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Comparative Study on the Primary Structure of the Iron-sulfur Subunit of Complex II in *Ascaris suum* Mitochondria

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Abstract

Complex II (succinate-ubiquinone oxidoreductase; SDH) is an important enzyme complex of both the tricarboxylic acid cycle and of the aerobic respiratory chain of mitochondria in eukaryotic cells and prokaryotic organisms. Complex II also catalyzes the reduction of fumarate (fumarate reductase; FRD), which is the reverse reaction of the SDH activity, in the anaerobic respiratory chain of *Ascaris*. The iron-sulfur subunit (Ip) of this enzyme complex contains three distinct types of iron-sulfur clusters, S-1, S-2 and S-3, and these prosthetic groups are essential for electron transfer in complex II.

In this study, the primary structure of the Ip subunit of complex II of *Ascaris* was determined using the peptide separated on Biogel P-60 in the presence of sodium dodecylsulfate. The alanine/threonine cluster, a conserved region in the amino terminus of the mammalian Ip subunit, was found in the *Ascaris* Ip subunit. Two of the three cysteine-rich segments generally conserved in the Ip subunit were identified by chemical modification of cysteine residues. Cysteine is thought to be an essential amino acid for the ligation of iron-sulfur clusters to the polypeptide. Modification was performed by alkylation of cysteine residues in the tryptic peptides of the Ip subunit with 4-vinylpyridine and subsequent separation of the peptides by high-performance liquid chromatography (HPLC). Two highly labeled peptides with typical sequences corresponding to S-1 and S-3 cluster binding sites were found. Peptides with an amino acid sequence similar to the region between the S-1 and S-2 cluster binding sites were also found in the separated peptides. From a comparative analysis of the sequences, the Ip subunit of complex II in *Ascaris* adult appears to be more closely related to those of bacterial SDH and mammalian complex II than to that of bacterial FRD, even though *Ascaris* complex II shows a high FRD activity.

Key words: Complex II, iron-sulfur protein, fumarate reductase, *Ascaris* mitochondria, anaerobic respiration

Introduction

Energy metabolism is one of the essential functions for the survival, growth, and reproduction of living organisms. A biochemical strategy to generate ATP, the key intermediate in the metabolism of parasites, is of particular importance. *Ascaris suum* is a parasitic nematode of swine and adult worm resides in the host's small intestine, where oxygen tension is quite limited. Adult *Ascaris* has the NADH-fumarate reductase system, an anaerobic respiratory chain, in which the reduction of fumarate to succinate is coupled to ATP synthesis. The fumarate reductase system was resolved into electron transfer complexes (Takamiya *et al.*, 1984, 1986). The fumarate reduction is catalyzed by complex II which unlike mammalian enzyme (succinate-ubiquinone oxidoreductase, SDH) functions in reverse direction as fumarate reductase (FRD) (Takamiya *et al.*, 1986; Kita *et al.*, 1988b and see reviews, Köhler, 1985; Oya and Kita, 1988; Kita

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et al., 1991). Since *Ascaris suum* is relatively large in size, mitochondria from adult body-wall muscle provide an excellent model system for biochemical study of anaerobic energy metabolism.

Mitochondrial complex II is generally composed of four polypeptides and appears to be a highly conserved enzyme complex (Ohnishi, 1987; Cole *et al.*, 1985). The largest flavoprotein subunit (Fp) with a molecular weight of about 70 kDa contains covalently bound flavin, and the second largest subunit (Ip) with a molecular weight of about 30 kDa contains iron-sulfur clusters. The Fp and Ip subunits are hydrophilic and form a catalytic portion of the enzyme complex that transfers reducing equivalents from succinate to water-soluble dyes such as 2, 6-dichlorophenol indophenol (SDH) or from reduced methyl viologen to fumarate (FRD). Two small hydrophobic membrane-anchoring polypeptides with molecular weights of about 15 kDa and 13 kDa (cytochrome *b* subunit; cybL and cybS) seem to be essential for the interaction between the complex and quinone species (Takamiya *et al.*, 1986, 1990; Kita *et al.*, 1988b, 1990a).

