [29], rhodopsin [30], photoreaction center [31], and cytochrome bc_1 complexes from five different species [25,26]. In a recent study, dodecyl maltoside and other maltosides with alkyl groups of varying lengths were compared to other chemically homogenous nonionic and zwitterionic detergents in terms of their effects on the activity of cytochrome oxidase. Alkyl maltoside detergents were superior, and dodecyl maltoside was nearly optimal in terms of alkyl chain length [32].

We have used dodecyl maltoside to purify a highly active succinate-ubiquinone oxidoreductase from *Paracoccus denitrificans*. This is the first report of the isolation of this enzyme complex from this organism.

Experimental procedures

Growth of bacteria and isolation of membranes

Paracoccus denitrificans (ATCC 13543) was obtained as a freeze-dried culture and grown in peptone/yeast extract medium [32]. To isolate membranes, 300 g *P. denitrificans* cell paste was suspended in 1.5 l 50 mM Tris-HCl (pH 7.5), homogenized, poured through several layers of cheesecloth and centrifuged at $20000 \times g$ for 15 min. The pellets were resuspended in the same buffer and washed two more times.

The cells were then suspended in 280 ml of buffer containing 1 mM diisopropylfluorophosphate and 500 units/l DNase I (Sigma D-5025), homogenized, and stirred at 4°C for 5 min. The cell suspension was put through a French pressure cell at 34.5 MPa piston in a precooled press into a cooled flask. The lysate was then centrifuged at $25000 \times g$ for 20 min to remove unbroken cells and cell debris, and the supernatant was centrifuged at $340000 \times g$ for 90 min. The reddish upper portion of the membrane pellet was recovered, leaving a hard white lower pellet behind, and resuspended in 250 ml of 50 mM Tris-HCl (pH 7.5), 10 mM EDTA. The membrane suspension was centrifuged at $324000 \times g$ for 90 min and resuspended in 50 mM Tris-HCl (pH 7.5) to a protein concentration of about 30 mg/ml. The washed membranes were stored at −80°C.

Isolation of succinate-ubiquinone reductase

The procedure for the isolation of succinate-ubiquinone reductase is summarized in the flow scheme in Fig. 1. All steps were performed at 4°C. Membranes were suspended at 10 mg/ml protein in 10 mg/ml dodecyl maltoside, 1 mM diisopropylfluorophosphate, 50 mM Tris-HCl (pH 7.5). The mixture was stirred for 60 min and centrifuged at $100000 \times g$ for 30 min. The supernatant was brought to 100 mM NaCl by adding 4 M NaCl, stirred for 30 min, and centrifuged at $100000 \times g$ for 30 min. Solid dodecyl maltoside was added to a final concentration of 20 mg/ml and stirred for 10 min. The supernatant was then applied to a 5 × 20 cm column of DEAE Se-

