

# THE DICYCLOHEXYLCARBODIIMIDE-BINDING PROTEIN OF THE MITOCHONDRIAL ATPase COMPLEX FROM BEEF HEART

## Isolation and amino acid composition

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### 1. Introduction

*N,N'*-Dicyclohexylcarbodiimide (DCCD) was introduced as an inhibitor of oxidative phosphorylation in beef heart mitochondria [1]. This hydrophobic carbodiimide specifically acts, like the antibiotic oligomycin, on the membrane-integrated part of the mitochondrial ATPase complex [2,3]. The irreversible mode of action allowed the identification of a DCCD-binding protein on the level of whole mitochondria [4] as well as of the purified oligomycin-sensitive ATPase [5]. This protein has been referred to as proteolipid due to its solubility in neutral chloroform/methanol [4]. The isolation of the DCCD-binding protein has been attempted [4], but it is uncertain whether it has been obtained in pure form, since it was never characterized in more detail.

In the meantime, DCCD-binding proteins have been identified in mitochondria from different sources [6–8], in chloroplasts [9] and in bacterial plasma membranes [10,11]. Some of them could be purified by a specific extraction with chloroform/methanol or with butanol. The bacterial protein was obtained in homogeneous form after DEAE-chromatography in the presence of chloroform/methanol [10].

The present communication describes the purification of the DCCD-binding protein from beef heart mitochondria. The procedure involves a gentle extraction with chloroform/methanol [10] followed by

CM-cellulose chromatography in these organic solvents. During purification, the protein was analyzed by phenol/formic acid gel electrophoresis [12], since it could not be completely disaggregated by dodecyl-sulfate [8]. The amino acid sequence of the beef heart protein will be communicated in a forthcoming paper.

### 2. Materials and methods

[<sup>14</sup>C]DCCD was synthesized from [<sup>14</sup>C]urea (NEN, 20 mCi/mmol) [8]. Carboxymethylcellulose (Servacel CM 23) was obtained from Serva, Heidelberg. Submitochondrial particles from beef heart were prepared from the total mitochondrial fraction isolated as in [13].

For labelling, the submitochondrial particles were suspended (15 mg/ml) in 10 mM Tris-acetate (pH 7.5) and incubated for 4 h at 0°C with [<sup>14</sup>C]DCCD (2.9 nmol × mg<sup>-1</sup> protein) [8]. Bound [<sup>14</sup>C]DCCD label was analyzed after extensively washing of the membranes with organic solvents [8].

The crude proteolipid fraction was isolated and concentrated essentially as in [10], then submitted to chromatography on CM-cellulose. The CM-cellulose was washed successively with glacial acetic acid, water, 25% ammonia and water, and was then equilibrated with chloroform/methanol (2/1) [10]. The CM-cellulose column was loaded with the crude proteolipid fraction, then equilibrated with chloroform/methanol/water (5/5/1) and finally developed with an ammonium acetate gradient as in [10], and detailed fig.2 legend.

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The protein fractions were concentrated as in [10].

The purification of the DCCD-binding protein was followed by gel electrophoresis in the presence of phenol/formic acid [12]. Slab gels were polymerized from acrylamide (10%), methylene-bisacrylamide (0.3%), tetramethylethylenediamine (1 ml/l) and ammonium peroxodisulfate (1 g/l) in water. Slits (10 × 1 × 2 mm) for application of the protein samples were preformed by projections in the mold (130 × 200 × 3 mm). The gel was equilibrated for 48 h with phenol/formic acid/water (2/1/1, w/v/v), with one change of the phenol medium after 24 h. Protein samples were dissolved in the same phenol medium. Horizontal slab-gel electrophoresis was performed at 300 V without cooling. Acetic acid, 10%, served as electrode buffer. The electrophoresis was stopped when cytochrome *c*, applied to the same gel slab (10 mg/ml), had migrated 6–7 cm. The gels were stained for 1 h with Coomassie Brilliant Blue R-250 [14], destained with first 10% acetic acid and then methanol/water/acetic acid (5/5/1). For analysis of radioactivity, the unstained gels were cut into slices of 1 mm. Protein was then eluted with 0.5 ml phenol medium at 50°C overnight. Radioactivity was measured by liquid scintillation counting.

Protein was determined by the Lowry method [15]. The protein samples were either dissolved in 2% dodecylsulfate or in 80% formic acid which was then neutralized with 5 N NaOH. N-Terminal amino acids were analyzed by the dansylation method [16]. Before amino acid analysis, the DCCD-binding protein was submitted to a chromatography on Bio-Gel P-30 (minus 400 mesh) in the presence of 80% formic acid. Aliquots were hydrolysed for 24 h, 48 h, 72 h and 120 h in 5.7 N HCl at 105°C. Cysteine and methionine were determined after performic acid oxidation [17].

