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Structural studies on three flavin-interacting regions of the flavoprotein subunit of complex II in *Ascaris suum* mitochondria

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The flavoprotein (Fp) subunit of mitochondrial complex II contains covalently bound FAD as a prosthetic group. In this study, the primary structure of the flavin-bound tryptic peptide from the Fp subunit of *Ascaris* complex II was determined and found to be highly similar to those of the corresponding flavin-binding regions of bovine heart and bacterial Fp subunits. Furthermore, the *Ascaris* Fp subunit was shown to contain two regions exhibiting striking sequence similarity to the segments that have been predicted to interact noncovalently with the AMP moiety of FAD in bacterial Fp subunits. The conservation of these two regions also in the mitochondrial Fp subunit suggests their functional importance.

Complex II; Flavoprotein; Flavin-binding domain; Fumarate reductase; Anaerobic respiration; (*Ascaris*)

1. INTRODUCTION

Complex II (succinate-ubiquinone oxidoreductase; EC 1.3.99.1), a membrane-bound flavoenzyme, catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle and is a component of the mitochondrial and bacterial respiratory chain (see [1] for review and [2] for the latest purification paper).

We have isolated complex II from muscle mitochondria of adult worms of *Ascaris* [3–6], inhabitants of the host's small intestine where the oxygen supply is limited. *Ascaris* complex II, unlike its mammalian counterparts, functions physiologically as the terminal oxidase in the NADH-fumarate reductase system, an anaerobic respiratory chain [3,5]. Indeed, *Ascaris* complex II exhibits high fumarate reductase (FRD) activity over succinate-ubiquinone oxidoreductase (SDH) activity (SDH/FRD = 0.05) [5]. It is a major component of *Ascaris* mitochondria (8% of mitochondrial protein) [7] and is composed of the following four subunits: a flavoprotein (Fp) subunit containing a flavin (M_r = 68 kDa), an Ip subunit associated with iron-sulfur centers (M_r = 26 kDa) and two hydrophobic, heme *b*-containing polypeptides called Cyb_L and Cyb_S (M_r = 15 and 13.5 kDa, respectively) [3,5]. In the case of bacteria, the genes of complex II have been cloned and sequenced from *Escherichia coli* (*sdh* and *frd*) [8–10], *Bacillus subtilis* (*sdh*) [11,12] and *Proteus vulgaris* (*frd*) [13]. Structural information on mitochondrial complex II is, however, still limited; only the primary structure of the flavin-binding region of the Fp subunit [14] and that of the Ip subunit [15] of bovine heart mitochondrial complex II have so far been reported.

We report here the primary structures of the domains of the Fp subunit of *Ascaris* complex II that are involved in flavin binding. A comparison of these sequences with those of the corresponding regions of bacterial complex IIs indicates that these domains are highly conserved between *Ascaris* and bacteria.

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2. MATERIALS AND METHODS

2.1. *Purification of the Fp subunit from* Ascaris *complex II*

Complex II was purified from *Ascaris* mitochondria as described by Takamiya et al. [3–5]. Purified complex II was treated with 20% (w/v) sodium dodecyl sulfate (SDS) and then subjected to gel chromatography on a Biogel P-60 (<400) column equilibrated with 2% (w/v) SDS [16].

2.2. *Protein chemical analysis of the purified Fp subunit*

The amino acid composition of the Fp subunit was determined in a Hitachi model 835 amino acid analyzer. For the preparation of tryptic peptides, the Fp subunit (550 μg) was digested with trypsin (5.5 μg) in 40 mM Na,K-phosphate buffer (pH 8.0) for 4 h at 37°C. After stopping the digestion by the addition of 0.3% trifluoroacetic acid (TFA), urea was added to the reaction mixture to a final concentration of 4 M to ensure complete dissociation. The tryptic peptides were then separated by high-performance liquid chromatography (HPLC) on an ODS-80TM column (4.6 × 150 mm, Toso) with a 20–80% gradient of acetonitrile in 0.1% TFA [17]. The elution of flavin-containing peptide was monitored by measuring emission of fluorescence at 550 nm (excitation at 360 nm). The N-terminal sequences of the Fp subunit and 20 out of the 40 separated tryptic peptides were determined by an Applied Biosystems model 470A protein sequenator. Sequence homology was analyzed using the database PRF-SEQ DB (Peptide Institute Protein Research Foundation,

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Osaka) which contains information on 12825 polypeptides including gene products of *E. coli sdh*, with a homology searching program PROSRCH.