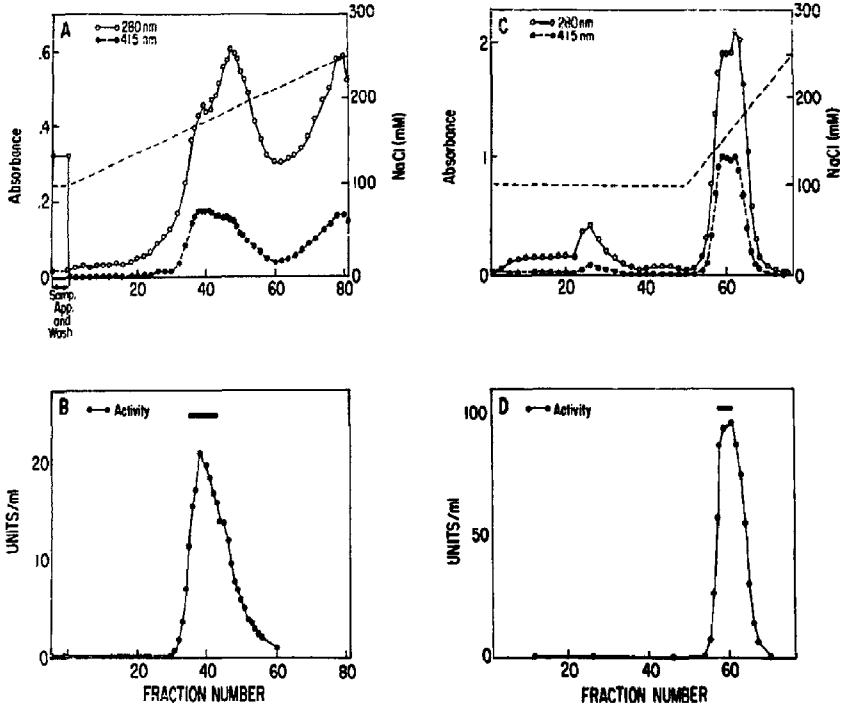

Fig. 1. DEAE Sepharose CL-6B profiles obtained during the purification of *P. denitrificans* succinate-ubiquinone reductase. The upper and lower left panels show the first column profile (A and B) and the two right panels show the second column profile (C and D). The upper panels depict the absorbance at 280 nm (protein), 415 nm (heme), and the NaCl gradient (dashed line). The lower panels show succinate-ubiquinone reductase activity and the solid bars indicate fractions that were pooled.

pharose CL-6B equilibrated with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mg/ml dodecyl maltoside, after which the column was washed with 1600 ml of equilibration buffer and then eluted with a 1600 ml linear gradient from 100 to 250 mM NaCl. Fractions were analyzed for absorbance at 280 and 415 nm for protein and heme, respectively, and for succinate-ubiquinone reductase activity. Fractions with maximal succinate-ubiquinone reductase activity were pooled and concentrated on an Amicon Diaflo PM 10 membrane, diluted with an equal volume of 20 mg/ml dodecyl maltoside in 50 mM Tris-HCl (pH 7.5), and stirred for 20 min.

This sample was then applied to a second 1 × 10 cm DEAE Sepharose CL-6B column equilibrated with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2 mg/ml dodecyl maltoside. Prior to sample application the column was washed with 8 ml of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mg/ml dodecyl maltoside. The sample was then applied, and the column was washed with 8 ml of the same buffer. The column was then washed with 80 ml of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2 mg/ml dodecyl maltoside and eluted with an 80 ml gradient of 100–250 mM NaCl in 50 mM Tris-HCl (pH 7.5), 0.2 mg/ml dodecyl maltoside. Fractions were pooled and concentrated on an Amicon Diaflo PM 30 filter, diluted with an equal volume of glycerol and stored at −20°C. On several occasions a third column, identical to the second, was run.

Analytical procedures

Protein was measured according to Lowry as modified by Markwell et al. [33]. Cytochrome *b* concentrations were determined from dithionite reduced-minus-oxidized difference spectra using the coefficient $E_{562-577} = 25.6\ mM^{-1} \cdot cm^{-1}$ [34] or by pyridine hemochrome spectra [35] using the coefficient $E_{557-540} = 23.98\ mM^{-1} \cdot cm^{-1}$. Succinate-ubiquinone reductase activity was measured in a mixture containing 49 μM DB, 52 μM dichlorophenolindophenol, 40 mM sodium phosphate, 20 mM sodium succinate, 0.02% Tween-80, 0.5 mM EDTA, 0.25 mM KCN (pH 7.5) [36]. The absorbance change was monitored at 600 nm and enzymatic rates calculated using a coefficient of $19.1\ mM^{-1} \cdot cm^{-1}$. Samples were routinely preactivated by incubation at 30°C in 50 mM succinate-Tris, 0.1 mg/ml dodecyl maltoside (pH 7.5), for 10–20 min. Succinate-ubiquinone reductase activity is expressed as μmol succinate oxidized per min (units) and turnover numbers as μmol succinate oxidized per s per μmol of non-acid extractable flavin [36].

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out according to Laemmli [37], except that sodium dodecyl sulfate was omitted from the gel and lower electrode buffer. Linear gradient gels (11–17%), stabilized within a 3–12% sucrose gradient, were made from a stock acrylamide solution of 30% T, 2.7% C. Samples for electrophoresis were first reduced in 30 mM dithiothreitol, sodium dodecyl sulfate was then added to 2%, and the samples denatured at 50°C for 10 min. 50% percent glycerol was then added to a concentration of 20%, and 0.2% bromphenol blue to a concentration of 0.004%. The 1.5 mm thick (14 cm width by 12 cm length) gels were run at 20 mA constant current through the 4% T stacking gel and at 30 mA constant current through the 11–17% T running gel. Gels were stained overnight with 0.125% Coomassie brilliant blue R-250 in 50% methanol, 10% acetic acid and destained in 25% methanol, 10% acetic acid. Fluorescence gel photographs were taken with gels agitated in 25% methanol, 10% acetic acid for 1 h. Gels were illuminated with a long-wavelength ultraviolet lamp and a green Wratten filter was used on the camera lens.