Iron-sulfur clusters on the Ip subunit are the essential prosthetic group for electron transfer in complex II. Three distinct types of iron-sulfur clusters are present in complex II: S-1; [2Fe-2S], S-2; [4Fe-4S], and S-3; [3Fe-4S] (Ohnishi, 1987). Spectroscopic analysis by Electron Spin Resonance shows a relationship between high fumarate reductase activity and the novel redox properties of the S-3 cluster in complex II of adult *Ascaris* (Hata *et al.*, 1988). The Em' value of S-3 clusters in mammalian complex II is +65 mV and the cluster is reported to be completely reduced by succinate. In the case of adult *Ascaris*, the S-3 clusters are only partially reduced by succinate, indicating that the Em' value of *Ascaris* S-3 is lower than that of the succinate/fumarate couple (+30 mV). A lower Em' value of the S-3 cluster is a common feature of the fumarate reductase system in *Wolinella succinogenes* (–24 mV) (Unden *et al.*, 1984) and *E. coli* (–70 mV) (Cole *et al.*, 1985). Clearly, the Ip subunit is a key subunit in complex II and much more information is required to understand its structure, function, and assembly. Complete amino acid sequences of complex II have been deduced from the DNA sequence of *E. coli* (*sdh* and *frd*) (Darlidson and Guest, 1984; Wood *et al.*, 1984), *Bacillus subtilis* (*sdh*) (Magnusson *et al.*, 1986; Phillips *et al.*, 1987) and *Proteus vulgaris* (*frd*) (Cole, 1987). However, structural information on mitochondrial complex II is still limited; only the primary structures of the Ip subunit from bovine (Yao *et al.*, 1986), human (Kita *et al.*, 1990) and *Saccharomyces cerevisiae* (Lombardo *et al.*, 1990) and partial amino acid sequences of the Fp subunit from bovine (Kenney *et al.*, 1972) and *Ascaris* (Furushima *et al.*, 1990) have been reported.

This paper reports the partial amino acid sequence of the Ip subunit in *Ascaris* adult mitochondria, with a special focus on the cysteine-rich segments thought to comprise the iron-sulfur clusters.

Materials and Methods

Preparation of Ip subunit from Ascaris adult complex II

Complex II was purified from *Ascaris* adult muscle mitochondria as described by Takamiya *et al.* (1986), except that the protease pretreatment was omitted. The Ip subunit was isolated from the complex II basically by the same method used for the purification of the Fp subunit of *Ascaris* complex II (Furushima *et al.*, 1990). Purified complex II from *Ascaris* mitochondria was precipitated in 50% saturated solid ammonium sulfate and dissolved in 20% (w/v) SDS containing 2% 2-mercaptoethanol. The sample was then subjected to gel filtration chromatography on a Biogel P-60 (<400) column equilibrated with 2% (w/v) SDS.

Tryptic digestion of the Ip subunit and chemical modification of cysteine residues

The isolated Ip subunit (210 μg) eluted from the Biogel P-60 column described above was dialyzed against 50% (v/v) ethanol for 48 hr at 4°C to remove the SDS and lyophilized. The sample was resuspended in 120 μl of 0.1 M Tris-HCl (pH 8.0) containing 2 M urea and

digested with TPCK-trypsin (2 μg) for 3 hr at 37°C.

For the chemical modification of cysteine residues (Friedman *et al.*, 1970; Fullmer, 1984), the tryptic peptides were first reduced by the addition of 2-mercaptoethanol (about 100 molar excess over total disulfides) and incubated for 30 min at 37°C. The free sulfhydryl groups were then exposed to 4-vinylpyridine (1:1 molar ratio with respect to all sulfhydryl groups) for 3 hr at 37°C with occasional mixing. The peptides were then separated by high-performance liquid chromatography (HPLC) on a Shimadzu Liquid Chromatograph LC-3A equipped with an ODS-80TM column (Toso, ϕ4.6 × 150 mm) with a 20 to 60% gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). The elution of cysteine-containing peptides was monitored by the absorbance at 254 nm derived from S-(4-pyridylethyl)-L-cysteine.

