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PURIFICATION AND CHARACTERIZATION OF DICYLCOHEXYLCARBODIIMIDE BINDING PROTEIN FROM MOUSE LIVER MITOCHONDRIA

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SUMMARY: The DCCD-binding protein from mouse liver Mt has been purified by chloroform:methanol method. The DCCD binding is drastically reduced when lipids are extracted from the proteolipid. The proteolipid as well as the lipid extracted protein migrate as single component with 7.8 K daltons molecular weight. The protein fraction yields a single band (pI 5.8) on isoelectric focusing gels. The DCCD binding protein is a product of Mt translation and contains Val as the N-terminal residue.

The mitochondrial (Mt*) ATPase complex from diverse organisms contains a soluble component (F_1) and a hydrophobic (F_o) fraction (1). The F_o fraction is believed to have an important role in the integration as well as in the function of the ATPase complex (1,2). One of the hydrophobic proteins, namely a proteolipid of 8 to 10 K daltons appears to be the site of attack of ATPase inhibitors such as 2-Nitro 4-Azido Carbonylcyanidephenylhydrazone (8). Recently, this proteolipid has received special attention because of its proposed role in proton translocation (1,6). Since the first observation of Cattell et al (3) that the DCCD binding proteolipid from beef heart is preferentially solubilized in chloroform:methanol, proteolipid fractions of varied purity have been isolated from several Mt systems. Recently, Sebald et al (5) have purified the DCCD binding proteolipid from Yeast and Neurospora Mt to electrophoretic homogeneity. In this paper, we report the purification of DCCD binding protein which is homogeneous with respect to electrophoretic migration, isoelectric separation and N-terminal amino acid residue. Results on N-terminal analysis suggest the possibility that the DCCD binding protein may be derived from a precursor.

*Abbreviations: Mt, mitochondrial; DCCD, dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Preparation of mitoplasts: Mouse liver Mt were prepared as described before (9), except that minced livers were homogenized in loose fitted teflon homogenizer (3 ups and 3 downs). The homogenate was made to 15% with Mt isolation buffer (0.3 M sucrose, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1 mg/ml bovine serum albumin), and Mt isolated by differential centrifugation. Crude Mt pellet was suspended in 5 volumes of Mt isolation buffer, and treated with digitonin (0.1 mg/mg protein). Resulting mitoplasts were pelleted at 10,000 X g and washed once with Mt isolation buffer.

Amino acid incorporation: Mitoplasts were incubated with radioactive amino acids as described before (10). The incubation mixture contained 0.25 M sucrose, 20 mM Tris-HCl (pH 7.6), 120 mM $MgCl_2$, 10 mM KH_2OP_4, 2 mM ATP, 2 mM GTP, 5 mM phosphoenol pyruvate, 15 ug/ml pyruvate kinase, 5 mM 2-mercaptoethanol, 300 ug/ml cycloheximide and 10 mg/ml mitoplast protein. Chloramphenicol, when added, was at 300 ug/ml. After 5 min incubation at 37°C, 15 uCi/ml ^{14}C amino acid mixture (Algal hydrolysate, New England Nuclear) and 20 uM each of the remaining unlabeled amino acids were added and the incubation was continued for 60 min. The incubated mitoplasts were washed 3 times with Mt isolation buffer and used for isolating the proteolipid.

Incubation with ^{14}C DCCD: Whole mitoplasts or isolated proteolipid were incubated with ^{14}C DCCD (50 uCi/mmole, Research Products, Inc.) according to the method of Sebald et al (5). The unbound DCCD was removed by washing with ether (5).