Antibodies to the *P. denitrificans* succinate-ubiquinone reductase-flavoprotein were raised in rabbits. Flavoprotein was electroeluted from electrophoresis gels, acidified and observed under longwave ultraviolet light to confirm the presence of flavin. These fractions were neutralized, concentrated on an Amicon Diaflo PM-10 filter, mixed with Freund's complete adjuvant, and 500 μg was injected into a rabbit subcutaneously. The rabbit was boosted after 21 days with 250 μg of protein in Freund's incomplete adjuvant and was bled after one month. The serum was complement-inactivated at 56°C for 30 min. The antibodies were purified by ammonium sulfate fractionation and DEAE cellulose chromatography [38]. Western blots [39] were probed with goat-anti-rabbit IgG conjugated to horse radish peroxidase and bands detected with 4-chloro-1-naphthol and hydrogen peroxide.

Non-acid-extractable flavin was determined after protease treatment [15,40]. FAD was purchased from Sigma (F-6625) and purified as described by Massey and Swaboda [41].

EPR spectroscopy

Samples for EPR spectroscopy were prepared from succinate-ubiquinone oxidoreductase which had been stored for several months at −80°C. The samples were thawed and rapidly refrozen by immersion of the EPR tubes in a 1:5 mixture of methylcyclohexaneisopentane at 81 K. EPR measurements were performed on a Varian E-109 spectrometer interfaced with an IBM personal computer for accumulation and manipulation of the spectra. The sample temperatures were controlled with a variable temperature cryostat (Air Products and Chemicals Inc., Ltd-3-110).

Reagents and Materials

Serum albumin (essentially fatty acid free), horse heart cytochrome *c*, and DEAE-Sepharose CL-6B were obtained from Sigma. Acrylamide and sodium dodecyl sulfate were obtained from Biorad. Dodecyl maltoside was obtained from Boehringer Mannheim. Ubiquinone analogues were synthesized and purified according to Wan and Folkers [42,43]. 3′-Methyl carboxin was a gift from Dr. G.A. White (Agriculture Canada, Research Institute Ontario, Canada). Goat anti-rabbit IgG conjugated to horse radish peroxidase was

obtained from Cooper Biomedical, Malvern, PA.

Results

Purification of succinate-ubiquinone reductase

Data summarizing the purification of succinate-ubiquinone reductase from cytoplasmic membranes of *Paracoccus denitrificans* is shown in Table I. Profiles of the two DEAE Sepharose CL-6B columns are shown in Fig. 1. The earlier eluting fractions are enriched in succinate-ubiquinone reductase activity and have a higher 415/280 nm absorbance ratio. Most of the contaminating protein remains tightly adsorbed to the first DEAE column and could be removed at higher salt concentrations (data not shown). This contamination consists primarily of the cytochrome *c*-552-oxidase complex [35] and the cytochrome bc_1 complex [25]. The former complex is apparent as a peak at the very end of the gradient. Some contaminating protein is removed in the eluates from the sample application and 100 mM NaCl wash.

The succinate-ubiquinone reductase fractions eluting from the first column at 165–185 mM NaCl (Fig. 2B) were combined. These fractions had an average 415/280 nm absorbance ratio of 0.37 and an estimated specific activity of 41 μmol of succinate oxidized per min per ml per absorbance unit at 280 nm.

The elution profile of the second DEAE Sepharose CL-6B column is shown in panels C and D. Contaminating proteins are eluted during the sample application and 100 mM NaCl column wash. Fractions eluting at 145–165 mM NaCl were combined. The pooled succinate-ubiquinone reductase