Amino acid sequence analysis

The amino acid sequences of the amino terminal of the Ip subunit eluted from Biogel P-60 and the tryptic peptides separated by HPLC were determined by automated Edman degradation using an Applied Biosystems Model 470A protein sequensor in the Central Laboratory of Medical Sciences, Division of Biochemical Analysis, at Juntendo University.

Other methods

The mixture of anti-Fp and anti-Ip subunit antibodies against bovine heart was prepared in rabbit by using purified bovine heart complex II as an antigen. Antibody against the *Ascaris* Ip subunit was obtained from rabbit immunized with isolated Ip subunit. Western blotting was performed according to the method described by Towbin *et al.* (1979) using an alkaline phosphatase system for detection. Homologies of the Ip subunits of the various species were maximized by the computer program GENETYX.

Chemicals

TPCK-trypsin and 4-vinylpyridine were purchased from Sigma and Aldrich, respectively. The gradient gel for SDS-polyacrylamide gel electrophoresis was obtained from Daiichi Pure Chemicals, and Biogel P-60 was obtained from Bio Rad Laboratories. Other chemicals mentioned in this study were of analytical grade.

Results

Properties and purification of the Ip subunit

In general, the Ip subunit of complex II is thought to be highly conserved subunit with respect to size, amino acid composition, and antigenic properties (Ma *et al.*, 1987; Kita *et al.*, 1991). However, when we cloned and sequenced the cDNA for the Ip subunit from human liver mitochondria by immunoscreening a λgt11 cDNA library (Kita *et al.*, 1990), we found that antibodies raised against the mammalian Ip subunit did not recognize the Ip subunit of *E. coli* complex II (SDH), although the amino acid sequences are highly homologous. In order to investigate the antigenic properties of the *Ascaris* Ip subunit, the cross-reactivity was analyzed using antibodies against the Ip subunits from *Ascaris* and bovine heart. In contrast to anti-bovine Fp subunit antibodies, anti-bovine Ip subunit antibodies did not cross-react with the Ip subunit of *Ascaris* (Fig. 1A). Antibodies against the *Ascaris* Ip subunit also did not recognize the bovine heart Ip subunit (Fig. 1B). This result indicates a difference in the epitopes on the polypeptides of bovine Ip and *Ascaris* Ip despite their similar amino acid compositions. This difference may be related to some unique feature of the Ip subunit of *Ascaris* complex II, such as a novel redox property of the S-3 center. Information on the primary structure of Ip is essential to understand the structure and function of the subunit.

Purification of the Ip subunit for protein chemical analysis was achieved satisfactorily on a Biogel P-60 column in the presence of SDS. The efficacy of this column has been shown especially in the case of the isolation of peptides from membrane associated enzyme complexes (Takamiya *et al.*, 1987). All four subunits of *Ascaris* complex II were separated from one another and the recovery of each peptide was greater than 80%. The isolated Ip subunit migrated as a single

