Volume 286, number 1,2, 121–124 FEBS 09928 July 1991

ADONIS 001457939100639C

Presence of an acyl carrier protein in NADH:ubiquinone oxidoreductase from bovine heart mitochondria

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Received 16 May 1991

The amino-acid sequence of a subunit of NADH:ubiquinone oxidoreductase from bovine heart mitochondria has been determined and is closely related to those of acyl carrier proteins that are involved in fatty acid biosynthesis in *Escherichia coli* and plants. Evidence for the presence of covalently attached pantetheine-4'-phosphate in the bovine protein has been obtained by determination of the molecular mass of the isolated subunit by electrospray mass spectrometry, before and after incubation of the protein at alkaline pH under reducing conditions. This decreased the molecular mass from 10751.6 to 10449.4, a difference of 302.2 mass units; the value calculated from the protein sequence with one covalently attached pantetheine-4'-phosphate is 10449.8. The acyl group which is removed by alkaline reduction, appears to be attached via a thioester linkage. By analogy with the bacterial protein it is likely that the attachment site of the pantetheine-4-phosphate is serine-44, which is found in a highly conserved region of the sequence. At present the function of the acyl carrier protein in mitochondrial complex I is not understood.

Complex I (bovine heart mitochondria); Acyl carrier protein

1. INTRODUCTION

One of the most striking features of the respiratory enzyme mitochondrial NADH : ubiquinone oxidoreductase (also known as complex I) is the large number of different subunits found in the assembly. The bovine heart enzyme now appears to contain at least 35 unique proteins, 7 of mitochondrial origin and the remainder the products of nuclear genes that are imported into the organelle after cytoplasmic translation. The sequences of many of the nuclear coded subunits have been determined recently [1–9] and have provided important clues to their structures and functions. For example, cysteine-rich sequence motifs in the 51, 75 and 23 kDa subunits suggest that they each form ligands with 4Fe-4S centers that probably participate in the transfer of electrons from NADH to ubiquinone [3,4,6], and a glycine-rich region of the 51 kDa subunit is likely to form part of the binding site for NADH [4]. The functions ascribed to some of these subunits fit in with earlier chemical [10,11] and EPR [12] evidence of transfer of electrons from an NADH binding site in the 51 kDa subunit via FMN and a series of spectroscopically characterized Fe-S centers to ubiquinone. In contrast, the subunit described below suggests that a hitherto unsuspected biochemical function is present in complex I. Its sequence is related to acyl carrier proteins that participate in fatty acid biosynthesis in bacteria and plants. Mass spectrometric measurements provide evidence that pantetheine-4'-phosphate is bound to this protein, probably via a conserved serine residue, as it is also in known acyl carrier proteins. However, at present there is no clearly defined function for an acyl carrier protein in complex I.

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2. MATERIALS AND METHODS

2.1. *Isolation and protein sequencing of the bovine acyl carrier protein*

Complex I was purified from bovine heart mitochondria [13]. Its subunits were separated by gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane and sequenced at their N-terminals. The SDAP subunit (which proved to be an acyl carrier protein) was also isolated from a water soluble fraction of complex I obtained by dissociation of the enzyme in 6 M guanidine hydrochloride, followed by dialysis against 0.4% ammonium bicarbonate solution. A precipitate was removed by centrifugation, and the supernatant (the water soluble fraction) was fractionated by HPLC on an Aquapore C_8 column (300 Å pore size, 7 μm particles; 100 mm × 2.1 mm i.d. Applied Biosystems, Warrington, UK) in 0.1% trifluoroacetic acid with an acetonitrile gradient. The SDAP subunit eluted at 52% acetonitrile. The purified protein was analyzed in an Applied Biosystems gas phase sequencer.

2.2. *Electrospray mass spectrometry*

Spectra were measured with a VG BIO-Q triple quadrupole instrument with electrospray ionization (VG Biotech, Altrincham, Cheshire, UK). Samples of the purified SDAP subunit (ca. 100 pmol dissolved in 10 μl of a solution of 1% acetic acid in 50% aqueous methanol) were delivered from an Applied Biosystems 120 liquid chromatograph at 4 μl/min and sprayed in the same solvent. Two aliquots of the purified SDAP subunit were also dissolved in a buffer containing 50 mM Tris-HCl, pH 9.0, and incubated in the absence and the presence of 20 mM dithiothreitol at 37°C for 30 min. In both cases, the whole sample was then chromatographed on the Aquapore

C_8 column in 0.1% trifluoroacetic acid and acetonitrile as described above. The protein that had been incubated in the presence of dithiothreitol eluted at 48% acetonitrile, whereas the one that had been incubated in its absence eluted at the same position as the untreated protein, namely at 52% solvent concentration. A similar change in the elution position of the protein was induced by incubation of the protein with 1 M hydroxylamine, pH 7.0, at room temperature for 90 min. In contrast, incubation of the protein in 20 mM dithiothreitol, 20 mM Tris, pH 7.0, under the same conditions left the protein unchanged. Both samples recovered from the first experiment were examined by electrospray mass spectrometry, in an instrument calibrated with horse heart myoglobin. The mass spectra were obtained from scans of mass to charge ratios between 600 and 1600 over 10 s intervals. The standard deviations of the values determined for the untreated and treated samples of the SDAP subunit were 0.2 and 0.5, respectively.