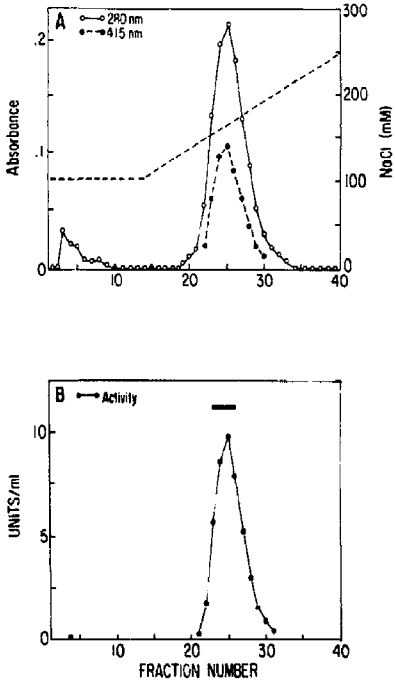

Fig. 2. Succinate-ubiquinone reductase chromatography on a third DEAE Sepharose CL-6B column. The column was as described in the legend to Fig. 2.

fractions had an average 415/280 nm absorbance ratio of 0.52, and an average activity per absorbance unit at 280 nm of 49.

The purification table (Table I) shows the increase in succinate-ubiquinone reductase specific activity as the purification proceeds. In the presence of 10 mg/ml dodecyl maltoside and after preactivation with succinate, cytoplasmic mem-

TABLE I

PURIFICATION OF SUCCINATE-UBIQUINONE REDUCTASE FROM *PARACOCCUS DENITRIFICANS*

Fraction	Protein (mg)	Activity (units)	Yield (%)	Purification (-fold)	Activity (U/mg)	Cytochrome *b* (nmol/mg)	FAD (nmol/mg)	*TN* (s^{-1})
Membranes	952	3494	–	–	3.67	–	–	–
Membranes & DM	952	6650	100	1	7.00	–	0.247	472
DM extract	806	6112	92	1.08	7.58	–	0.312	406
DM extract & NaCl	782	5696	86	1.04	7.28	–	0.326	372
1st DEAE column	42.6	2680	40	8.99	62.9	2.55 (2.29)	3.05	345
2nd DEAE column	10.1	1018	15	14.4	100.8	3.73 (3.79)	5.62	305

branes produced from French pressure cell extracts had a specific activity of 7.00 units/mg protein. Membranes prepared by the lysozyme-EDTA procedure had a specific activity of 3.22 units/mg protein. The former procedure was thus utilized for the work presented here. There is a slight decrease in turnover number of the enzyme from membranes (472 s^{-1}) to the final purified

TABLE II

COMPARISON OF SUCCINATE-UBIQUINONE REDUCTASE PREPARATIONS

Species	Number of polypeptides	Major polypeptides (kDa)	FAD [e] (nm/mg)	Cyt *b* [f] (nm/mg)	SQR [k] specific activity	*TN* (s^{-1})
Bovine heart [2,46,47]	10	73; 24 [b]; 13.7; 12.5	4.6–5.0	4.5–4.8 [g,i]	50–55 [b,q]	167–183
Bovine heart [8]	4–5	–	5.8	1.2 [g,i]	17.5 [l,o]	50
Bovine heart [48]	4–5	74; 26 [c]; 15.8; 14.9	5.9–6.2	1.2–2.5 [g]	28 [o]; 40 [p]; 54 [q,l]	83 [o]; 150 [q]
Bovine heart [a]	6	68.5; 27.5 [c]; 11.7; 10.8	–	1.03 [g]	18.0	–
Neurospora crassa [14]	3	72; 28 [d]; 14	6.0	5.0	–	–
Escherichia coli [49]	4	71; 26 [c]; 17; 15	–	present	–	–
Micrococcus luteus [50,51]	4	72; 30 [c] 17; 15	–	present [g]	3.1 [n,o]	–
Bacillus subtilis [15,17]	3	65; 28 [d]; 19	–	present [h,j]	–	–
Paracoccus denitrificans (this paper)	4	64.9; 28.9 [c]; 13.4; 12.5	5.6	3.7 [g,i]	103.0 [m,p]	305
Rhodobacter sphaeroides SDH [52]		68; 30 [c]				
Rhodospirillum rubrum SDH [53]		60: 25 [b]				
Ipomoea batas (sweet potato) mitochondria SDH [54]		65;26 [b]				

[a] Sample donated by C.A. Yu.
[b] Weber-Osborn gel system [55].
[c] Laemmli gel system [37].
[d] Modified Laemmli gel system.
[e] FAD determined as acid non-extractable flavin.
[f] Not reducible by succinate.
[g] Not reducible by succinate.
[h] Reducible by succinate.
[i] Not reactive with CO.
[j] Reactive with CO.
[k] Succinate-ubiquinone reductase (SQR) activity expressed as μmole succinate oxidized per s per mg protein.
[l] Activity measured with Q_2 as acceptor.
[m] Activity measured with 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB) as acceptor.
[n] Activity measured with phenazine methosulfate (PMS) as acceptor.
[o] Activity assayed at 25°C.
[p] Activity assayed at 30°C.
[q] Activity assayed at 38°C.
[r] Turnover numbers (*TN* s^{-1}) expressed as μmol succinate oxidized per s per μmol acid non-extractable FAD.