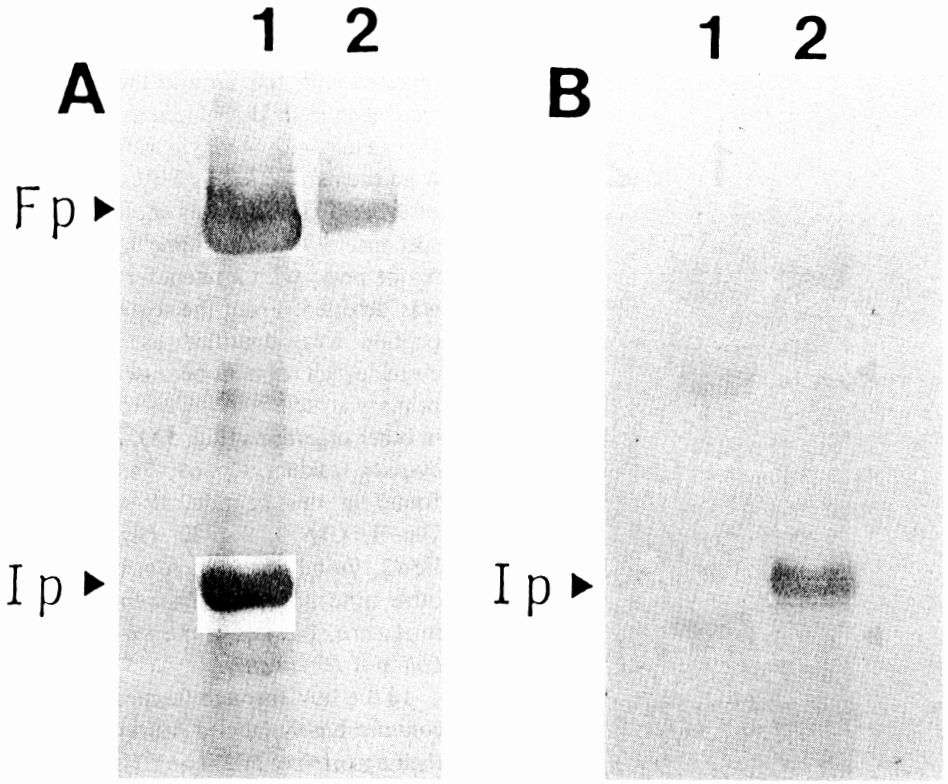

Fig. 1 Western blot with anti-Ip subunit antibodies. Lane 1, bovine heart complex II (9 μg). Lane 2, *Ascaris* adult complex II (9 μg). (A) Western blot with a mixture of anti-Fp and anti-Ip subunit antibodies against bovine heart. Arrows indicate Fp (68 kDa) and Ip (26 kDa) subunit. (B) Western blot with anti-Ip subunit antibody against *Ascaris* adult. Arrows indicate Ip subunit.

band on SDS-polyacrylamide gel electrophoresis (Fig. 2) and was used for the following studies.

Amino terminal sequence of the Ip subunit

The amino acid sequence of the amino terminal portion of the *Ascaris* Ip subunit eluted from Biogel P-60 was determined by automated Edman degradation. A thirty amino acid sequence was obtained and compared with those of human liver (Kita *et al.*, 1990b), bovine heart (Yao *et al.*, 1986) and *S. cerevisiae* (Lombardo *et al.*, 1990) mitochondria, as well as bacterial succinate dehydrogenase and fumarate reductase (Cole, 1987; Darlidson and Guest, 1984; Cole *et al.*, 1982) (Fig. 3, Ala-1 to lys-30). The entire amino terminal region of mitochondrial Ip is rich in alanine and sequence conservation was found. Compared with the mammalian sequence, two additional amino acids, alanine and serine, were found in the amino terminal of the *Ascaris* Ip subunit. The presence of two extra amino acids in the terminal was previously suggested from a comparison of the DNA sequences of the yeast and bovine Ip subunits (Lombardo *et al.*, 1990) and is shown directly by this study.

The binding site for iron-sulfur clusters

Nearly all iron-sulfur clusters are ligated to the polypeptide chain by covalent linkages with the sulfhydryl groups of cysteine residues (Cole *et al.*, 1985). From a comparison of the primary structures of Ip subunits and ferredoxins (Adman *et al.*, 1973), both of which contain iron-sulfur cluster prosthetic groups, three cysteine-rich

segments in the Ip subunits have been thought to comprise the iron-sulfur clusters of the enzyme. To analyze the cysteine-rich segments of the *Ascaris* Ip subunit, the isolated peptide was digested with trypsin and the cysteine residues were chemically derivatized to S-(4-pyridylethyl)-L-cysteine with 4-vinylpyridine. The peptides were then separated by HPLC (Fig. 4). Elution of labeled peptides was monitored by the absorbance of 4-vinylpyridine at 254 nm. Only one major peak, with a retention time of 21.5 min, was detected. From the sequence analysis, this peptide was identified as the S-1 binding, cysteine-rich segment because of its similarity in primary structure to the corresponding segment in other organisms (Fig. 5A). All four conserved cysteine residues, Cys-69, -74, -77 and -89, were found in this peptide. It was observed that Glu-71, Gly-72, Asn-80, Gly-83, Asn-85, and Ile-93, found in the *E. coli sdh B* product and other mitochondrial Ip subunits, are conserved in *Ascaris,* but not in the *frd B* products of *E. coli* and *P. vulgaris.*

