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Electron-transfer complexes of *Ascaris suum* muscle mitochondria. III. Composition and fumarate reductase activity of Complex II

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Complex II of the anaerobic respiratory chain in *Ascaris* muscle mitochondria showed a high fumarate reductase activity when reduced methyl viologen was used as the electron donor. The maximum activity was 49 μmol/min per mg protein, which is much higher than that of the mammalian counterpart. The mitochondria of *Ascaris*-fertilized eggs, which require oxygen for its development, also showed fumarate reductase activity with a specific activity intermediate between those of adult *Ascaris* and mammals. Antibody against the *Ascaris* flavoprotein subunit reacted with the mammalian counterparts, whereas those against the *Ascaris* iron-sulfur protein subunit did not crossreact, although the amino acid compositions of the subunits in *Ascaris* and bovine heart were quite similar.

Cytochrome *b*-558 of *Ascaris* complex II was separated from flavoprotein and iron-sulphur protein subunits by high performance liquid chromatography with a gel permeation system in the presence of Sarkosyl. Isolated cytochrome *b*-558 is composed of two hydrophobic polypeptides with molecular masses of 17.2 and 12.5 kDa determined by gradient gel, which correspond to the two small subunits of complex II. Amino acid compositions of these small subunits showed little similarity with those of cytochrome *b*-560 of bovine heart complex II. NADH-fumarate reductase, which is the final enzyme complex in the anaerobic respiratory chain in *Ascaris*, was reconstituted with bovine heart complex I, *Ascaris* complex II and phospholipids. The maximum activity was 430 nmol/min per mg protein of complex II. Rhodoquinone was essential for this reconstitution, whereas ubiquinone showed no effect. The results clearly indicate the unique role of *Ascaris* complex II as fumarate reductase and the indispensability of rhodoquinone as the low-potential electron carrier in the NADH-fumarate reductase system.

Introduction

Complex II is a flavoenzyme that is located in the inner membrane of mitochondria and the cytoplasmic membrane of aerobic micro-organisms. Under aerobic conditions, complex II catalyzes the oxidation of succinate to fumarate and transfers its reducing equivalent to ubiquinone. Complex II has been isolated from mitochondria [1–4] and bacteria [5–7], and the genes for complex II (*sdh*) have been cloned and sequenced in *Escherichia coli* [8,9] and *Bacillus subtilis* [10,11].

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Abbreviations: F_p, flavoprotein subunit of complex II; I_p, Iron-sulphur protein subunit of complex II; TTFA, 2-thenoyltrifluoroacetone; E_m', oxidation–reduction potential at neutral pH; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; QPs, a protein complex that converts succinate dehydrogenase into succinate-ubiquinone reductase; HPLC, high-performance liquid chromatography.

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Complex II of mitochondria is generally composed of four polypeptides. The largest flavoprotein subunit, (F_p) with a molecular weight of about 70000, contains covalently bound flavin and the second largest subunit, (I_p) with a molecular weight of about 30000, contains iron-sulphur. Two small hydrophobic polypeptides, with molecular weights of approx. 15000 and 13000, supply the binding sites for the two large subunits to the membrane. Succinate dehydrogenase activity is located on the two large subunits (F_p and I_p), and the two small subunits seem essential for converting succinate dehydrogenase into succinate-ubiquinone reductase [12–14]. Complex II of animal mitochondria contains *b*-type cytochrome with an equimolar amount of flavin, and its α-absorption peak splits into two peaks at low temperatures [4,13,15]. The participation of this cytochrome in electron transfer in mitochondrial complex II has not been made clear.

Many bacteria which can grow under anaerobic conditions possess a similar enzyme complex, fumarate reductase, which catalyzes the reduction of fumarate. Fumarate reductases have been isolated form *E. coli* [16], *Wolinella succinogenes* [17] and other bacteria, and is well characterized (for a review, see Ref. 18). In *E. coli*, the *frd* operon, which is distinct from the *sdh* operon, has been cloned and sequenced [19–21]. The *frd* operon is induced anaerobically and is strongly repressed under aerobic conditions, whereas the *sdh* operon is induced under aerobic conditions. Thus, fumarate reductase in anaerobic bacteria grown in the presence of fumarate functions as the terminal oxidase which oxidizes reducing equivalents from substrates via menaquinone.

With regard to the mitochondrial anaerobic respiratory chain, electron transfer from NADH to fumarate has been reported in intertidal bivalves [22], fresh water snails [23], parasitic helminths [24,25], and protozoa [26], organisms that accumulate succinate and/or propionate as the end-product of carbohydrate metabolism during anoxia. However, little information is available on the molecular properties of fumarate reductase in anaerobic mitochondria.

