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Short sequence-paper

Cloning of a cDNA encoding the small subunit of cytochrome b_{558} (cybS) of mitochondrial fumarate reductase (complex II) from adult *Ascaris suum* [1]

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Abstract

Complex II in the mitochondria of the adult parasitic nematode, *Ascaris suum*, exhibits high fumarate reductase activity in addition to succinate dehydrogenase activity and plays a key role in the anaerobic energy metabolism of the worm. In this study, the amino acid sequence of the small subunit of cytochrome b_{558} (cybS) in adult complex II was deduced from the cDNA isolated by immunoscreening an *A. suum* muscle cDNA library. Histidine residues, which are possible heme axial ligands in cytochrome b_{558}, were found in the second transmembrane segment of the subunit. This is the first report of the primary structure of the small subunit in the two-subunit cytochrome *b* in mitochondrial complex II from a multicellular eukaryote.

Keywords: Complex II; Fumarate reductase; Cytochrome *b*; cDNA; (*Ascaris suum*)

Complex II (succinate-ubiquinone oxidoreductase) catalyzes the oxidation of succinate to fumarate (succinate dehydrogenase: SDH) and transfers its reducing equivalents to ubiquinone [1,2]. Complex II also catalyzes the reduction of fumarate (fumarate reductase: FRD), which is the reverse of the reaction catalyzed by SDH, in the respiratory chain of anaerobic bacteria and in mitochondria of facultative anaerobic animals such as adult *Ascaris suum* [3,4]. Complex II is generally composed of four polypeptides, with the largest flavoprotein (Fp) subunit of 70 kDa containing covalently bound FAD. The second-largest subunit, the iron-sulfur protein (Ip) subunit, has an apparent molecular mass of about 30 kDa and contains three different types of iron-sulfur centers. The Fp and Ip subunits comprise the catalytic portion of complex II and catalyze electron transfer from succinate to artificial electron donors such as phenazine methosulfate (PMS), or from reduced methylviologen to fumarate. The amino acid sequences of Fp and Ip are highly conserved among species, and the structural and functional relationships in the binding of prosthetic groups and substrate recognition have been well-characterized [1–4]. The presence of a two-subunit cytochrome *b* composed of large (cybL) and small (cybS) subunits acting as hydrophobic membrane-anchor peptides, is a general feature of complex II in mitochondria and bacteria [1,2]. Exceptions are the enzymes from *Bacillus subtilis* SDH [5] and *Wolinella succinogenes* FRD [6], which contain two cytochrome *b* components bound to a single large hydrophobic subunit, and *Saccharomyces cerevisiae* SDH [7] and *Escherichia coli* FRD [8], which do not contain heme *b*. The cytochrome *b* in complex II seems to be essential for the interaction between the complex and quinone species. Evidence for the quinone binding site on these hydrophobic peptides has been reported for *E. coli* FRD [9] and bovine heart SDH [10,11].

Recently, we found a mammalian-type isoform of complex II showing only SDH activity in free-living second

Abbreviations: SDH, succinate dehydrogenase; FRD, fumarate reductase; cybL, large subunit of cytochrome *b* in complex II; cybS, small subunit of cytochrome *b* in complex II.

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[1] The nucleotide sequence data reported in this paper are available in the DDBJ, EMBL, and NCBI databases under the accession number D78158.