succinate-ubiquinone reductase (305^{-1}). However, the specific activity and turnover number for the pure succinate-ubiquinone reductase are the highest yet reported from any species (Table II). The levels of cytochrome *b* and non-acid extractable FAD increased when the pooled succinate-ubiquinone reductase was further purified on a second column. The pyridine hemochrome values are similar to the values determined from optical spectra of the native cytochrome *b* (in brackets).

If the succinate-ubiquinone reductase from the second column was applied to a third DEAE Sepharose CL-6B there appeared to be a slight increase in purity of the final preparation, as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, but the turnover number decreased (data not shown). The profile for such a column is shown in Fig. 2. A small amount of contaminating protein is removed in the column wash, as in the second column, and the succinate-ubiquinone reductase activity (Fig. 2B) is symmetrically associated with the absorbance at 280 and 415 nm (Fig. 2A). The reason for the decreased turnover number resulting from repeated ion-exchange chromatography is not known.

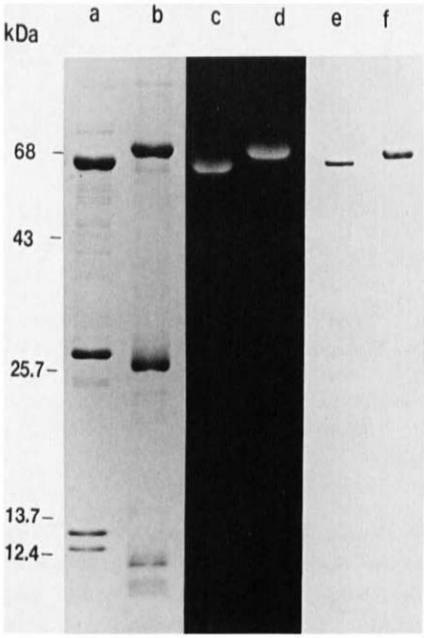

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showing purification of *P. denitrificans* succinate-ubiquinone reductase. Lane a, lysozyme-EDTA membranes (50 μg); b, French press membranes (50 μg); c, dodecyl maltoside extract of French press membranes (50 μg); d, succinate-ubiquinone reductase from the first DEAE Sepharose CL-6B column (16 μg); e, succinate-ubiquinone reductase from the second DEAE Sepharose CL-6B column (10 μg); f, same as lane e, but sample denatured at 100 °C for 3 min.

The protein composition of samples during purification is shown on the sodium dodecyl sulfate gel electrophoresis gel in Fig. 3. The pure complex (lanes e and f) appears to consist of four subunits having apparent molecular masses of 64.9, 28.9, 13.4 and 12.5 kDa. The substantial purification from the first to second columns is apparent in lanes d and e. There appears to be a significant difference in the polypeptide profiles of membranes prepared with the French press compared to those prepared by the lysozyme-EDTA procedure, with higher levels of succinate-ubiquinone reductase polypeptides in the former preparation (lanes a and b), consistent with the differences is specific activity of the two membrane preparations (see above). There does not appear to be any specific solubilization of succinate-ubiquinone reductase with dodecyl maltoside, as the detergent seems to extract all of the proteins from the membranes (lanes b and c)

Optical and EPR spectra

Optical spectra of the pure succinate-ubiquinone reductase are shown in Fig. 4. The dithionite reduced spectrum has peaks typical of cytochrome *b*, and a shift in the cytochrome Soret absorption peak from 412.7 nm to 424.1 nm is apparent upon dithionite reduction. The shoulder at 450 nm in the air oxidized spectrum is most likely due to flavin. The reaction of the dithionite-reduced succinate-ubiquinone reductase cytochrome *b* with CO is shown in Fig. 5. Even after 10 min of exposure to CO there is only a slight decrease in the absorbance at 558 nm. By this criterion we infer that this cytochrome *b* is not auto-oxidizable. After reduction with a minimum amount of dithionite, addition of 0.35 mM fumarate reoxidized more than 50% of the reduced cytochrome.