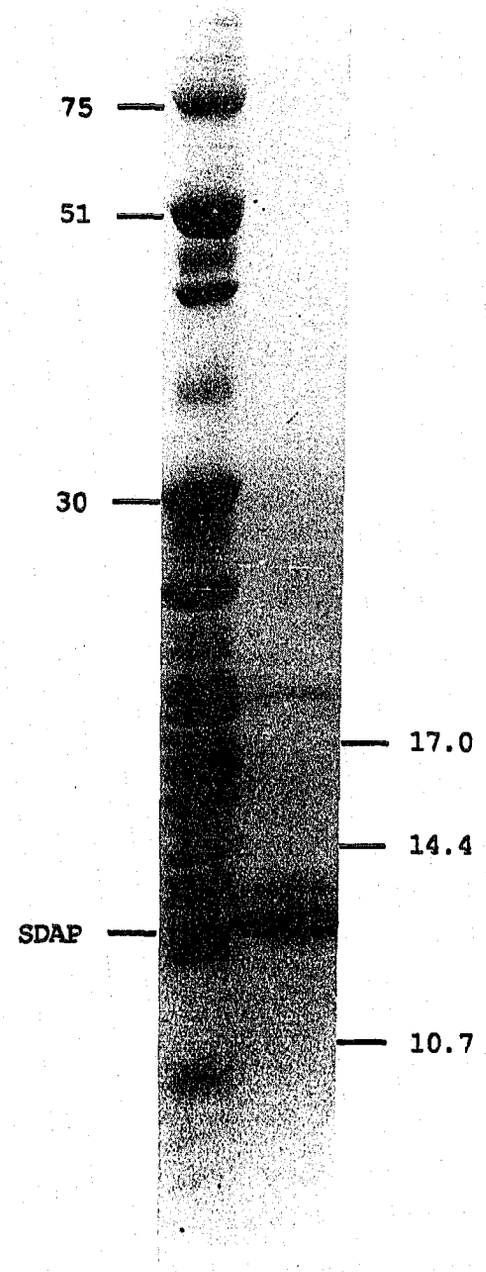

Fig. 1. Polyacrylamide gel electrophoresis in the presence of SDS of complex I from bovine heart mitochondria and of isolated SDAP subunit (acyl carrier protein). The system described by Schägger and Von Jagow [26] was employed. The positions of the 75, 51, 30 and SDAP subunits of the enzyme complex and molecular weight markers are indicated on the left and right hand sides, respectively.

2.3. *Isolation and sequencing of cDNA clones for the SDAP subunit*

cDNAs for the SDAP subunit were amplified from total bovine heart cDNA by the polymerase chain reaction (PCR) using mixtures of synthetic oligonucleotides based upon the known protein sequence as primers and hybridization probes. Details of similar experiments conducted on other subunits of complex I and of DNA sequence analysis have been described elsewhere [4,6–9]. See also the legend to Fig. 2 for additional details. The DNA sequence was determined completely in both senses of the DNA.

3. RESULTS AND DISCUSSION

3.1. *Sequence of the SDAP subunit*

Various subunits of complex I that had been fractionated by gel electrophoresis (see Fig. 1) and had been transferred subsequently to a PVDF membrane, were subjected to Edman degradation. In this way the sequence of amino acids 1–22 of the SDAP subunit were determined (see Fig. 2), and the same sequence was obtained on samples of chromatographically purified protein. Segments of this partial sequence were then used as the basis of 3 synthetic oligonucleotide mixtures (see Fig. 2). Two of them (based on amino acids 1–6 and 13–18, respectively, and referred to as primers 1F and 1R in Fig. 2) were employed as forward and reverse primers in a polymerase chain reaction with total bovine heart cDNA as template, to produce a partial cDNA encoding amino acids 1–18. This partial cDNA was recognized by hybridization with the third synthetic oligonucleotide (based on amino acids 7–12). The partial cDNA clone was then extended to the 3′ poly A tail and in the 5′ direction in two further polymerase chain reactions. In both reactions oligo dT was used as one of the primers; in the 3′ extension it hybridised with the 3′ poly A tail derived from the mRNA, and in the 5′ extension poly A had been added to the 5′ end of the cDNA population with terminal transferase. In the 3′ extension the second primer was based on the central part of the short partial cDNA, and in the 5′ extension the two unique nested primers 3RA and 3RB were employed.