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stage larvae, in contrast to adult complex II which catalyzes FRD activity and functions as a terminal oxidase in the anaerobic NADH-fumarate reductase system [12]. Oxygen is required for larval development, and their respiratory chain is similar to that of the aerobic mammalian host. The major quinone in larvae is ubiquinone (+110 mV) which accepts reducing equivalents from larval complex II (SDH), while the major quinone in adults is low potential rhodoquinone (−63 mV), which is the electron donor for adult complex II (FRD) [13]. In addition, we have found that larval cybS is not recognized by antibody raised against adult cybS [12]. Clearly, cytochrome *b* is a key component in *A. suum* complex II and much more information is required to understand its structure and function. The cytochrome b_{558} of adult *A. suum* complex II was separated from the Fp and Ip subunits by gel filtration column chromatography in the presence of Sarkosyl [14]. The isolated cytochrome b_{558} is the typical two-subunit cytochrome *b* of complex II comprising cybL (17.2 kDa) and cybS (12.5 kDa) [14]. However, only partial amino-terminal sequences have been determined from purified peptides of both subunits [14,15]. The complete amino acid sequence of mitochondrial cybL has been deduced from the bovine cDNA sequence [16,17] and from the DNA sequence of the *SDH3* (CYB3) gene of *S. cerevisiae* [18,19]. However, no information about the primary structure of mitochondrial cybS has been available except for the sequence of the *SDH4* gene product of *S. cerevisiae* complex II [20], which does not contain heme *b*. In this study, the amino acid sequence of cybS of adult *A. suum* complex II (FRD) was deduced from the sequence of the cDNA isolated by immunoscreening a muscle cDNA library, and compared with the sequences of the *S. cerevisiae SDH4* product and its bacterial counterparts, the *E. coli sdhD* and *frdD* gene products [21,22].

In cloning the cDNA for adult *A. suum* cybS, we were unable to apply the homology probing strategy used successfully to clone adult *A. suum* Fp [23], because the amino acid sequences of the cytochrome *b* component in complex II from different species show little conservation, and only the deduced amino acid sequence of *S. cerevisiae SDH4* [20] was available for mitochondrial complex II. Therefore, we prepared a monoclonal antibody raised against adult cybS using purified complex II, and used it to screen an adult muscle cDNA library. From 5×10^4 recombinants, 4 positive clones were obtained, and the largest cDNA insert of about 700 bp was sequenced. This cDNA contained an open reading frame of 468 nucleotides encoding 156 amino acids, including residues 26-55 which were identical to the first 30 residues of the amino terminal of the *A. suum* cybS peptide [15] (see Fig. 1). The open reading frame ends with a TAG termination codon fol-

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                                                                  GGTTTAATTACCCAAGTTTGAG      -1

1     M  L  S  A  V  R  R  A  I  P  L  S  A  R  I  L  R  T  S  L  I  Q  R  C  A          25
1     ATG CTG TCG GCG GTA AGA CGT GCA ATC CCT TTG AGT GCC CGC ATC CTG AGG ACA TCG TTG ATT CAA CGT TGT GCC    75

26    G  A  T  S  A  A  V  T  G  A  A  P  P  Q  F  D  P  I  A  A  E  K  G  F  K          50
76    GGA GCA ACG TCG GCA GCA GTT ACC GGA GCG GCT CCT CCT CAA TTC GAT CCT ATC GCT GCG GAG AAA GGA TTT AAG   150

51    P  L  H  S  H  G  T  L  F  K  I  E  R  Y  F  A  A  A  M  V  P  L  I  P  A          75
151   CCT CTA CAT AGC CAT GGA ACT CTC TTC AAA ATA GAG CGC TAC TTC GCC GCT GCG ATG GTA CCA CTC ATT CCA GCT   225

76    A  Y  F  I  H  G  R  E  M  D  L  C  L  A  L  A  L  T  L  H  V  H  W  G  V          100
226   GCC TAT TTC ATC CAT GGT CGT GAA ATG GAT CTG TGC CTA GCG CTC GCA CTT ACT CTG CAC GTC CAC TGG GGT GTT   300

101   W  G  V  V  N  D  Y  G  R  P  F  V  L  G  D  T  L  A  A  A  V  R  V  G  A          125
301   TGG GGT GTC GTT AAC GAT TAT GGT CGT CCG TTT GTG TTG GGA GAT ACC TTG GCG GCG GCA GTG CGC GTT GGT GCG   375