The physiological significance of the NADH-fumarate reductase system has been well established in the energy metabolism of the parasitic nematode, *Ascaris suum* [27,28]. This organism resides in the host's small intestine where oxygen tension is fairly limited. It is generally accepted that complex I (NADH-ubiquinone reductase) is the only one site for ATP formation in *Ascaris* adult mitochondria, coupled to electron transfer from NADH to fumarate. This may because these mitochondria contains a small amount of Complex III and an even smaller amount of cytochrome oxidase. We have isolated and characterized complexes I–III (NADH-cytochrome *c* reductase) [29] and complex II from *Ascaris* adult mitochondria [4]. The results revealed that cytochrome *b*-558, a major constituent cytochrome of *Ascaris* mitochondria, is associated with complex II. This *b*-cytochrome is reducible by NADH, α-glycerophosphate, and succinate and reoxidized rapidly by fumarate [4,57] indicating that cytochrome *b*-558 in complex II participates in the electron transfer to fumarate. The participation of cytochrome *b*-560 in electron transfer in bovine heart complex II is controversial.

Complex II is one of the major components in *Ascaris* adult mitochondria (8% of mitochondrial protein), and shows high fumarate reductase activity [30]. This paper reports the properties of complex II as fumarate reductase, the reconstitution of heterogous NADH-fumarate reductase activity from isolated mammalian complex I and *Ascaris* complex II, and the indispensability of rhodoquinone in this system.

Materials and Methods

Preparation of mitochondria and isolation of complex II from Ascaris. Muscle mitochondria of adult *Ascaris suum* and complex II were prepared as described by Takamiya [4,23]. Mitochondria of fertilized eggs were prepared by the method reported by Rodrick [31]. Complex II of bovine heart mitochondria was purified in almost the same manner as for *Ascaris* muscle [15].

Measurement of enzymatic activity. Fumarate reductase activity was measured as the change of absorbance by the oxidation of reduced methyl viologen ($\varepsilon = 6\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ at 550 nm) at 25°C in an anaerobic cuvette as described previously [30]. NADH-fumarate reductase was assayed by monitoring the oxidation of NADH at 340 nm.

The reaction mixture consisted of the reconstituted complexes, 0.1 mM NADH, 10 mM fumarate and 30 mM phosphate buffer (pH 7.5) in a total volume of 3 ml. The reaction was initiated by the addition of fumarate. Succinate-ubiquinone reductase was measured as described [4].

ESR measurement. ESR spectra were obtained with an X-band Bruker ER 200D spectrometer (Bruker, F.R.G.) equipped with a liquid-helium cryostat (Model ESR-900, Oxford Instruments, U.K.). Aliquots of the air-oxidized sample (0.2 ml) were transferred to an ESR sample tubes and kept frozen in liquid nitrogen until use. Measurement conditions are given in Fig. 4.

Separation of cytochrome b-558 by high performance liquid chromatography. Purified complex II was subjected to freezing and thawing more than 5 times using liquid nitrogen, and a solution of 10% (w/v) Sarkosyl was added to produce a final concentration of 3% (w/v). Separation of cytochrome *b*-558 by HPLC with a gel permeation column of TSK gel-G3000SW was achieved as previously described [32]. Absorption of eluted proteins was monitored and analyzed with a photodiode array UV-VIS detector (Model SPD-MIA, Shimazu Ltd., Kyoto). In this system, the spectral properties of eluted proteins can be recorded from 200 to 699 nm without stopping the flow.

Reconstitution of NADH-fumarate reductase activity. A mixture (50 μl) of acetone-washed soybean phospholipid (asolectin, 0.45 mg), various amounts of rhodoquinone or ubiquinone-10, bovine heart complex I (90 μg), *Ascaris* complex II (14 μg) and 30 mM phosphate buffer (pH 7.5) was incubated for 5 min in the cuvette at 25 °C. Then, 30 mM phosphate buffer (pH 7.5) was added to produce the final volume of 3 ml for the assay. Complex I was isolated according to the method of Hatefi [33] and dialyzed against 30 mM phosphate buffer (pH 7.5) before use. The NADH-ubiquinone reductase activity of complex I was 3.8 μmol/min per mg protein at 25 °C, when ubiquinone-2 was used as the electron acceptor.