### 3. Results and discussion

The ATPase activity of submitochondrial particles from beef heart is maximally inhibited by DCCD at 2–3 nmol/mg protein [4,8] after an incubation period of 4 h at 0°C. When [<sup>14</sup>C]DCCD is applied at this concentration range, about 1 nmol is found to be bound/mg membrane protein as demonstrated in table 1, and described in [4,8]. Gel electrophoresis in the presence of phenol/formic acid reveals that the bound [<sup>14</sup>C]DCCD label migrates as a single peak (fig.1A). The electrophoretic mobility appears to be unusually low considering an ~8000 mol. wt of the polypeptide, and may be explained by the unusually low content of basic amino acids (table 2). When the same labelled membranes were separated on dodecyl-sulfate gels, the [<sup>14</sup>C]DCCD label appears in several polypeptide fractions which apparently represent aggregates [4,8]. The tendency to aggregate is even more pronounced in the purified DCCD-binding protein. In contrast, DCCD-binding proteins from other sources migrate on dodecylsulfate gels as a single band corresponding to an app. mol. wt ~8000 [8,10].

The [<sup>14</sup>C]DCCD-labelled protein is extracted to about 40% by neutral chloroform/methanol (table 1) with a concomitant 7.5-fold enrichment of the specific [<sup>14</sup>C]DCCD content (nmol/mg protein). The extraction and the concentration of the crude proteolipid fraction was performed essentially as in [18] for isolation of brain proteolipids and in [10] for the isolation of the DCCD-binding protein from *Escherichia coli*. Analysis of the crude proteolipid fraction by phenol/formic acid gel electrophoresis showed the presence of at least 8 polypeptide bands (fig.1B), and one peak of the bound [<sup>14</sup>C]DCCD label migrating in

Table 1  
Purification of the DCCD-binding protein from beef heart mitochondria

Protein fraction	Total protein (mg)	Total <sup>14</sup> C radioactivity (cpm)	Specific [ <sup>14</sup> C]DCCD content (nmol DCCD/mg protein)
Submitochondrial particles	880 (= 100)	19.8 × 10 <sup>6</sup> (= 100)	1.12 (= 1)
Crude proteolipid	46 ( 5)	7.6 × 10 <sup>6</sup> ( 39)	8.3 ( 7.5)
DCCD-binding protein	5 ( 0.6)	3.9 × 10 <sup>6</sup> ( 20)	39 ( 35)

the same position as in the whole membrane protein. It may be mentioned that the DCCD-binding protein from beef heart is no longer extracted by chloroform/methanol when the membranes were prewashed with 90% methanol or chloroform/methanol/ether (2/1/12). After such a prewash the corresponding protein from

Table 2  
Amino acid composition of the DCCD-binding protein from beef heart mitochondria

Amino acid	Amount (mol/100 mol)	Best integral residue
Aspartic acid	4.0	3 ( 3.18)
Threonine	3.9	3 ( 3.05)
Serine	6.3	5 ( 5.01)
Glutamic acid	3.9	3 ( 3.08)
Proline	1.1	1 ( 0.89)
Glycine	14.7	12 (11.6 )
Alanine	17.4	14 (13.7 )
Cysteine	1.4	1 ( 1.13)
Valine	5.2	4 ( 4.13)
Methionine	3.6	3 ( 2.87)
Isoleucine	8.9	7 ( 7.02)
Leucine	11.9	9 ( 9.38)
Tyrosine	2.6	2 ( 2.06)
Phenylalanine	10.3	8 ( 8.11)
Histidine	—	0
Tryptophan	—	0
Lysine	2.6	2 ( 2.01)
Arginine	1.2	1 ( 0.97)
Total residues		78