3. RESULTS AND DISCUSSION

When *Ascaris* complex II was subjected to Biogel P-60 gel chromatography as described by Takamiya et al. [16], all four subunits, including the two smaller ones (Cyb_L and Cyb_S) having similar molecular sizes, were separated clearly from one another (Fig. 1). Upon gel electrophoresis in the presence of urea and SDS [16], the separated Fp subunit migrated as a single band (Fig. 1B) together with a fluorescent material characteristic of a flavin (emission at 520 nm when excited at 360 nm), indicating that the flavin in this subunit is covalently attached to the polypeptide, as is the case for bovine heart and bacterial Fp subunits [18,19]. The amino acid composition of the *Ascaris* Fp subunit was similar to that of the bovine heart Fp subunit [20] and those deduced from nucleotide sequences of Fp subunits of *E. coli sdhA* [8] and *frdA* [10] (Table I). This similarity is reflected by the finding that antibodies raised against the *Ascaris* Fp subunit cross-reacted with the subunit of bovine heart complex II and that of *E. coli* complex II (*sdhA*) (data not shown). The polarity index of Capaldi and Vanderkooi [21] was calculated to be 48.3% for the *Ascaris* Fp subunit, a value which is similar to those determined for Fp subunits from other sources. This polarity index suggests that the Fp subunit is not embedded in the membrane but is extruded to the aqueous phase, permitting the access of its soluble substrates (succinate and fumarate).

In the bovine heart Fp subunit, FAD is covalently bound to the protein by a linkage between the 8α-methyl group of the isoalloxazine ring and the *tele*-N atom of a histidyl residue [18]. The amino acid sequence around the histidyl residue is highly conserved between the bovine heart Fp subunit on the one hand and those of bacterial *sdhA* [8] and *frdA* [10,13] on the other. To see if the amino acid sequence of the flavin-binding site of the *Ascaris* Fp subunit is also conserved, this subunit was digested with trypsin and the flavin-containing peptide was isolated and sequenced. Although histidine could not be detected in this study, the primary structure thus determined was found to be very similar to those of bovine heart and bacterial Fp subunits (Fig. 2A). It was observed that the residues Glu-49, Ile-52 and Gly-57 in *E. coli sdhA* are conserved in *Ascaris*, but not in *frdA* of *E. coli* and *P. vulgaris*.

From a comparison of the primary structures of *E. coli frdA* and glutathione reductase, which also contains FAD as a prosthetic group, and of three-dimensional structural data for them [8,10,22], it has been predicted that the AMP moiety of FAD in bacterial Fp subunits interacts noncovalently with two