Protein chemical analysis of cytochrome b-558. Each subunit was separated by electrophoresis in a 12.5% gel in the presence of SDS, and eluted from the gel by two methods. The first method was electroelution, and the second was elution by formic acid. In the latter case, the gel was cut into small cubes and immersed in 1 ml of formic acid for 48 h. The eluted materials by both methods were dialyzed against pure water (Millipore) for 48 h, and the molecular weights of each subunit was checked again before analysis. Amino acid compositions of the materials eluted by formic acid were analyzed by Hitachi 835. The amino terminal sequence of the third subunit ($M_r =$ 15000) of *Ascaris* complex II was determined by automated Edman degradation using an Applied Biosystems model 470A sequenator. In addition to the two samples obtained by the different elution methods described above, a sample prepared by HPLC in the presence of SDS was analyzed. Separation was carried out in a buffer containing 0.1% (w/v) SDS and 200 mM phosphate buffer (pH 7.5) at a flow rate of 1 ml/min with a gel permeation column of TSK gel-G3000SW. All three preparations showed an identical amino-terminal sequence. The recovery in each step was sufficient to allow quantitative determination of amino acid composition and sequence. Sequence homology was analyzed using the database 'PRF/SEQDB' (Peptide Institute Protein Research Foundation, Osaka).

Other methods. Antibodies against each subunit of *Ascaris* complex II were prepared from rabbits. Each subunit was separated by electrophoresis and eluted from the gel by the same procedure as for protein chemical analysis, and was further purified by HPLC (gel permeation column) in the presence of SDS under the same conditions as described above. Specificity of each antibody was checked by Western Blotting. Polyacrylamide gel electrophoresis in the presence of SDS in a normal gel system and the determination of concentration of quinones and protein were carried out as described previously [34]. Western Blotting was done according to the method reported by Towbin [35] using a peroxidase-antiperoxidase system (CAPPEL).

Chemicals. Rhodoquinone of *Rhodospirillum rubrum* was a generous gift from Dr. S. Okayama, Kyushu University. Ubiquinone-10, and methyl viologen were purchased from Sigma. Gradient gel for electrophoresis (SDS-PAGE plate 10/20) was obtained from Daiichi Pure Chemicals (Tokyo).

Other chemicals mentioned in this study [4,30,34] were of analytical grade.

Results

Fumarate reductase activity of Ascaris Complex II

Isolated *Ascaris* complex II showed high fumarate reductase activity when the reduced form of methyl viologen was used as the electron donor. The activity was not observed when ubiquinol or naphthoquinol was used. Complete removal of oxygen and addition of a minimal amount of freshly prepared dithionite are essential for this assay to obtain a reproducible result because of the low E'_m in this system.

The enzyme activity was proportional to the amount of complex II added, and this dose dependency was linear up to 150 nmol methyl viologen reduced/min in 3 ml of the reaction mixture. Table I shows a summary of the kinetic properties of the fumarate reductase of *Ascaris* complex II. The maximum specific activity was 49 μmol/min per mg protein with a turnover number of 6000 min^{-1}, calculated on the basis of protein. The K_m for fumarate was 3.09 mM which was similar to that of mitochondria (3.3 mM). The pH optimum was 7.5, and activity decreased to 80% at pH 8.5. Under acidic conditions (below pH 6.5), the activity decreased apparently, probably due to the pH-dependence of the redox potential of dithionite. The fumarate reductase activity of *Ascaris* complex II was insensitive to the malonate and TTFA which are potent inhibitors of succinate dehydrogenase activity of mammalian complex II.

In general, the mitochondria from aerobic sources have a high ratio of succinate dehydrogenase/fumarate reductase, and the ratios decrease as the environment becomes more anaerobic [36]. The ratio obtained in our experiment on *Ascaris* adult mitochondria and complex II was low (0.05) (Table II), and was constant during purification. This value is comparable to that of the fumarate reductase purified from *E. coli* (0.053) [16]. In contrast, aerobic mitochondria of bovine heart and rat liver showed high ratios, although the specific activity of succinate-ubiquinone reductase was almost identical to that of *Ascaris* mitochondria.

TABLE I

KINETIC PROPERTIES OF *ASCARIS* COMPLEX II AS FUMARATE REDUCTASE

V_{max}	49.0 μmol/min per mg
K_m (fumarate)	3.09 mM
pH Optimum	7.0–8.0
Inhibitors:	
malonate (2 mM)	100 % [a]
TTFA (30 μM)	92.9%
(500 μM)	85.8%

[a] Percentage of control without inhibitor.