In the flow through fraction from the HPLC column, highly labeled peptides were found by their absorbance at 254 nm. The mixture of these peptides was sequenced directly because the individual peptides were difficult to resolve. Eight residues were successfully sequenced and most of

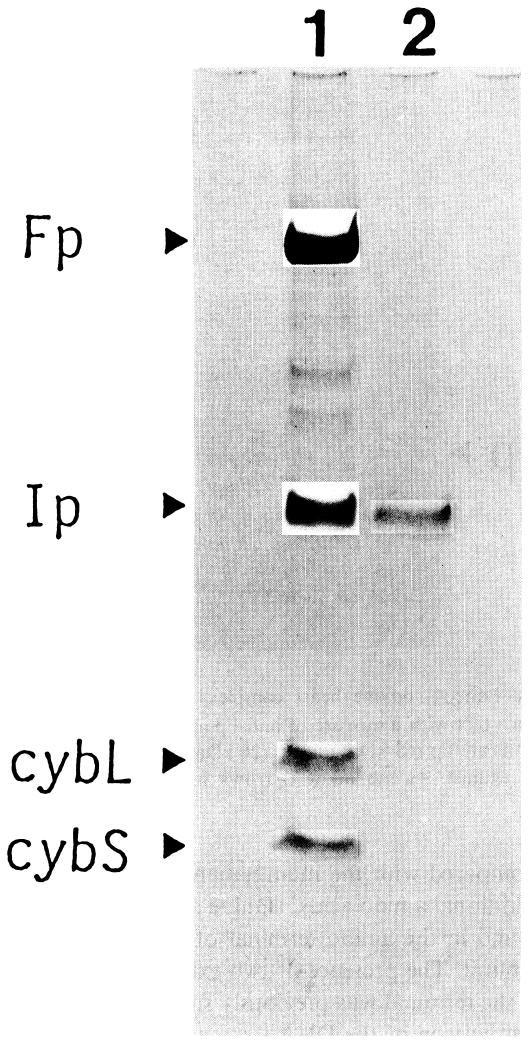

Fig. 2 Polyacrylamide gel electrophoresis of *Ascaris* adult complex II and the Ip subunit using a gradient gel (15–25%) in the presence of SDS. Lane 1, Purified complex II (9 μg). Complex II was isolated from *Ascaris* muscle. Arrows indicate the positions of each subunit: Fp (68 kDa); Ip (26 kDa); cybL (15 kDa) and cybS (13.5 kDa). Lane 2, Purified Ip subunit (9 μg). Arrow indicates the Ip subunit isolated by Biogel P-60 column chromatography.

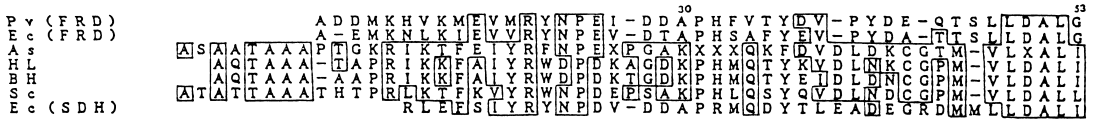

Fig. 3 A comparison of the amino terminal sequences of Ip subunits. As, *Ascaris suum;* HL, Human liver (Kita *et al.*, 1990b); BH, Bovine heart (Yao *et al.*, 1986); Sc, *S. cerevisiae* (Lombardo *et al.*, 1990); Ec, *E. coli* (Darlidson and Guest, 1984; Cole *et al.*, 1982); and Pv, *P. vulgaris* (Cole, 1982). Identical residues are boxed in all cases. The numbering is according to the *Ascaris* sequence. The sequence of *Ascaris* Ip from Ala-1 to Lys-30 was determined by amino terminal analysis, and the sequence from Gln-34 to Ile-53 was determined for the tryptic peptide eluted in Peak 3 (Fig. 4).