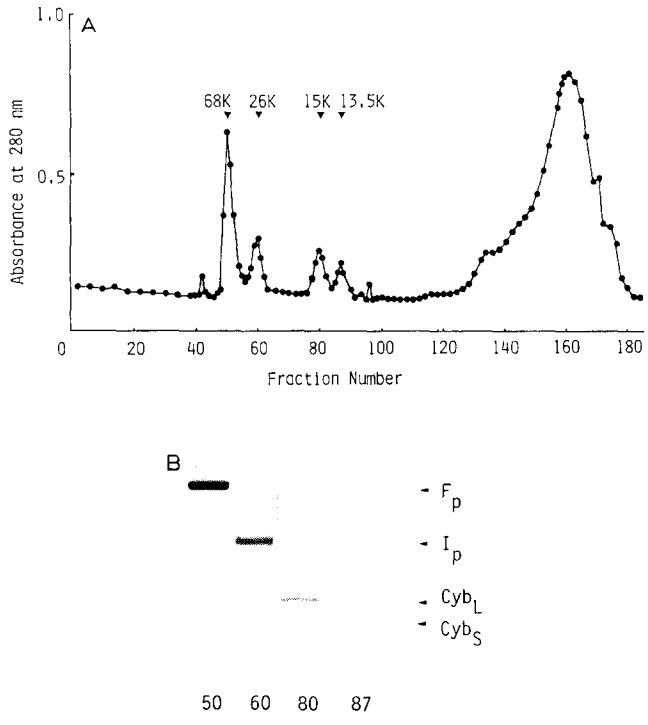

Fig. 1. (A) Elution profile of four subunits of *Ascaris* adult muscle complex II on Biogel P-60 (<400). Complex II (10 mg protein) was applied on a column (1.5 × 105 cm) equilibrated with 2% SDS and eluted at a flow rate of 1.0 ml/h. Absorbance at 280 nm (—●—) was monitored. 68K, 26K, 15K and 13.5K indicate the position of subunits having these molecular sizes. (B) Urea SDS-polyacrylamide gel electrophoresis [16] of the peak fractions.

regions of the Fp polypeptide. One such region is called the βA-αA-βB fold (Rossman nucleotide binding fold) [24], which is located near the NH_2-terminus (e.g. it starts from the 9th residue of *E. coli* SDH). This region consists of a sequence of five hydrophobic residues ending with a glycine residue (βA), followed by a stretch of residues having short side chains (αA) and another hydrophobic segment (βB). The amino acid sequence from the amino terminus of the *Ascaris* Fp polypeptide eluted from Biogel P-60 was directly determined and compared with those of bacteria. A typical sequence of βA (Val-Val-Ile-Ile-Gly) was found in *Ascaris* Fp (Fig. 2B). Moreover, the first residue of the αA, Gly-15, was found to be conserved among the Fp subunits so far sequenced (Fig. 2B).

Another segment interacting with the AMP moiety is the stretch from residues 357 to 386 in *E. coli sdhA* [8] and from residues 355 to 374 in *B. subtilis sdhA* [11]. To see if this segment is also detectable in the *Ascaris* Fp subunit, 20 out of the 40 tryptic peptides of the Fp subunit were partially sequenced, since the Lys residues in the amino terminal part of the segment in bacterial Fp (Lys-343 in *E. coli sdhA* and Lys-341 in *B. subtilis sdhA*) are well-conserved and trypsin specifically digests the C-terminal of Lys and Arg residues in the

Table I

Comparison of the amino acid composition of F_p subunits from various sources

Amino acid	No. of residues			
	Ascaris	Beef heart[a]	*E. coli* SDH[b]	*E. coli* FRD[c]
Lysine	34.5	26	22	27
Histidine	18.2	17	19	21
Arginine	32.8	41	40	38
Aspartic acid	54	57	32	35
Asparagine			25	23
Threonine	39.3	38	33	36
Serine	29.2	37	29	20
Glutamic acid	52.6	67	43	44
Glutamine			21	22
Proline	25.6	30	24	22
Glycine	60.9	65	59	66
Alanine	48.4	59	56	61
Valine	28.9	45	41	41
Methionine	6.1	14	15	20
Isoleucine	30.3	31	26	23
Leucine	44.2	55	51	48
Tyrosine	13.9	21	14	17
Phenylalanine	19.8	22	19	20
Polarity (%)[d]	48.3	44	45	44
Molecular weight	68000	70000	64268	65835

[a] From the results reported in [20]
[b] From the results reported in [8]
[c] From the results reported in [10]
[d] Calculated according to [21]