Egg mitochondria and immunological cross reactivity

The mitochondria of *Ascaris*-fertilized eggs, which require oxygen for development, also showed fumarate reductase activity, but the ratio of succinate-ubiquinone reductase (1.05) ws intermediate between those of the adult *Ascaris* and mammals. As described in the introduction, *E. coli* has two distinct enzymes, a succinate-ubiquinone reductase and a fumarate reductase. The relative ratio of these two enzymes varies depending upon the growth conditions, and each

TABLE II

COMPARISON OF RATIOS BETWEEN SUCCINATE-UBIQUINONE REDUCTASE AND FUMARATE REDUCTASE

Preparation	(μmol/min per mg protein)		SDH/FRD
	Succinate-ubiquinone reductase	fumarate reductase	
Ascaris			
Isolated complex II	2.04	40.8	0.050
Muscle mitochondria	0.136	2.72	0.050
Egg mitochondria	0.0547	0.0521	1.05
Bovine heart mitochondria	0.135	0.00422	32
Rat liver mitochondria	0.154	0.0077	20

corresponding subunit of isozyme is different in size even though the number of subunits is identical [18]. To analyze the polypeptides of complex II in egg mitochondria, we used antibodies raised against the subunits of Complex II from adult *Ascaris*. For egg mitochondria and adult mitochondria, cross-reacting bands with the same molecular weight were observed (Fig. 1). No additional cross-reacting band of the isozyme was detected near the bands in the egg mitochondria. The subunit with a molecular weight of 15000, the intensity of the band in egg mitochondria was faint because of low antibody titer. Anti-F_p subunit antibody cross-reacted with the F_p in complex II of bovine heart, rat liver, and cytoplasmic membrane of aerobically grown *E. coli* giving a single band with molecular weight around 70000 in blotting. However, antibodies against I_p and the two small subunits were specific for *Ascaris* (data not shown).

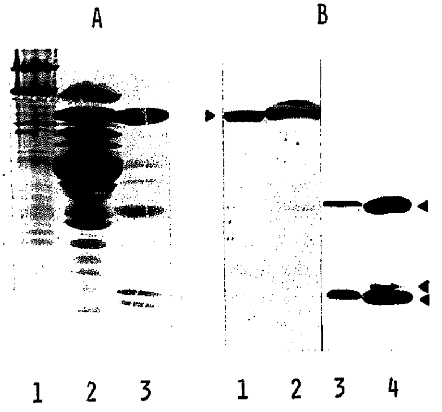

Fig. 1. (A) SDS-polyacrylamide gel (12.5%) of *Ascaris* mitochondria stained for protein. Lane 1, *Ascaris*-fertilized egg mitochondria (11 μg); lane 2, *Ascaris* muscle mitochondria (20 μg); lane 3, complex II isolated from *Ascaris* muscle (2.5 μg). Proteins were stained by Coomassie brilliant blue R250. (B) Western Blot with anti-*Ascaris* complex II antibodies. Lanes 1 and 3, *Ascaris* fertilized egg mitochondria (11 μg); lanes 2 and 4, *Ascaris* muscle mitochondria (20 μg). Lanes 1 and 2 were immunoblotted with anti-*Ascaris* F_p subunit antibody. Lanes 3 and 4 were immunoblotted with a mixture of antibodies against the I_p subunit and the two small subunits of complex II. Arrows indicate the positions of each subunit with molecular weights of 68000 (F_p), 26000 (I_p), 15000 and 13500 (two small subunits) from the top, determined with protein staining of purified complex II.

TABLE III

COMPARISON OF AMINO ACID COMPOSITION OF I_p IN COMPLEX II

	Ascaris	Bovine heart [a]
Amino acids (mol per mol polypeptides)		
Lysine	18.2	23
Histidine	3.31	3
Arginine	8.0	12
Aspartic acid	21.3	28
Threonine	12.8	14
Serine	11.2	15
Glutamic acid	20.9	23
Proline	12.7	13
Glycine	13.6	12
Alanine	18.8	20
Valine	5.44	5
Methionine	7.02	9
Isoleucine	13.4	18
Leucine	16.9	20
Tyrosine	7.02	14
Phenylalanine	8.79	5
Polarity (%) [b]	47.4	46.8
M_r	26000	28655

[a] From the result reported by Yao et al. [37].

[b] Calculated according to Capaldi et al. [56].

The amino acid composition of the *Ascaris* I_p was determined and was found very similar to that of the bovine heart (Table III). It is known that the primary sequence of I_p, as well as of F_p, show marked homology among different animal species and bacteria [9,11,20,37].

Subunit structure of cytochrome b-558

Cytochrome *b*-560 of bovine heart complex II was isolated and characterized first by Hatefi and Galante [13], and by Ackrell et al. [12]. Yu and coworkers recently showed the presence of cytochrome *b*-560 in QPs [38]. In the case of *Ascaris*, it is very difficult to obtain enough cytochrome using similar methods, so we established a micro-analytical system for the cytochromes of mitochondria using HPLC [32]. With this system, cytochrome *b*-558 could be separated from F_p as shown in Figs. 2 and 3. Cytochrome *b*-558 with absorption peak at 413 nm was eluted at 15.4 min, and F_p with broad absorption peaks at 445 and 470 nm was eluted at 16.8 min. The retention times of