the sequence cycles contained more than two amino acids. In this mixture of peptides, a well-matched sequence to the S-3 binding cysteine-rich segment was found (Fig. 5B, underlined). Considering the high incorporation of 4-vinylpyridine and the identity in sequence, this peptide seems to be the S-3 cluster binding site of the Ip subunit of *Ascaris* complex II. It should be noted that a sequence corresponding to the S-3 binding site of bacterial fumarate reductase was not found in the mixture of peptides.

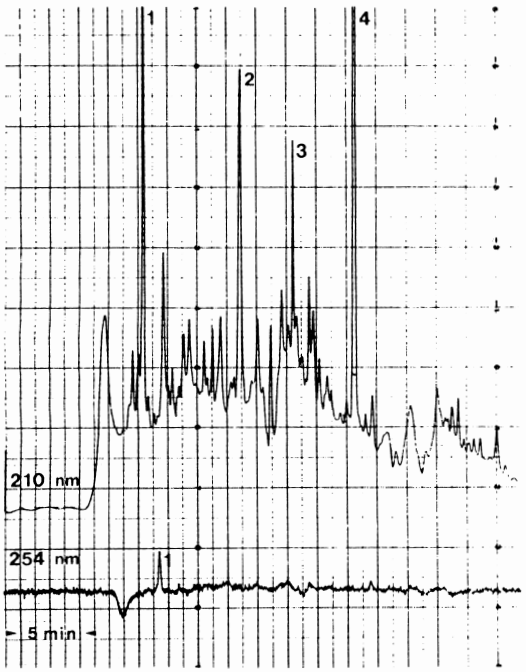

Fig. 4 HPLC elution profile of the tryptic peptides of the *Ascaris* Ip subunit. Experimental details are described in Materials and Methods. Elution of peptides was monitored by the absorbance at 210 nm, and that of labeled peptides was monitored at 254 nm to detect S-(4-pyridylethyl)-L-cysteine. Peak 1 (retention time, 21.5 min), which exhibits a strong signal at 254 nm, contains the peptide corresponding to the S-1 cluster. Retention times of other major peaks are: Peak 2, 29.2 min; Peak 3, 34.2 min; Peak 4, 39.5 min.