126   Y  I  F  T  A  C  L  L  A  G  L  L  Y  F  N  E  H  D  V  G  L  T  R  A  F          150
376   TAC ATC TTT ACA GCG TGT CTC CTC GCC GGT CTA TTG TAT TTC AAC GAG CAT GAT GTG GGG CTG ACG CGT GCA TTC   450

151   E  M  V  W  E  L
451   GAA ATG GTG TGG GAG CTT TAG
472   GCTTTCGGAGTCGTCTCGTCTCGTATTGAAATTGAAGCCCTTCTAGTGTCCACTTTTGCTGTCATGCGCTCTTTTTATTGTCTTACCATAAGGACTCTC   570
571   GATTACTTCTTGTCACTTTTATCCCAGCGTGTGTTGTGTGAATTGGTTCGTTGACTCAGCGATTGTTGTTGTAATGGGTTTTGCCTCCGATAAACATGC   669
670   CTCGTTTAAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide sequence of the cDNA for the cybS subunit from adult *A. suum* and its deduced amino acid sequence. The amino-termial sequence of the mature cybS protein [15] is underlined. Screening of an *A. suum* cDNA library by anti-*A. suum* monoclonal antibody was carried out as described previously [37], and the DNA sequences were determined with an automated DNA sequencer, DSQ-1 (Shimadzu).

lowed by a 205 nucleotide 3′-untranslated region. A nucleotide sequence, ATAAA, similar to the consensus AATAAA polyadenylation signal [24], was found 17 nucleotides upstream of the poly(A) tail. The five nucleotides at the 5′-end of this cDNA clone, TTGAG, are identical to the 5 nucleotides at the 3′-end of the conserved nematode spliced leader sequence [25]. The presence of the spliced leader sequence in the mRNA of adult *A. suum* cybS was confirmed by cDNA-PCR using a primer designed from the sequence as described previously [23]. A putative initiation codon, ATG, located just downstream of the spliced leader sequence and the first 25 amino acid residues appear to be a mitochondrial presequence. This sequence is rich in the basic amino acid arginine, and contains the hydroxylated amino acid serine. This is characteristic of the cleavable amino acid terminal presequences that are essential for the import of mitochondrial proteins encoded by nuclear DNA [26]. Thus, the mature cybS contains 131 amino acids with a calculated molecular mass of 14217 Da. This value is similar to that estimated from a gradient gel electrophoresis in the presence of SDS (~ 12500 Da) [14]. The polarity index calculated according to Capaldi and Vanderkooi [27] was 28.3% for *A. suum* cybS. This low polarity is consistent with its hydrophobic characteristic as a membrane-anchor for the complex.

A comparison of the amino acid sequence of the cybS peptide from adult *A. suum* with those of the *S. cerevisiae* peptide and the gene products of *E. coli sdhD* and *frdD* is presented in Fig. 2. In contrast with the highly conserved features of the Fp and Ip subunits, the amino acid sequence of *A. suum* cybS shows little similarity to the sequences of the other species. The homology of *A. suum* cybS with the *S. cerevisiae SDH4* gene product was 18%. The similarity between *A. suum* cybS and the cybS of *E. coli* FRD was much lower (17% with the *frdD* product) than that between *A. suum* cybS and the cybS of *E. coli* SDH (24% with the *sdhD* product), even though *A. suum* complex II exhibits high FRD activity. It is noteworthy that the similarity between *A. suum* cybS and the *E. coli sdhD* product in the region from Asp-60 to Tyr-82 of *A. suum* cybS (48%) is higher than that of the entire peptide. The conservation between mitochondrial and bacterial cybS in this segment suggests that these regions are functionally important. In fact, histidine residues, which are candidates for the heme *b* ligand, as discussed later, are found in this region and a critical role for His-81 in the cybS of *E. coli* FRD in quinone binding has been reported [9].