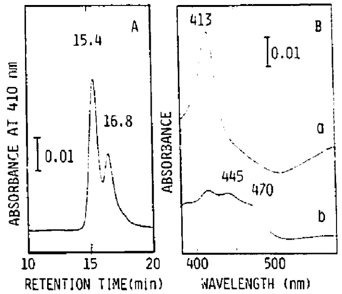

Fig. 2. (A) Elution profiles of cytochrome *b*-558 and F_p from HPLC. After freezing and thawing, complex II (25 μg) was dissociated by 3% (w/v) Sarkosyl and separated on a gel permeation column (TSK gel-G3000SW) as described in Materials and Methods. Elution was monitored by a photodiode array detector. (B) Absolute spectra of peak fractions. (a) Peak at 15.4 min.; (b) peak at 16.8 min.

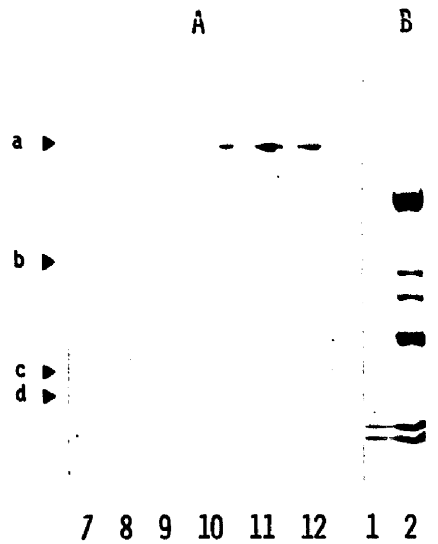

Fig. 3. SDS-polyacrylamide gel electrophoresis of HPLC elute. (A) Fractions eluted from HPLC (Fig. 2) were subjected to electrophoresis in 12.5% polyacrylamide. Fraction no. 8 corresponded to the peak at 15.4 min, and no. 11 to the peak at 16.8 min. Arrows indicate the positions of each subunit of native complex II before dissociation. (a) F_p; (b) I_p; (c) and (d), small subunits with molecular weights of 15000 and 13500, respectively. (B) Cytochrome *b*-558 (fraction No. 8) was subjected to electrophoresis in a gradient gel (10–20%); lane 1, Cytochrome *b*-558. Lane 2, complex II isolated from *Ascaris* muscle. Proteins were stained by Coomassie brilliant blue R250.

cytochrome *b*-558 and F_p corresponded to apparent molecular weights of 90000 and 70000, respectively. For the separation of cytochrome *b*-558, complex II had to be purified from mitochondria prepared with Nagarse treatment, and at least five series of freezing and thawing of complex II was essential before treatment with Sarkosyl. By freezing and thawing, the protein band of I_p in complex II decreased gradually, whereas those of other subunits remained intact. This can be explained by the residual proteolytic activity in the preparation, which may either remove or degrade I_p from complex II. It is indicative, therefore, that I_p plays an important role in the assemblage of *Ascaris* complex II.

In 12.5% polyacrylamide gel, a broad protein band with a molecular weight of 16000, which was slightly larger than that of the third subunit (M_r = 15000: arrow C in Fig. 3A) of *Ascaris* native complex II was observed in fractions containing cytochrome *b*-558 (Fig. 3A, lane 8). The subunit structure of cytochrome *b*-558 was analyzed further using gradient gel (Fig. 3B). Two protein bands with molecular weights of 17200 and 12500 were observed which corresponded to the two small subunits of the native complex II. Identification of each of the subunits was done using antibodies monospecific for each peptide (data not shown). Thus, the migration of these two subunits were affected by the electrophoresis system, and these phenomena were also reported about cytochrome *b*-560 in bovine heart complex II [14,39].

The amino acid composition of the subunits in cytochrome *b*-558 was determined and compared with those of cytochrome *b*-560 as reported by Hatefi and Galante [13] (Table IV). High leucine and glycine content in the large subunit (M_r = 15000) and high leucine, glycine, and alanine in the small subunit (M_r = 13500) are properties similar in the *Ascaris* and the bovine heart. The amino acid composition of the subunit with a molecular weight of 15000 in bovine heart complex II, reported by Capaldi [39] and Yu [14] was also similar with the exception of the content of aspartic acid and glutamic acid. On the contrary, composition of polar amino acids, especially serine, was somewhat different. The calculated polarity of these peptides was low, and the tendency for smaller subunits to be more hydrophobic than