Purification of DCCD binding protein: The method was modified from Kuzela et al (4) and Sebald et al (5). About 150 ug mitoplasts or 20 mg electron transfer particles (2), were suspended in 3.0 ml 0.25 M sucrose, 0.01 M Tris-acetate (pH 7.5) and sonified 3 times (20 sec each with 1 min interval). The suspension was pre-extracted 3 times with neutral $CHCl_3$-CH_3OH (2:1) and cold ether (5). The residue was extracted with 20 ml neutral $CHCl_3$-CH_3OH at 50°C for 20 min. (4). The mathanol-chloroform extract was dried with a jet of N_2. The dry material was dissolved in 3 ml of methanol-chloroform and precipitated with about 15 volumes of ether. Resultant proteolipid was stored at -70°C. In some experiments, the proteolipid was treated with 0.5 N NaOH at 37°C for 18 hr to solubilize the lipid, and the protein was precipitated with ether (11). This treatment removes over 80% of the lipid from the proteolipid.

Polyacrylamide gel electrophoresis: The electrophoretic procedure was as described by Laemmli (12). The separating gel (0.3 cm X 10 cm) contained 10% acrylamide, 0.27% bis-acrylamide, 0.2% SDS and 8 M urea. The stacking gel (0.3 cm X 1 cm) contained 3% acrylamide, 0.08% bis-acrylamide, 0.2% SDS and 8 M urea. The staining conditions were as described by Weber and Osborn (13).

Other procedures: The N-terminal amino acid residues in the proteolipid and the lipid extracted protein was determined by N-dansylation and two dimensional analysis of dansyl-amino residues on pre-coated polyamide thin layer plates (14,15). Isoelectric focusing on 0.3 cm X 10 cm polyacrylamide-urea gels was carried out according to O'Farrell (16).

RESULTS

The proteolipid isolated from either the Mt inner membrane fraction or from the electron transfer particles migrate as a single component with a molecular weight of 7.8 K daltons (see Fig. 1A). Amido black stains the proteolipid with higher efficiency as compared to Coomassie blue. It can also be stained with lipid specific stain Sudan black B (see Fig. 1C). The proteo-

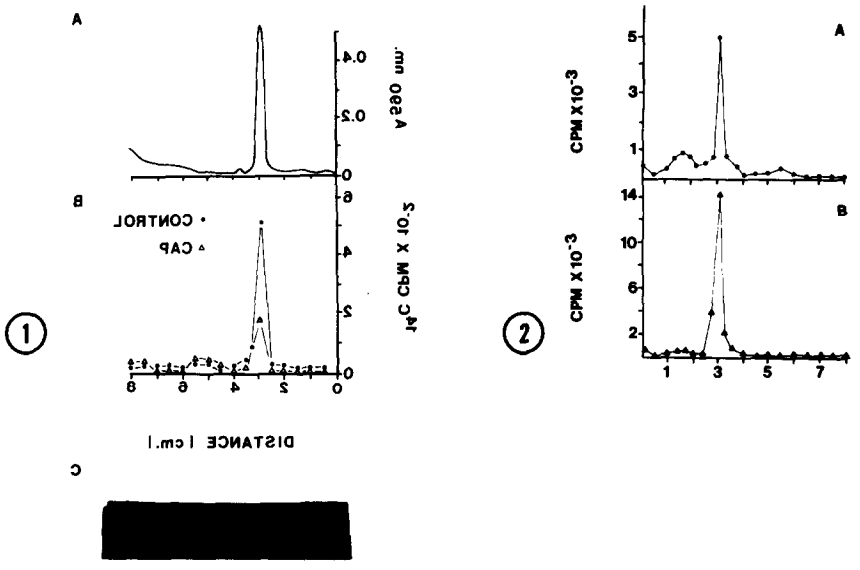

Fig. 1. Electrophoretic Analysis of the Proteolipid: Electrophoresis on 10% polyacrylamide-8M urea gels was carried out as described in the Materials and Methods. A, 7.5 ug proteolipid was electrophoresed and the gel was stained with Amido black; B, purified mitoplasts (25 mg each) were labeled with ^{14}C amino acid mixture with and without added chloramphenicol. After 60 min of labeling, proteolipid fractions were isolated and electrophoresed as described in A; C, photograph of a gel stained with Sudan black B.