EPR spectra of the purified succinate-

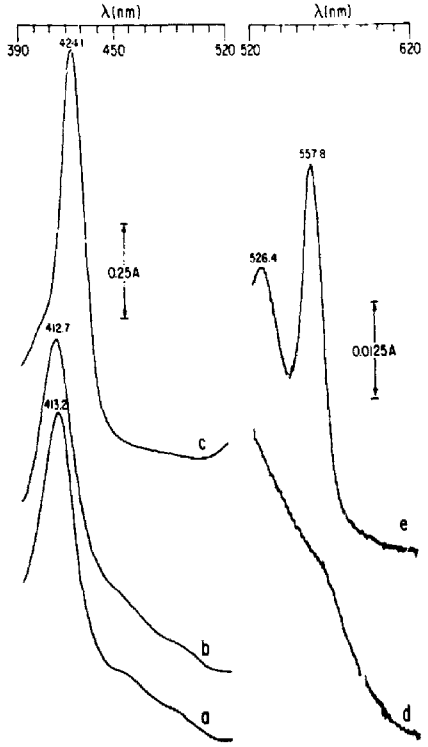

Fig. 4. Optical spectra of pure succinate-ubiquinone reductase. For a, b, and c, protein was at 1.25 mg/ml (390–520 nm scan); and for d and e at 0.4 mg/ml (520–620 nm scan). Spectra a and d are of the preparation as obtained from the purification: b, ferricyanide oxidized; c and e, dithionite reduced.

ubiquinone reductase are shown in Fig. 6. Iron-sulfur centers S-1 and S-3 in the isolated ubiquinone reductase elicit EPR spectra having line shapes which are analogous to those of their mitochondrial counterparts [44]. Albracht and co-workers [45] reported previously that the g_x line shape of the cluster S-1 in situ varies, depending on different membrane preparations, with values ranging from 1.901 to 1.916. The g_x, g_y and g_z values observed here (1.916, 1.934, and 2.025) are thus very similar to the g values reported for cluster S-1 in the cytoplasmic membrane. In the preparation shown in Fig. 6 even prolonged incubation with succinate resulted in only partial reduction of the cluster S-1, and the spin relaxation rates of cluster S-1 in the succinate and dithionite reduced enzyme are almost identical over a wide temperature range. These observations suggest that the *P. denitrificans* succinate-ubiquinone oxidoreductase is less stable during storage than the mitochondrial enzyme. The EPR spectrum of cluster S-2 was not detectable, due to the low concentration of enzyme available.

Polypeptide composition

A sodium dodecyl sulfate electrophoresis gel comparing the *P. denitrificans* and bovine heart succinate-ubiquinone reductases is shown in Fig. 7 and a densimetric scan of the gels in Fig. 8. The polypeptide profiles of the two complexes are

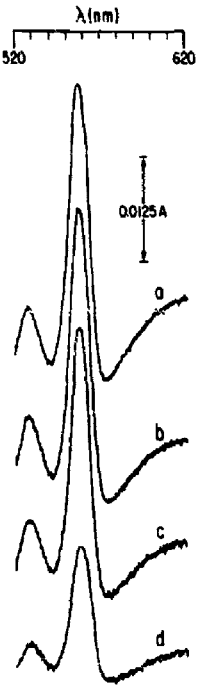

Fig. 5. Effect of CO on the reduced minus oxidized spectra of pure succinate-ubiquinone reductase. All spectra are dithionite reduced minus enzyme as obtained from the purification (oxidized) with protein at 0.4 mg/ml. Spectra a, control (dithionite reduced minus oxidized); b, after stirring in a sealed cuvette with CO for 2 min; c, after stirring in the presence of CO for 10 min; d, the same sample as in c with 0.35 mM fumarate.