TABLE IV

COMPARISON OF AMINO ACID COMPOSITION OF TWO POLYPEPTIDES OF *b*-CYTOCHROME IN COMPLEX II

Amino acid	Large polypeptide		Small polypeptide	
	cytochrome *b*-558 (*Ascaris*)	cytochrome *b*-560 (bovine heart) [a]	cytochrome *b*-558 (*Ascaris*)	cytochrome *b*-560 (bovine heart) [a]
Amino acids (mol per mol polypeptide):				
Lysine	6.64	5.05	3.03	5.31
Histidine	3.50	5.79	4.69	4.71
Arginine	6.62	3.22	4.39	2.03
Aspartic acid	8.60	4.28	6.71	6.98
Threonine	8.10	9.09	5.29	7.44
Serine	5.44	14.23	2.12	9.40
Glutamic acid	8.18	7.26	6.75	3.79
Proline	7.94	6.21	5.55	3.33
Glycine	12.5	13.41	10.6	10.17
Alanine	8.76	12.1	15.9	17.03
Valine	7.21	8.28	8.00	9.81
Methionine	2.67	4.79	2.60	1.97
Isoleucine	8.13	5.95	4.34	3.25
Leucine	11.0	21.78	13.5	17.55
Tyrosine	2.88	1.46	4.21	4.68
Phenylalanine	9.40	4.96	3.56	3.30
Polarity (%) [b]	39.9	37.5	30.8	35.1
M_r	15000	15500	13500	13500

[a] From the result reported by Hatefi [13].

[b] Calculated according to Capaldi [56].

larger subunits is also characteristic for the corresponding bovine heart peptides [13] and the gene products of *E. coli*, *sdh C D* [8] and *frd C D* [21].

The amino-terminal amino acid sequence of the subunit with a molecular weight of 15000 in 12.5% gel was determined by automated Edman degradation (Table V). No homology was found between this sequence and amino acid sequences of bacterial counterparts such as the gene products of *sdh C* [8] and *frd C* [21] of *E. coli*, and that of *sdh C* of *B. subtilis* [10]. Also, no homologous sequence was found in a database containing 10021 polypeptides. The amino-terminal sequence of the smallest subunit ($M_r = 13500$) could not be determined, possibly due to blockage.

EPR spectroscopy of cytochrome b-558 in complex II

An EPR spectrum of an air-oxidized form of *Ascaris* complex II recorded at 8 K is shown in Fig. 4. Signals can be detected at g values of 6.0, 4.3 and 3.6. The broad signal at a *g* value of 3.6 may be assigned to a ferric low-spin heme resonance by the analogy of the corresponding spectra of complex II from bovine heart mitochondria [38,40] and cytochrome *b*-558 from *Bacillus subtilis* [41]. This broad signal may indicate that the interaction between cytochrome *b*-558 and succinate dehydrogenase (F_p and I_p) in the isolated *Ascaris* complex II is intact as discussed by Yu et al. [38], although the *g* value is slightly different from that of the bovine heart cytochrome *b*-560. The $g = 3.6$ signal disappeared after reduction by succinate. The signal at a *g*-value of 6.0 seemed to be from ferric high-spin heme and was not observed in all preparations. Cytochrome *b*-566 of complex II of *E. coli* also showed this kind of high-spin signal [42]. The signal with a *g*-value of 4.3 is likely due to free ferric ions as discussed previously [42]. In the spectrum of the air-oxidized form, a $g = 2.03$ signal from iron sulfur cluster – 3

TABLE V

AMINO ACID SEQUENCE OF A LARGE SUBUNIT OF CYTOCHROME *b*-558 FROM AMINO-TERMINAL

X, not determined.

Residue number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Amino acid	X	Ala	Glu	Lys	Thr	Pro	Ile	Gln	Val	X	Gly	Pro	Asp	Tyr

Residue number	15	16	17	18	19	20	21	22	23	24	25	26	27
Amino acid	Leu	Met	X	Gln	Ala	Ala	Leu	Lys	X	Pro	X	Ala	Pro

was observed. This signal was partially reducible by succinate (Kita, K., unpublished results).

Reconstitution of NADH-fumarate reductase activity and effect of rhodoquinone

In anaerobic respiration by *Ascaris* mitochondria, reducing equivalents from NADH have been thought to enter the electron-transport chain at the site of complex I and ultimately transferred to fumarate by the fumarate reductase [28]. However, no substantial information on the electron transport from NADH to fumarate and the participating components in the chain has been documented except for our report on the participation of cytochrome *b*-558 [4,29]. Reconstitution of NADH-fumarate reductase activity from purified complexes I and II was therefore attempted. For the reconstitution, complex I purified from bovine heart mitochondria was used because it is difficult to obtain *Ascaris* complex I with high NADH-ubiquinone reductase activity and high purity. Rhodoquinone, which is the major quinone in *Ascaris* mitochondria [43], and phospholipid were essential for the reconstitution. The dependency of rhodoquinone and the small effect of ubiquinone are shown in Fig. 5. The dose response of rhodoquinone was linear up to 35 nmol per reaction mixture. Maximum specific activity was 430 nmol/min per mg of complex II, and which is about 15% of the specific activity in *Ascaris* mitochondria calculated on the amount of complex II. One of the reasons for low specific activity of reconstituted NADH-fumarate reductase can be ascribed to the low reduction rate of rhodoquinone by bovine heart complex I (Kita, K., unpublished results). It was required to incubate the reaction mixture at high concentration otherwise no activity was reconstituted. The NADH-fumarate reductase activity obtained without rhodoquinone may be due to trace amount of endogenous rhodoquinone in purified complex II