Fig. 2. ^{14}C DCCD Binding to Proteolipid Sonicated mitoplasts (A) and purified proteolipid (B) were labeled with ^{14}C DCCD as described by Sebald et al (5). The labeled Mt particles and the proteolipid were electrophoresed on 10% polyacrylamide-8M urea gels as described in Fig. 1. The slices (1 mm) were swollen in NCS and counted with a toluene based scintillation mixture as described before (9).

lipid is a product of Mt translation in the mouse liver system since it is labeled with relatively high efficiency (10^5 CPM/mg) when intact mitoplasts are incubated with ^{14}C amino acid mixture in the presence of cycloheximide. Chloramphenicol, which is known to inhibit Mt ribosome dependent translation reduces the labeling by about 70% (see Fig. 1B).

It is well-known that ATPase inhibitor, DCCD, exclusively binds to a low molecular weight proteolipid in both Mt and chloroplast systems (1-7,17). To ascertain that the proteolipid under study is the DCCD binding protein, experiments were carried out using both the intact mitoplasts and purified proteolipid. It is seen from Fig. 2A that when total mitoplasts are incubated with

Table 1. Extent of DCCD binding to proteolipid and lipid extracted protein.

Fraction	nmoles DCCD Bound/mg Protein	Molar Ratios Protein/DCCD
Proteolipid	127	1.04
Lipid Extracted Protein	0.18	722

Isolated proteolipid and lipid extracted protein were labeled with ^{14}C DCCD as described by Sebald et al (5). The molar ratios were calculated taking into consideration that protein contents of proteolipid and lipid extracted protein were 40% and 80%, respectively.

^{14}C DCCD, only the component migrating as 7.8 K daltons is labeled. Similarly, the proteolipid purified by chloroform-methanol extraction binds ^{14}C DCCD which migrates as a single component with electrophoretic mobility identical to the proteolipid (Fig. 2B). The molar ratios of bound DCCD and the protein is nearly 1 indicating a single DCCD binding site on the protein (2,5). The extent of DCCD binding is drastically reduced when lipid is removed from the proteolipid by NaOH extraction (see Table 1). These results suggest that the hydrophobic lipid environment may be essential for DCCD binding.

The purity of DCCD binding protein was further investigated by isoelectric focusing on urea-polyacrylamide gels (16). The gel scan presented in Fig. 3 shows that the proteolipid contains a single component having an isoelectric pH of 5.8. Essentially a similar pattern was obtained when lipid extracted protein was subjected to isoelectric focusing.

A recent report on the bovine Mt system showed that the DCCD binding proteolipid fraction isolated by chromatography on Sephadex LH-60 in $CHCl_3$-CH_3OH solvent system could be resolved into three components of a similar size class by reverse phase HPLC (7). The isoelectric focusing method used in our experiments is expected to detect such microheterogeneity since proteins with one charge difference are resolved into distinct components in this system (16). We have sought additional evidence on the homogeneous nature of the DCCD bind-