Cytochrome b_{558} of adult *A. suum* complex II is reducible by succinate [28], and has been shown to have an E'_m of −34 mV [29], which is much higher than the E'_m of the cytochrome b_{560} in bovine heart complex II (−185 mV) [30]. The rather positive E'_m of cytochrome b_{558} may facilitate electron transfer from rhodoquinone (−63 mV) to the succinate/fumarate couple (+30 mV), although the

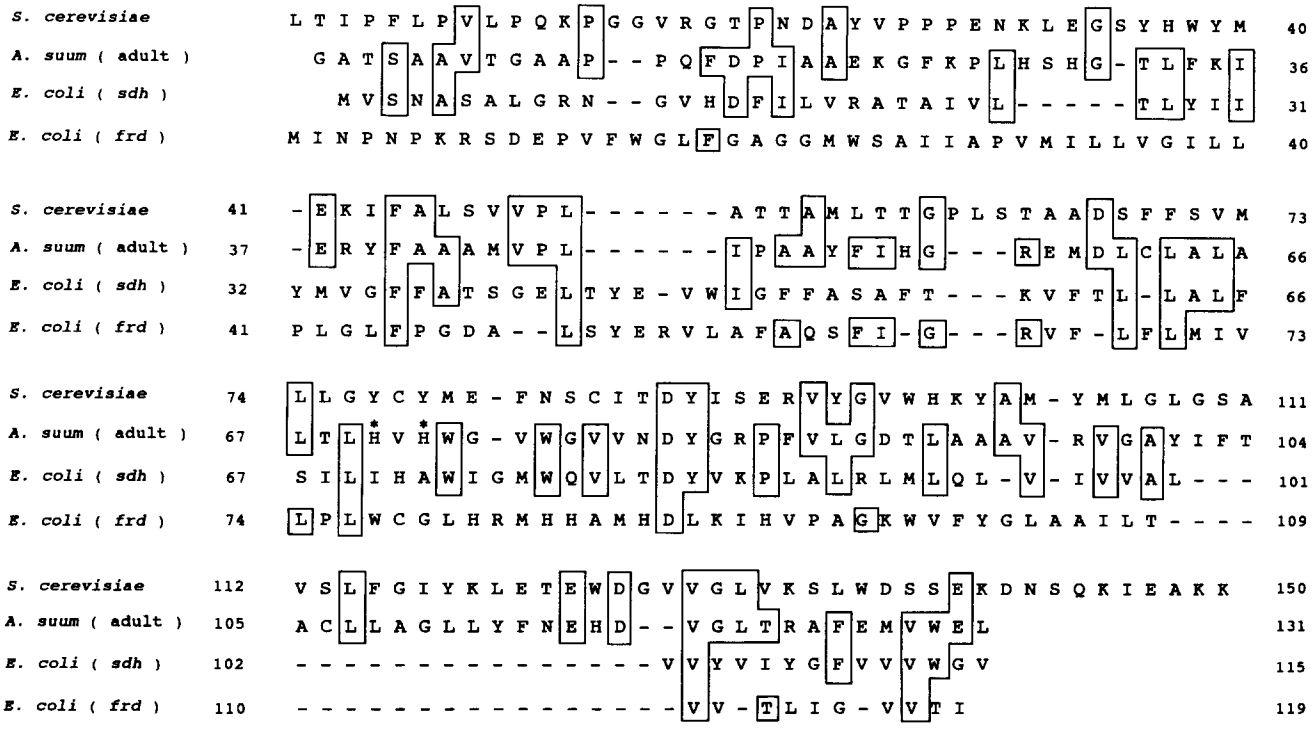

Fig. 2. Comparison of the deduced amino acid sequences of cybS subunits from various species. *S. cerevisiae* [20]; *A. suum* (adult complex II) [this study]; *E. coli*. SDH [21]; and *E. coli*. FRD [22] are presented. Similarity in the sequences of the various species was maximized with the computer program GENETYX. Gaps introduced to maximize similarity are shown in dashes. Amino acids identical to those in *A. suum* cybS are boxed. The histidine residues in the region homologous to cybS of *E. coli* SDH are indicated by asterisks.