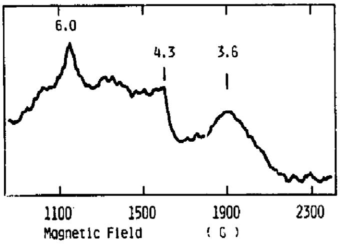

Fig. 4. EPR signals from air-oxidized *Ascaris* complex II. The complex II (3.9 mg/ml) was air-oxidized. The cytochrome *b* content of the preparation used was 30 μM. Conditions were as follows: microwave power, 5 mW; modulation amplitude, 10 G at 100 kHz; response time, 320 ms; magnetic field, 1500 G ± 1000 G; accumulation, 16 times; temperature, 8 K.

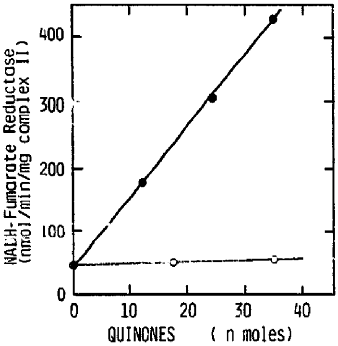

Fig. 5. Reconstitution of NADH-fumarate reductase activity and the effect of quinones. Reconstitution and assay were carried out as described in Materials and Methods. ●——●, reconstituted with rhodoquinone; ○——○, reconstituted with ubiquinone-10.

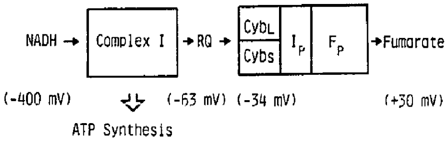

Fig. 6. Arrangement of components in NADH-fumarate reductase system in *Ascaris* muscle mitochondria. The values in parentheses are E_m' of the components. RQ, rhodoquinone; F_p, flavoprotein subunit; I_p, Iron-sulphur subunit; Cyb_L, large subunit of cytochrome *b*-558 ($M_r = 15000$); Cyb_S, small subunit of cytochrome *b*-558 ($M_r = 13500$).

or low potential ubiquinone in complex I as reported by King and Suzuki [44]. No rhodoquinone and enzymatic activities of the individual complexes were detected in the supernatant when the reconstituted mixture was centrifuged, indicating that they were all bound to the liposomes.

This reconstitution provided direct evidence that complexes I and II and rhodoquinone are the only components required for electron transport from NADH to fumarate.

Discussion

From our previous observations [4,29,30] and the result presented in this work we propose a linear sequential order of the respiratory components in the NADH-fumarate reductase system in *Ascaris* muscle mitochondria, as shown in Fig. 6.

Complex I of *Ascaris* mitochondria functions as the only one coupling site in anaerobic cells, and a similar composition of peptides to that bovine heart was reported from the analysis of purified complexes I–III [29].

Ubiquinone is the most widely distributed benzoquinone in nature and accepts reducing equivalents from complexes I and II in the mammalian respiratory chain. Rhodoquinone has been found only in a few micro-organisms and parasitic helminths that are capable of fumarate reduction [43]. *Ascaris* mitochondria has been reported to contain only rhodoquinone-9 instead of ubiquinone-10 [43], and a specific requirement of rhodoquinone for electron transfer in *Ascaris* mitochondria have been shown by extraction and re-incorporation experiment [45]. In rhodoquinone, the methoxy group of ubiquinone is substituted with an amino group (2-amino-3-methoxy-6-methyl-5-isoprenyl-1,4-benzoquinone), and the E_m' (−63 mV) [46] is more negative than that of ubiquinone (+110 mV). In the case of bacteria, reducing equivalents from dehydrogenases are transferred to fumarate reductase via low-potential naphthoquinone (menaquinone; −74 mV), and ubiquinone cannot restore the deficiency of menaquinone [18]. *Ascaris* complex II is able to react with ubiquinone because specific activity of succinate-ubiquinone reductase is similar to that in mammals, suggesting that specificity of rhodoquinone in reconstitution is due to the difference in E_m' between rhodoquinone and ubiquinone rather than the structural differences. The data presented here clearly demonstrate the indispensability of rhodoquinone as the low potential electron carrier in the *Ascaris* NADH-fumarate reductase system.