3.2. *Relationship of subunit SDAP to acyl carrier proteins*

The cDNA sequence presented in Fig. 2 encodes the mature SDAP subunit. The protein sequence is 88 amino acids long and is closely related to that of the *E. coli* acyl carrier protein and plant acyl carrier proteins (exemplified by the protein from *Brassica campestris*) which function in fatty acid biosynthesis (see Fig. 3). The most highly conserved region is around amino acids 39–58 and includes the conserved serine residue 44, the site to which pantetheine-4′-phosphate is known

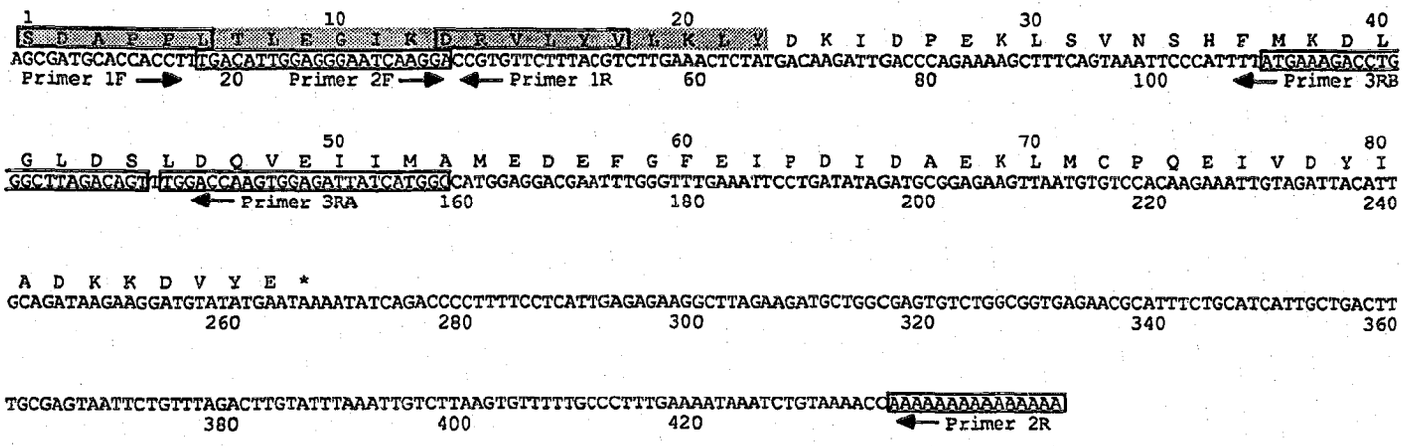

Fig. 2. Analysis by protein and DNA sequencing of the SDAP subunit of complex I from bovine heart mitochondria. The shaded region of the sequence of the protein (residues 1–22) was determined by N-terminal analysis. Overlapping cDNAs were isolated from total bovine heart cDNA by means of a strategy based on the polymerase chain reaction. The positions of protein sequences used in the design of mixed oligonucleotide primers and the positions of unique oligonucleotide primers employed in these experiments are boxed. The arrows indicate the senses of the various primers. For further details see the text. DNA sequence was determined in a 5′ direction beyond nucleotide 1, but this sequence is not presented here.

to be bound by a phosphodiester linkage in the *E. coli* protein [14]. The sequence of the bovine protein is also related to the N-terminal sequence determined on a protein isolated from *N. crassa* mitochondria which was labelled with [^{14}C]pantothenic acid [15,16]. It has been shown recently that this protein forms part of complex I in the fungus (H. Weiss, personal communication).

3.3. *Presence of pantetheine-4′-phosphate in bovine subunit SDAP*

In order to obtain evidence that the SDAP subunit of bovine complex I functions as an acyl carrier protein, it was isolated from purified complex I and its molecular mass was determined by electrospray mass spectrometry. The value measured by this means, 10751.6, was 642.1 mass units greater than the value calculated from the sequence. On incubation of the protein at pH 9.0 in the presence of dithiothreitol, the molecular mass of the protein was reduced by 302.2 mass units to 10449.4. This is close to the calculated value of 10449.8