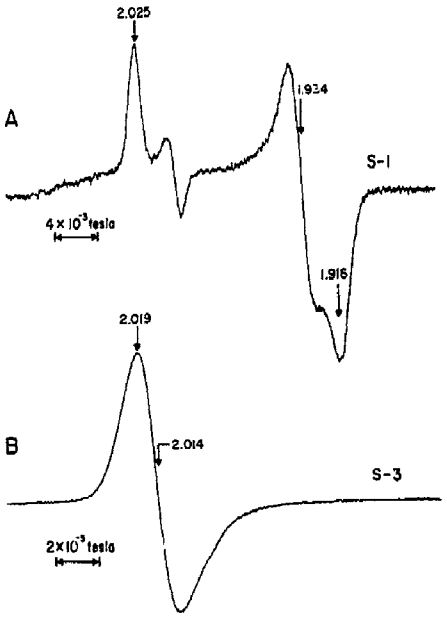

Fig. 6. EPR spectra of iron-sulfur clusters in purified *P. denitrificans* succinate-ubiquinone reductase. (A) Reductase reduced with 5 mM dithionite. The flavin concentration was 17.4 μM. Spectrum was taken at 5 mW microwave power; modulation amplitude, $1.25 \cdot 10^{-3}$ tesla; time constant, 0.128 s; sweep rate, $1.0 \cdot 10^{-2}$ tesla/min; and sample temperature of 25 K. (B) No reductant was added. EPR spectrum taken a. 0.2 mW microwave power; time constant, 0.064 s; sample temperature, 12 K. Enzyme concentration and other EPR conditions were the same as in (A).

quite similar (Fig. 7, lanes a and b). The flavoprotein subunit was identified by low pH fluorescence (Fig. 6, lanes c and d). Antibodies raised against the *P. denitrificans* flavoprotein crossreacted with the bovine heart counterpart (Fig. 7, lanes e and f). The 28.9 kDa polypeptide is tentatively assigned as the iron-protein (IP) subunit on the basis of its molecular weight when compared to bovine heart and other enzyme complexes (Table II).

The lower molecular weight polypeptides of the *P. denitrificans*-succinate ubiquinone reductase are more distinct than their bovine heart counterparts in this gel system. Whether one or both of these polypeptides is cytochrome *b* has yet to be established. Stoichiometries for the four polypeptides of the *P. denitrificans* complex, estimated from scans of the stained gels (Fig. 8) and normalized for their apparent molecular masses, were 1:1:1:0.6 for the 64.9:28.9:13.4 and 12.5 kDa polypeptides, respectively.

Effects of inhibitors and ubiquinone analogs on ubiquinone reductase activity

The pure succinate-ubiquinone reductase was tested for sensitivities to various succinate-ubiquinone reductase inhibitors. The K_i for 3-methyl-carboxin, determined from Dixon plots (data not shown), was 13.2 μM, and for TTFA was 5.52 mM. The I_{50} for mersalyl was 0.25 mM with 20 mM succinate in the assay mixture.

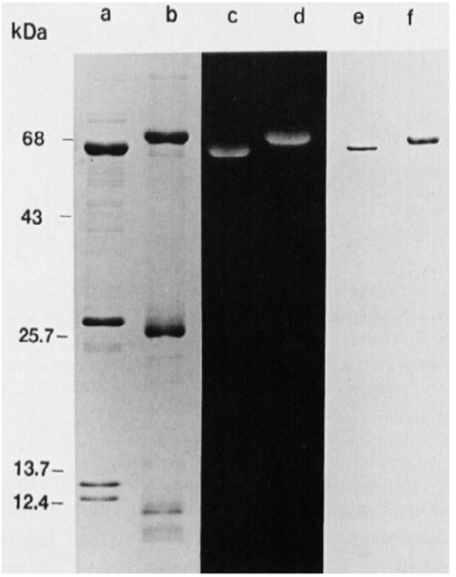

Fig. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis comparison of *P. denitrificans* and bovine heart succinate-ubiquinone reductase (sample generously donated by Dr. C.A. Yu). *P. denitrificans* succinate-ubiquinone reductase in lanes a, c, and e; bovine heart succinate-ubiquinone reductase in lands b, d, and f. Lanes a and b show 10 and 8 μg of protein, respectively, after staining with Coomassie blue; lanes c and d show fluorescence at low pH; lanes e and f show an immunoblot on nitrocellulose of the *P. denitrificans* and bovine heart enzymes probed with polyclonal antibodies raised against the bacterial succinate-ubiquinone reductase flavoprotein and detected with peroxidase conjugated anti-rabbit IgG second antibody.

A series of ubiquinone analogues containing alkyl side chains of various lengths was synthesized and tested in order to optimize the succinate-ubiquinone reductase assay. As shown in Fig. 9 the five, seven, nine, and ten carbon analogs were of similar efficacy for both membranes and the isolated enzyme, with the nine carbon homolog being slightly optimal. The succinate-ubiquinone reductase activity with the thirteen and fifteen carbon chain analogs was lower, probably due to the hydrophobicity of these longer chain analogs, which may inhibit their ability to transfer electrons to the soluble dichlorophenolindophenol.