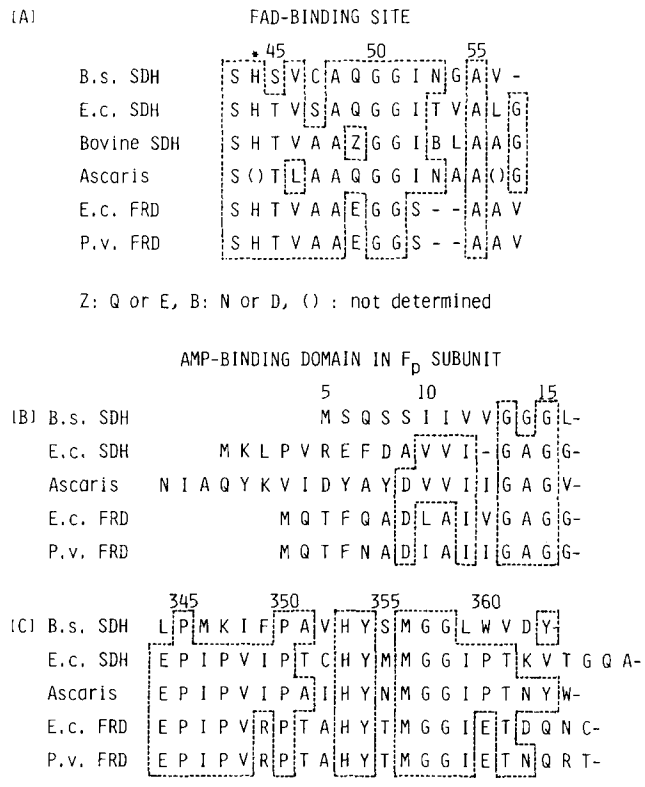

Fig. 2. The amino acid sequences of the flavin-interacting region of Fp subunits. Partial sequences from various species are aligned. B.s. SDH, *B. subtilis* SDH [11]; E.c. SDH, *E. coli* SDH [8]; Bovine SDH, bovine heart SDH [14]; *Ascaris*, this work; E.c. FRD, *E. coli* FRD [10] and P.v. FRD, *P. vulgaris* FRD [13]. The numbering is according to the *E. coli* SDH sequence [8]. The initiating methionine residue is not included. Identical residues are boxed in all cases. (A) Flavin-binding peptides of various species. The asterisk denotes the site of attachment of the flavin. (B) Amino acid sequences of the amino terminal portion of various Fp subunits which contain the Rossman nucleotide binding fold. (C) Alignment of residues of the AMP-interacting domain.

peptide. It was thus possible to find a peptide showing high sequence similarity to the aforementioned segments of bacterial Fp subunits (Fig. 2C). Although the location of the segment in the *Ascaris* Fp subunit is unknown at present, the high sequence similarity assures that the *Ascaris* segment is also involved in the interaction with the AMP moiety. The existence of two putative AMP-binding regions in the *Ascaris* Fp subunit suggests further that the flavin in this subunit is also FAD. In any case, the occurrence of the two segments that interact with the AMP moiety of FAD has not yet been reported for the Fp subunit of mitochondrial complex II. The conservation of these segments between mitochondrial and bacterial Fp subunits suggests that these regions are functionally important.

As can be seen in Fig. 2, the amino acid sequences of the flavin-binding peptide and the two AMP-interacting regions of the *Ascaris* Fp subunit are more similar (though only slightly) to those of *E. coli sdhA* than to those of *frdA* of the same organism. We have recently cloned and sequenced a cDNA for the Ip subunit of human complex II [24]. Its deduced amino acid sequence is highly similar to those of *E. coli sdhB* (50.8%) [8] and bovine heart Ip subunit (94.1%) [15], whereas the primary structure of the human Ip subunit is only 28% similar to that of *E. coli frdB* [10]. Complex II having succinate dehydrogenase activity in aerobic organisms has been suggested to have evolved from soluble fumarate reductase in anaerobic bacteria [25]. From the standpoint of the evolution of mitochondrial fumarate reductase, it is of interest that the Fp subunit of complex II of *Ascaris* mitochondria is more related to that of *E. coli sdh* than to that of *frd* of the same organism, even though *Ascaris* complex II shows a much higher fumarate reductase activity than succinate dehydrogenase activity.

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