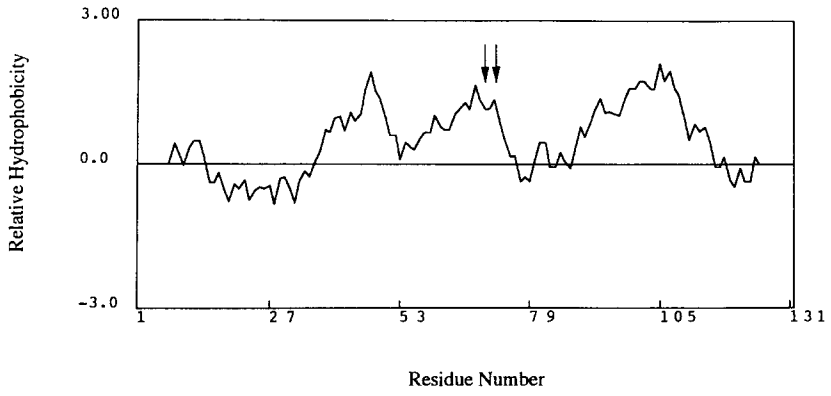

Fig. 3. Hydrophobicity analysis of cybS of adult *A. suum* complex II. The cybS sequence was analyzed with the algorithm of Kyte and Doolittle [36]. A 15-residue sliding window was used. The three major hydrophobic regions (residues 36-52, 53-76, 88-115) are potential transmembrane segment. The histidine residues (His-70 and His-72) in the region homologous to cybS of *E. coli* SDH are indicated by arrows.

participation of heme *b* in electron transfer in the complex has not yet been proved. An EPR spectrum of the air-oxidized form of cytochrome b_{558} in complex II showed a ferric low-spin signal at $g = 3.6$ [14], which is similar to that of cytochrome b_{560} in bovine heart complex II [30]. Recent analyses by EPR and near-infrared magnetic circular dichroism (MCD) suggest a bis-histidine ligation with the heme *b* in cytochrome b_{558} in *B. subtilis* complex II [31,32], in cytochrome b_{556} of *E. coli* SDH [33], and in cytochrome b_{560} of bovine complex II [34]. In addition, we have shown the indispensability of both cybL and cybS subunits for heme *b* ligation to form the two-subunit cytochrome *b* in *E. coli* SDH [35], and, based on experiments using site-directed mutants, His-84 in cybL and His-71 in cybS appear to be possible axial ligands in cytochrome b_{556} (C. Vibat et al., manuscript in preparation). Interestingly, two histidine residues, His-70 and His-72 (indicated by asterisks in Fig. 2), are found in *A. suum* cybS in the same conserved region that contains His-71 in cybS of *E. coli* SDH. From hydrophobicity analysis [36], *A. suum* cybS appears to have three transmembrane segments, and His-70 and His-72 are both found in the second transmembrane segment as shown in Fig. 3. The location of the heme ligand histidine in the transmembrane segment has been demonstrated in cytochrome b_{558} of *B. subtilis* complex II [31,32]. The results of the present study together with these observations indicate that His-70 or His-72 in *A. suum* cybS is the ligand for heme *b* in the cytochrome b_{558} of adult complex II. The absence of a histidine residue in this region in the *SDH4* gene product of *S. cerevisiae* complex II, which does not contain heme *b* [7], is consistent with this idea. At present, it is difficult to predict which histidine residue, His-70 or His-72, is the heme *b* ligand, because this is the first report of the primary structure of cybS in a mitochondrial complex II with a two-subunit cytochrome *b*. However, it is likely that heme *b* bridges two histidine residues in the cybL and cybS hetero-dimer of the two-subunit cytochrome *b* in mitochondrial complex II. Sequence analysis of *A. suum* cybL and its expression together with cybS in *E. coli* are now in progress.

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