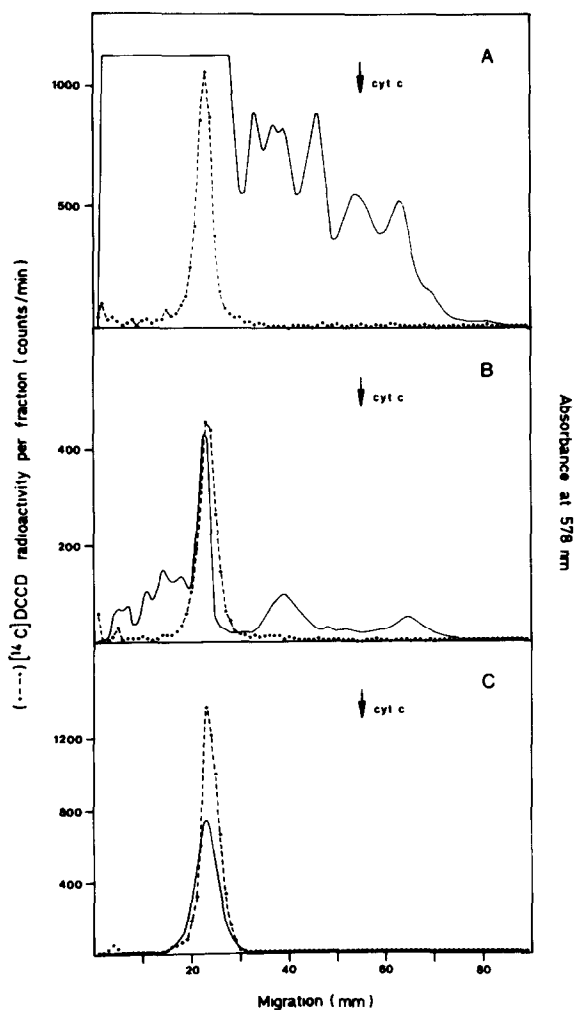


Fig.1. Phenol/formic acid gel electrophoresis of [ $^{14}\text{C}$ ]DCCD-treated (A) submitochondrial particles, (B) crude proteolipid fraction and (C) purified DCCD-binding protein. Two samples of each of the protein fractions described in table 1 were separated in parallel on the same gel slab. The densitometric trace (—) was obtained from the stained gel of 1 aliquot. The distribution of [ $^{14}\text{C}$ ]DCCD label (●—●) was determined in the second unstained gel. The position of a cytochrome *c* marker is indicated by the arrows.

*Neurospora* and yeast could be extracted in good yield and in pure form [8].

DEAE-cellulose chromatography in the presence of chloroform/methanol has been successfully applied for the isolation of the DCCD-binding protein from *E. coli*, and the DCCD-modified form and the free form of the polypeptide have been eluted from the column at different salt concentrations [10,11]. The corresponding protein from beef heart could not be purified by this procedure, since the DCCD-binding protein and several other proteins present in the crude proteolipid fraction were not bound to DEAE-cellulose. In contrast, the whole proteolipid fraction is bound to CM-cellulose equilibrated with chloroform/methanol (fig.2). The DCCD-binding protein was eluted in pure form (see below) at low ionic strength (12–14 mM ammonium acetate). Free and DCCD-modified form of the polypeptide are eluted at the same position. The other proteins, eluted at higher salt concentrations, up to 500 mM, did not contain any [ $^{14}\text{C}$ ]DCCD label. 40–50% of the applied protein and [ $^{14}\text{C}$ ]DCCD label stayed bound to the column (table 1). The specific DCCD content of the pure protein (nmol/mg