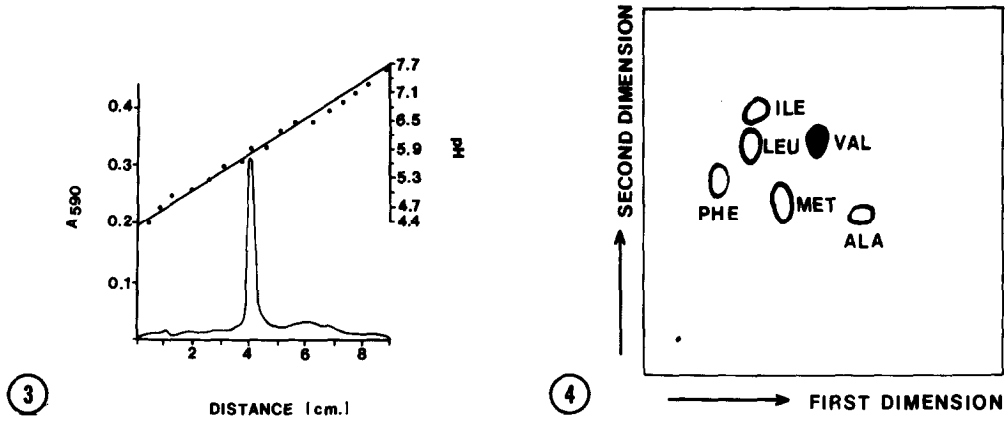

Fig. 3. Isolelectric Focusing of Purified Proteolipid: About 5 ug of proteolipid was subjected to isoelectric focusing on polyacrylamide-urea gels as described in Materials and Methods. Gels were stained with Amido black.

Fig. 4. N-Terminal Amino Acid Analysis of DCCD-Binding Protein. About 50 ug of proteolipid or 20 ug of lipid extracted proteins were subjected to N-dansylation as described in the Materials and Methods. The protein was hydrolyzed with 6 N HCl, the hydrolyate was extracted with 50 ul of ethyl acetate and spotted on 20 X 20 cm precoated polyamide think layer plates. Open spots represent the position of known markers. Shaded spot represents the N-terminal residue of the proteolipid.

ing proteolipid from mouse liver system. N-terminal amino acid analysis by N-dansylation shows that Val is the exclusive amino acid residue in the DCCD binding protein (see Fig. 4). Although not shown here, deformylation and hydrozinolysis of the proteolipid did not alter the N-dansylation patterns.

DISCUSSION

The chloroform-methanol extraction procedure used in the present paper yields about 50 mg proteolipid from 1 to 1.2 gm crude Mt (about 0.8 gm mitoplasts). The proteolipid contains 60% lipid and 40% protein.

As reported for other Mt systems (3-7), the ATPase inhibitor DCCD exclusively binds to the proteolipid both under *in vivo* (intact mitoplasts) and *in vitro* conditions. The proteolipid as well as the lipid extracted protein migrate as a single electrophoretic peak on SDS-urea polyacrylamide gels. The purified component appears to contain homogeneous polypeptides as tested by isoelectric focusing on urea-polyacrylamide gels as well as by N-terminal amino acid analysis. The isolation procedure used in our studies appears to facilitate

the extraction of a single DCCD binding component (4,5). In contrast, chromatographic separation in presence of methanol-chloroform (3) appears to yield multiple components in the range of 7.2 to 8 K daltons (7).

The DCCD binding protein in mouse liver Mt system is a product of Mt translation as shown in Fig. 1B. Incubation of purified mitoplasts with ^{14}C amino acids under conditions which permit only Mt ribosome specific translation leads to an efficient labeling of the proteolipid. Furthermore, the labeling is sensitive to chloramphenicol. These results are consistent with the Yeast (2,5) A. nidulans (18) and rat liver (4) systems. In Yeast and A. nidulans the N-terminal residue is f-Met suggesting negligible N-terminal processing. In Neurospora, however, the DCCD binding protein is translated on the cytoplasmic ribosomes and contains Tyr at the N-terminal position (5,19). It is now well recognized that Mt translation involves f-Met as the initiator (see ref. 20). In view of this, our results showing Val as the N-terminal residue of a mitochondrially translated protein suggests that the proteolipid in the mouse liver Mt system is derived from a precursor. The N-terminal processing of a large number of proteins formed on the cytoplasmic ribosomes has been well documented (21). It has also been proposed that such processing may serve as a signal for the transport of peptides across the membranes (22). Although the precise nature as well as the extent of N-terminal processing of DCCD binding protein is unknown, our results strongly suggest that the Mt translation products may be subjected to post translational cleavage. Experiments are underway to isolate and characterize the putative precursor.

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