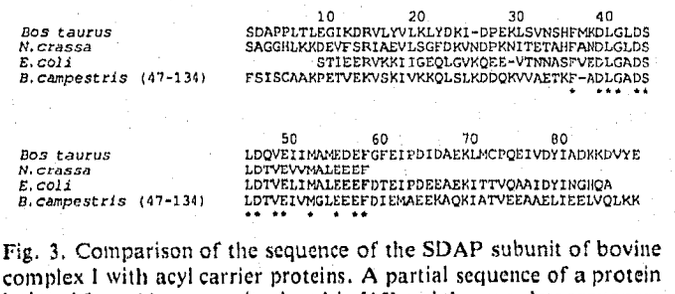

Fig. 3. Comparison of the sequence of the SDAP subunit of bovine complex I with acyl carrier proteins. A partial sequence of a protein isolated from *N. crassa* mitochondria [16] and the complete sequences of the *E. coli* [14] and *B. campestris* [27] acyl carrier proteins are aligned. Amino acids that are identical in all four sequences are denoted by asterisks. In the *E. coli* protein, pantetheine-4′-phosphate is covalently attached to serine-44 (bovine numbering) by a phosphodiester linkage.

for the SDAP protein covalently modified with one pantetheine-4′-phosphate per protein molecule. By analogy with the *E. coli* acyl carrier protein it is likely that this pantetheine-4′-phosphate moiety is attached to the conserved serine-44, although further experimentation is needed to establish this point. The precise nature of the acyl group also is unknown at present. However, its molecular mass shows that it cannot be 3-hydroxytetradecanoate (molecular mass 243), the acyl group which is proposed to be attached to the acyl carrier protein isolated from *N. crassa* mitochondria [16]. The acyl group can be removed by incubation with hydroxylamine or dithiothreitol at pH 9.0, but not with dithiothreitol at pH 7.0, and therefore appears to be attached via a thioester linkage.

3.4. *Implications for the function of complex I*

The evidence that the acyl carrier protein is a *bona fide* subunit of bovine mitochondrial complex I is provided by its co-purification with the enzyme complex. It has been detected by sequence analysis in several different preparations of the enzyme made by the procedure of Hatefi [13], in the same preparations further purified by chromatography to remove low levels of contaminants such as transhydrogenase and cytochrome oxidase, in preparations of the enzyme made by an independent procedure based upon ion-exchange chromatography in detergents (M. Finel, unpublished work) and in defined fragments of complex I produced by disruption of the intact complex with mild detergents (M. Finel and J.E. Walker, unpublished work). Moreover, the SDAP subunit (acyl carrier protein) is found in approximately stoichiometric amounts with other subunits (for example, the 51 kDa, 30 kDa and 24 kDa subunits) that are accepted as intrinsic components of the enzyme. The presence of substantial

amounts of the SDAP subunit is also evident in polyacrylamide gel analyses of complex I, and its position has been verified by N-terminal sequence analysis after transfer of the subunits to PVDF membranes. The acylated form of the protein, which is the form present in complex I, has an apparent molecular weight of about 8 kDa estimated by gel electrophoresis in the presence of SDS. On deacylation this apparent molecular weight increases to 13 kDa, but the deacyl subunit has not been detected in intact complex I. A similar apparent increase in molecular weight on deacylation has been noted with the acyl carrier protein isolated from *N. crassa* mitochondria [16].

The precise location of the acyl carrier protein in complex I is unknown at present. Its sequence contains no hydrophobic segments that could be folded into membrane-spanning α-helices, and it is more likely to be an extrinsic rather than an intrinsic membrane protein. It is not found in either the flavoprotein (FP) or the iron-sulphur protein (IP) fractions (J.E. Walker, J.M. Skehel and I.M. Fearnley, unpublished work), and it can be assumed to be a component of the residual (HP) fraction.

The major remaining unsolved question is: what is the function of the acyl carrier protein in complex I? Acyl carrier proteins are associated with fatty acid and polyketide biosynthesis [17], in the biosynthesis of the bacterial peptide antibiotic, gramicidin [18], in the citrate lyase complex in *Klebsiella* [19] and *Rhodopseudomonas* [20] and in nodulation in *Rhizobium meliloti* [21]. The acyl carrier proteins involved in fatty acid biosynthesis in chloroplasts and bacteria are discreet and monofunctional and act as acyl group carriers. The *E. coli* protein also acts as an acyl donor in membrane phospholipid biogenesis [22]. In yeast and animals, fatty acid biosynthesis acyl carrier domains are present on one or two multifunctional proteins found in the cytoplasm of the cell, and synthesis of fatty acids is not associated with the mitochondrion. It has been suggested before [16] that the *N. crassa* mitochondrial acyl carrier protein could be involved in formation of very long chain fatty acids, the elongation of unsaturated fatty acids, in the formation of uncommon fatty acids or in β-oxidation of fatty acids. There is no evidence to support any of these functions, and the association of the bovine homologue with complex I offers none either. Finally, it is worth pointing out that pantetheine-4-phosphate has been proposed to be associated with both mitochondrial ATPase and cytochrome oxidase [23–25].

Acknowledgements: We thank Prof. H. Weiss for communicating information about the acyl carrier protein associated with complex I in *N. crassa* mitochondria, and Mr. T.V. Smith and Mrs J. Fogg for synthesizing oligonucleotides.

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