Sequence similarity of Ip subunits from Ascaris and other organisms

In addition to the peptides labeled with

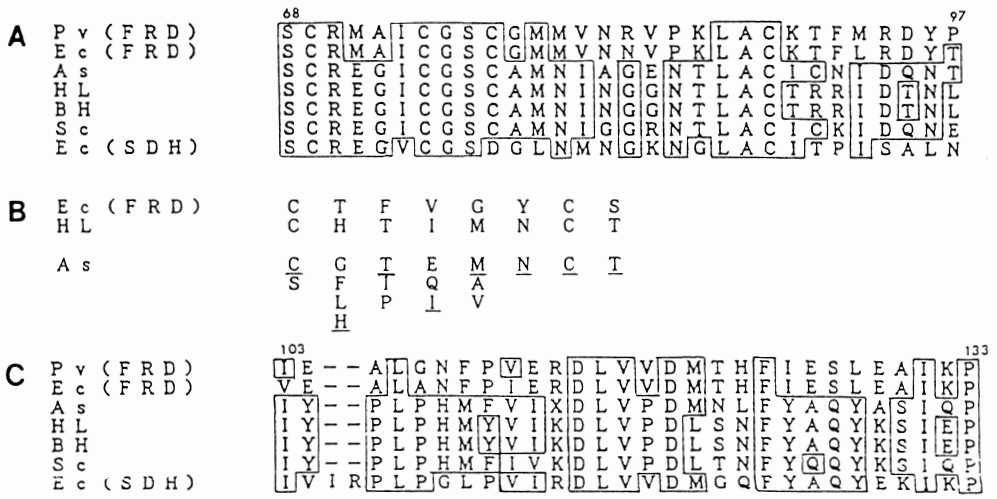

Fig. 5 Comparison of the partial amino acid sequence of the *Ascaris* Ip subunit with the sequences of the Ip subunits from various species (For abbreviations, see Fig. 3). (A) Sequence corresponds to the binding site for cluster S-1. (B) The sequence corresponding to S-3 cluster binding sites of *E. coli frd B* and human liver are compared with the amino acid sequence of the mixture of peptides in the flow through fraction from HPLC. The 1st cysteine of *E. coli* (*FRD*) is Cys-213 and the 1st cysteine of human liver is Cys-219. (C) Sequences corresponding to the region between S-1 and S-2 cluster binding sites. The numbering in (A) and (C) is according to the *Ascaris* sequence.

4-vinylpyridine, some of the other tryptic peptides separated by HPLC were sequenced in order to obtain further information on the primary structure of the *Ascaris* Ip subunit. The peptides with amino acid sequences corresponding to the region between the S-1 and S-2 clusters binding sites were found among the major peaks eluted from the HPLC column. The sequences of the peptides contained in peak 2 (Ile-103 to Ile-114) and peak 4 (Asp-116 to Pro-133) are aligned in Fig. 5C. Residue 115 may be arginine or lysine because trypsin cleaves on the carboxyl side of arginine and lysine residues, and in all other organisms this residue is either arginine or lysine. A peptide corresponding to Pro-31 to Ile-53 of the yeast Ip subunit was also found in peak 3, with a retention time of 34.2 min (Fig. 3 and 4). The first 3 amino acids residues of this peptide could not be determined because of high background levels during amino acid analysis.

Thus, about half of the amino acid sequence of *Ascaris* Ip subunit (255 amino acids in the human Ip subunit) was determined in this study; a comparison of the partial amino acid sequences of *Ascaris* and Ip subunits from other organisms is summarized in Table 1. The Ip subunit of complex II is a highly conserved protein, suggesting its functional importance in complex II. The homology between the Ip subunits of *Ascaris* and *S. cerevisiae* in their S-1 cluster binding domains was higher (86.7%) than those between *Ascaris* and human or bovine (76.7%). A similarity between *Ascaris* and *S. cerevisiae* was also found in the amino terminal region of the subunit in the conservation of Thr-16, Asn-23, Pro-27, Ala-29 and in the presence of two additional amino acids. It should be noted that the similarity between the Ip subunit of *Ascaris* and bacterial fumarate reductase was lowest of the Ip subunits analyzed, although *Ascaris* complex II shows high fumarate reductase activity.

Discussion

Recent advances in biochemical techniques, including new purification methods and apparatus for peptide microsequencing, allows studies on the primary structure of proteins isolated from parasites, even though many parasites are very difficult to obtain in large quantity. Biogel P-60 column chromatography in the presence of SDS is useful for the separation of hydrophobic peptides of similar size. Another advantage of this method is that the amino terminal of the peptide remains free after the separation, making it possible to directly sequence *Ascaris* Ip from amino terminal. Previously, we determined the amino terminal sequence of the cyb subunits of *Ascaris* complex II using a sample eluted from a gel after electrophoresis (Kita *et al.*, 1988b, 1990a). However, this protocol was not useful for the analysis of the Ip subunit because of the blockage of the amino terminal during electrophoresis. The amino terminal of the Fp subunit from *Ascaris* complex II was also determined using a peptide separated on a Biogel P-60 column (Furushima *et al.*, 1990).