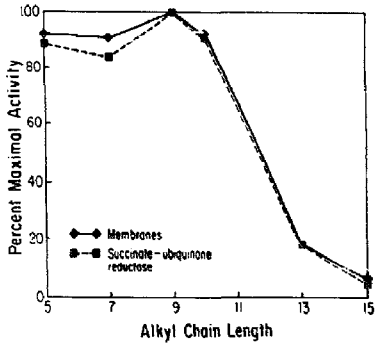

Fig. 9. Succinate-ubiquinone reductase activity of succinate-ubiquinone reductase complex measured with homologous ubiquinone analogues of varying lengths of alkyl side chain.

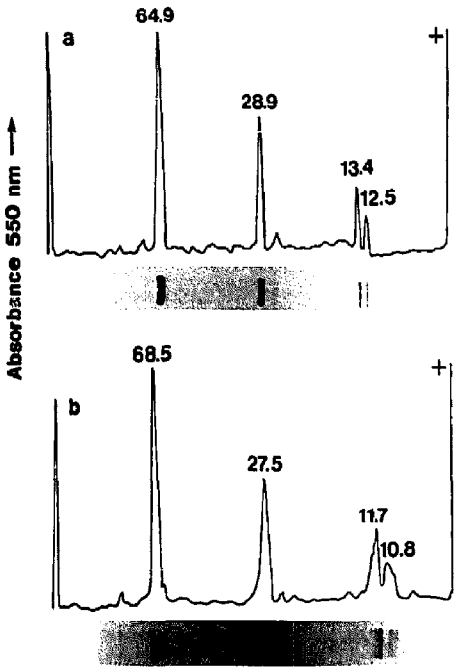

Fig. 8. Sodium dodecyl sulfate polyacrylamide gel scan comparisons of *P. denitrificans* and beef heart succinate-ubiquinone reductase (sample generously donated by Dr. C.A. Yu). The upper panel is *P. denitrificans* succinate-ubiquinone reductase (10 μg protein) and the lower panel is the bovine heart enzyme (18 μg protein). Photographs of the gels are positioned below the respective scans. Scans were performed on an E-C Apparatus Corporation Densitometer interfaced with a Hewlett Packard 33390A integrator.

Discussion

A highly active succinate-ubiquinone reductase preparation has been purified for the first time from *Paracoccus denitrificans*. The turnover number and specific activity of the *P. denitrificans* complex are the highest reported for this enzyme from any species. Whether this is an inherent property of this enzyme in this organism, or reflects less damage to the current preparation during purification, is not known.

Succinate-ubiquinone reductase preparations from different species are shown in Table II. There is a great similarity in the number and molecular weights of subunits. The little variation in molecular weights and subunit numbers is probably due, in part, to the electrophoresis systems utilized. For example, when the bovine heart complex was analyzed by sodium dodecyl sulfate gel electrophoresis in this study, the calculated molecular weights for all four subunits were less than has been previously reported. The last three species have been included for comparison of the FP and IP subunit molecular weights. The levels of FAD are quite constant between species analyzed, but the succinate-ubiquinone reductase specific activities vary from different preparations and at different temperatures. It appears that the preparations with the highest activities have higher

cytochrome *b* levels. In this regard it is significant that the enzyme from *P. denitrificans* is enriched in cytochrome *b*, and that the cytochrome is not CO-reactive.

The lack of reaction with CO also indicates that the succinate-ubiquinone reductase cytochrome is a *b*-type cytochrome, as opposed to an *o*-type, in addition to not being grossly denatured. Reoxidation of this cytochrome by fumarate suggests that the heme group is electronically connected to at least some of the other redox centers of the enzyme. The pathway of electron transfer through the enzyme and the involvement of the heme therein remain to be established. In this regard it may be useful to point out that lack of reduction of a cytochrome by substrate under equilibrium conditions does not preclude involvement of that cytochrome in the redox pathway of an enzyme. In the cytochrome bc_1 complex cytochrome *b*-566 is only transiently reduced by a low-potential ubisemiquinone [43]. It is conceivable that a similar low-potential reductant of cytochrome *b* is transiently generated during the catalytic cycle of the succinate-ubiquinone reductase.

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