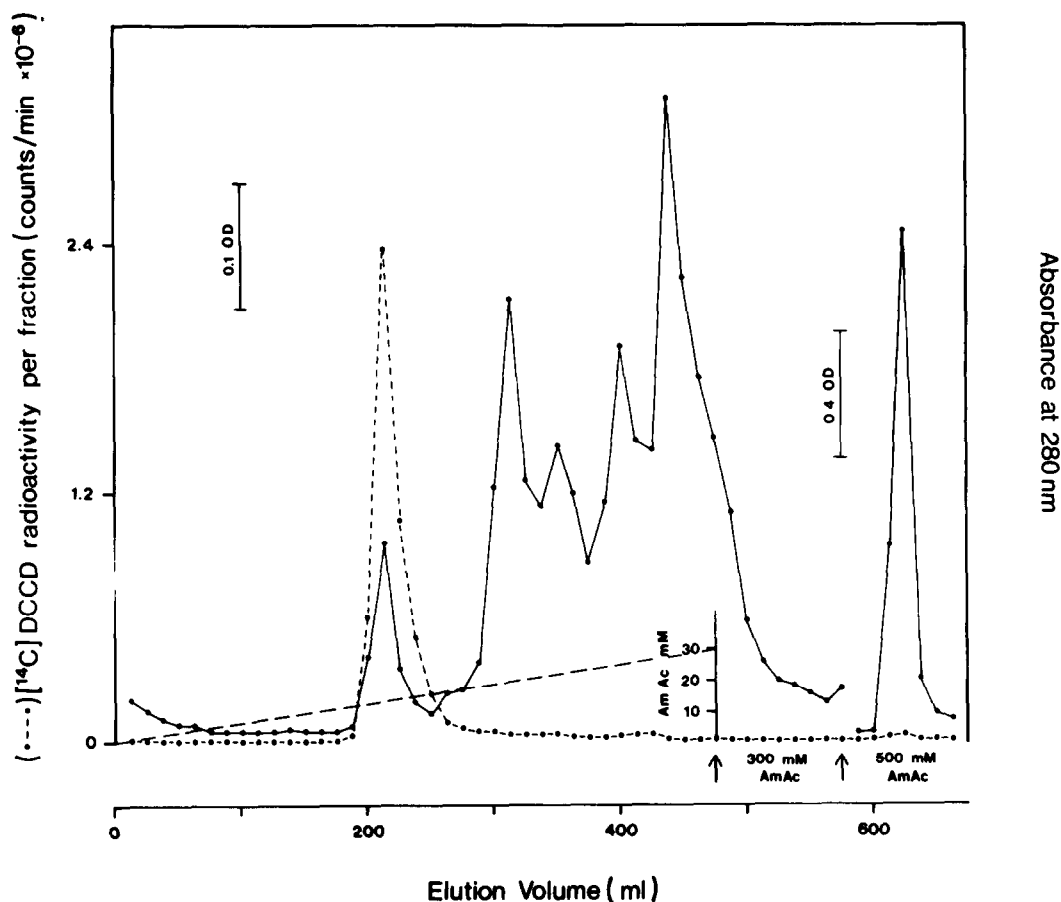


Fig.2. CM-cellulose chromatography of the crude proteolipid fraction isolated from [<sup>14</sup>C]DCCD-labelled submitochondrial particles of beef heart. The crude proteolipid (46 mg) dissolved in 46 ml chloroform/methanol (2/1) was applied to a CM-cellulose column of 40 ml bed volume. The column was then washed successively with 50 ml chloroform/methanol (2/1), 100 ml chloroform/methanol (1/1) and 50 ml chloroform/methanol/water (5/5/1). The proteins were then eluted with, first 500 ml of a linear ammonium acetate gradient (0–30 mM) at a flow rate of 50 ml/h, secondly 100 ml 300 mM ammonium acetate and, thirdly 100 ml 500 mM ammonium acetate, all in the presence of chloroform/methanol/water (5/5/1).  $A_{280}$  (●—●) and [<sup>14</sup>C]DCCD radioactivity (●—●) were determined in 12.5 ml fractions.

protein) is enriched 35-fold compared to the whole membrane protein.

The CM-cellulose chromatography also can be applied for purification of the DCCD-binding protein from spinach chloroplasts and *E. coli* (W.S., unpublished observation).

The purified DCCD-binding protein migrates as a single band during gel electrophoresis in the presence of phenol/formic acid (fig.1C). It is uncertain, whether the slight displacement between the distribution of

protein and [<sup>14</sup>C]DCCD label is significant. Only aspartic acid was found as N-terminal amino acid by the dansylation method. No further N-terminal residues were detected when the protein was pretreated with methanolic HCl. This treatment is known to release the formyl group from formylmethionine [19]. It may be mentioned that the N-terminal residue of the DCCD-binding protein from *Neurospora* and yeast is tyrosine and formylmethionine, respectively [8]. The amino acid composition (table 2) strongly

suggests that the polypeptide consists of 78 residues. The resultant ~8000 mol. wt could be confirmed by P-30 chromatography in the presence of 80% formic acid (data not shown) and is in the same range as the molecular weights determined for the corresponding proteins from *Neurospora* [8], yeast [8] and *E. coli* [10]. As in these other proteins, the content of hydrophobic residues is extremely high. Most abundant is glycine, alanine and leucine. No histidine and tryptophan are present, and only two lysines and one arginine occur.

Evidence has been provided that the DCCD-binding subunit of the *Neurospora* and yeast ATPase occurs as oligomer, probably as hexamer [8,20]. A similar conclusion may be drawn from the present experiments with beef heart. The specific DCCD content (nmol/mg protein) of the purified protein is enriched 35-fold compared to whole submitochondrial particles. This could indicate that 1 mg particles contain 2.9% or 3.5 nmol DCCD-binding protein. The molar amount of the ATPase in the particles appears to be 6–7-times lower based on the assumption that the ATPase occurs in similar amounts [21] as the cytochromes (0.5–0.6 nmol/mg protein) [22]. It is uncertain, however, whether both the DCCD-modified and the free form of the protein are recovered in the same yield by the described purification procedure.

Under the described experimental conditions (see section 2) the purified protein has been labelled to an extent corresponding to 39 nmol bound DCCD/mg Lowry protein. An only 2% higher specific DCCD content is calculated when protein is determined by amino acid analysis. This indicates that only about 30% of the protein has been modified by the DCCD, since after a 1:1 reaction 125 nmol DCCD should be bound per mg of the 8000 dalton protein. The dose-binding curve indicates that a high inhibition (80%) of the ATPase activity already is obtained when about 0.6 nmol DCCD are bound/mg membrane protein [8]. The specific DCCD content of the membranes in the present experiments (table 1) was about 2-fold higher. This suggests that the modification of 1 of the 6–7 DCCD-reactive proteins of the ATPase complex is sufficient for inhibition of the enzymatic activity.

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