As with other mammalian Ip subunits, an alanine/threonine cluster was also found in the amino terminal region of the *Ascaris* Ip subunit. This stretch of uncharged amino acid residues has been suggested to function as an anchor domain of the peptide to the inner membrane of mitochondria (Clarkson *et al.*, 1991), but no such stretch is found in the amino terminal of Ip subunits from bacterial complex II (Darlison *et al.*, 1984; Cole, 1987; Philips *et al.*, 1987). Before the spacial arrangement of the enzyme complex in the membrane and the interactions between each subunit can be fully understood, many problems remain to be solved. As shown in Fig. 1, the *Ascaris* Ip subunit dose not cross-react with antibodies against mammalian Ip, and bovine Ip was not recognized by antibodies against *Ascaris* Ip, although their sequence similarities are high. The amino terminal segment is one candidate for the epitope recognized by the antibodies because the homology in this region is lower than that found in other regions (Table 1).

Two of the three cysteine-rich segments generally conserved in Ip subunits were identified by chemical modification of cysteine residues, thought to be the essential amino acid for the ligation of iron-sulfur clusters to the polypeptide. The highly labeled peptide eluted in peak 1 was identified as the binding site for cluster S-1 from

Table 1 Comparison of the partial amino acid sequence of the Ip subunit between *Ascaris* and other species

Species	Homology (%)			
	N-terminal region*	S-1†	S-1–S-2‡	Ref.
Human liver	65.9	76.7	78.6	16
Bovine heart	61.4	76.7	78.6	33
S. cerevisiae	59.6	86.7	75.0	19
E. coli (SDH)	41.2	53.3	64.3	6
E. coli (FRD)	29.7	43.3	32.1	5
P. vulgaris (FRD)	29.7	40.0	35.7	3

* The residues from 1 to 53 (Fig. 3).
† from 68 to 97 (Fig. 5A).
‡ from 103 to 133 (Fig. 5C).

its typical sequence Cys x x x x Cys x x Cys-11 residues-Cys with the uncharged polar small amino acids, glycine and serine, between the second and third cysteines. This sequence is similar to that found in [2Fe-2S] clusters of plant and cyanobacterial ferredoxins (Tsukihara *et al.*, 1982; Adman *et al.*, 1973). The peptide in the flow through fraction appears to contain the binding site for cluster S-3. An association between cluster S-3, containing an [3Fe-4S] cluster, and this domain has been suggested by analogy to the trinuclear iron-sulfur cluster in *Azotobacter vinelandii* Fd I (Ohnishi, 1987). It is of interest to note that the sequence found in the mixture of peptides (Fig. 5B) was identical to that of human liver mitochondrial Ip, which functions as a succinate-ubiquinone oxidoreductase, while a sequence similar to bacterial fumarate reductase was not found, although the Em' of the S-3 cluster in the *Ascaris* Ip subunit is low and comparable to that of bacterial fumarate reductase. Changes in amino acid residues of the Ip subunit or some other subunit in the complex II may account for the observed difference in the redox properties of the *Ascaris* Ip subunit. Based on the sequence analysis of the Ip subunit in this study together with our previous work on the Fp subunit (Furushima *et al.*, 1990), we conclude that complex II of adult *Ascaris* is more closely related to those of *E. coli sdh* and mammalian mitochondria than to that of *E. coli frd,* even though *Ascaris* complex II shows a much higher fumarate reductase activity than succinate dehydrogenase activity. These results suggest that *Ascaris* complex II evolved from complex II of aerobic mitochondria, which has succinate dehydrogenase activity, and fumarate reductase activity of *Ascaris* complex II was acquired during parasitic adaptation.

A peptide with a sequence corresponding to that of the binding site for cluster S-2 was not found in this study. This may be explained by the accessibility of the reagent, because the cysteine residues in this segment are located in relatively hydrophobic domain of the Ip polypeptide (Kita *et al.*, 1990b). It is difficult to determine the whole amino acid sequence of the *Ascaris* Ip subunit from its peptides because of the limited amount of material. However, sequence information on the subunit is quite useful for further study, even if it is partial. A set of primers was designed from the amino acid sequence determined in this study, and a product with the expected size was obtained from the cDNA-PCR of *Ascaris* mRNA. Sequence analysis is now in progress.

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