Ascaris adult complex II showed high fumarate reductase activity and a low succinate dehydrogenase/fumarate reductase ratio. Low ratio of succinate dehydrogenase/ fumarate reductase (0.05) is consistent with the suggestion that the activity ratio is low in anaerobic organisms [36], and is similar to those of obligate anaerobes. The intermediate properties of *Ascaris* egg mitochondria stimulate our interest about whether there are two different enzymes, namely a succinate dehydrogenase and a fumarate reductase similar to in *E. coli*. If *Ascaris* indeed has both enzymes, then the relative ratio should change during the life cycle. However, there is no evidence for the occurrence of two separate enzymes in mitochondria. The apparent molecular weight and antigenic properties of each subunit were found identical in adult muscle and egg mitochondria. In *Ascaris*, the reversible reaction by a single enzyme, succinate dehydrogenase, has been suggested by Kemtec and Bueding [27]. To clarify this point, properties of complex II of larvae which aerobically metabolize and have an active cyanide-sensitive oxidase [47], have to be elucidated.

The association of *b* cytochrome to complex II is a general feature in mitochondria and bacteria [1–7], and the splitting of the α-absorption peak in *b*-cytochrome in animal mitochondria, such as bovine heart [1] and *Ascaris* [4], has also been confirmed in *Paragonimus westermani* [15]. Cytochrome *b*-560, isolated from bovine heart, contains two small polypeptides [13]. These polypeptides were also identified in *Ascaris* cytochrome *b*-558 as shown in Fig. 3, though the polypeptides associated with *b*-heme have not been determined. The content of histidine, which is generally thought to be ligand for heme is as low in these subunits (Table IV) as is the case of *E. coli* [8]. Cammack has suggested the possibility that the heme is bound between the two small subunits [48] of complex II. Cytochrome *b*-556, which is the *b*-cytochrome in complex II of *E. coli*, was purified [49] and identified by the present authors [50] as the third subunit ($M_r = 14200$) of the complex. Positive E'_m (-34 mV) of *Ascaris* cytochrome *b*-558 (Takamiya, S. et al., unpublished data) compared with that of cytochrome *b*-560 of bovine heart (-185 mV) [38] is favorable to the direction of electron transfer from rhodoquinone (-63 mV) [46] to the succinate/fumarate couple ($+30$ mV), which can explain the reducibility of cytochrome *b*-558 by succinate.

Cytochrome *b*-560 of *Ascaris* muscle, which also has split peaks at low temperatures with molecular weight of 14000, was purified and characterized as a soluble protein by Cheah [51]. This cytochrome *b*-560 of *Ascaris* is distinct from cytochrome *b*-558 in complex II because the amino acid composition is different, and the polarity of cytochrome *b*-560 (53%) is much higher than that of cytochrome *b*-558 (39.9 and 30.8%).

The requirement of two small subunits in electron transport in complex II has been suggested from capability in converting succinate dehydrogenase to succinate-ubiquinone reductase [12–14]. The essential role for these subunits in quinone reduction is also shown in the fumarate reductase system in *E. coli* [52,53]. Differences in the molecular weight, antigenic properties, and amino acid compositions were observed in the two small subunits of *Ascaris* and bovine heart. In contrast, antibody against *Ascaris* F_p cross-reacted with F_p's of other sources. Amino acid compositions of *Ascaris* F_p (to be presented elsewhere) and I_p (this work) closely resemble those of the bovine heart. In *E. coli*, no sequence homology in the two small subunits between succinate dehydrogenase and fumarate reductase was observed, whereas F_p and I_p of both enzymes showed a marked homology in their total amino acid sequence [8]. Based on our observation and evidence from bacterial systems, it can safely be said that the two small subunits play important roles in electron transfer between quinone and substrate, in determining the direction of electron transfer in complex II as well as their anchoring function to the membrane.

As described above, *Ascaris* complex II of adult muscle mitochondria functions as the terminal oxidase and donates electrons to fumarate, which is the terminal acceptor under anaerobic conditions. On the other hand, spectroscopic detection of cytochrome oxidase in *Ascaris* larvae has been done by Hayashi et al. [54], and the presence of cytochrome oxidase was confirmed recently by the nucleotide sequence of *Ascaris* mitochondrial DNA [55]. Thus, *Ascaris* adapts itself to the changes of environmental and physiological conditions in its life cycle by modulating energy metabolism including terminal oxidase systems. To better understand the mechanisms of adaptation to anoxia, more intensive study for the structure and function of complex II as entrance (succinate dehydrogenation) and exit (fumarate reduction) of the respiratory chain in *Ascaris* mitochondria is needed.

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