Short Communications

The Isolation of Dicyclohexylcarbodi-imide-Binding Proteins from Mitochondrial Membranes

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DCCD* is a potent inhibitor of oxidative phosphorylation. It has been suggested that its site of action may be an intermediate of ATP synthesis with which it forms a covalent bond (Beechey, Robertson, Holloway & Knight, 1967). Therefore it is desirable to isolate and characterize the component(s) in the mitochondrion with which DCCD interacts. Knight, Holloway, Robertson & Beechey (1968) have shown that the radioactivity in [¹⁴C]DCCD-treated mitochondria or submitochondrial particles was recovered with two proteolipid fractions when the mitochondrial preparations were subjected to the procedures described by Folch-Pi (1963) for the preparation of white-matter proteolipids. However, these procedures were time-consuming and resulted in an intractable product. In this paper we describe the application of the methods used by Soto, Pasquini, Plácido & La Torre (1969) to isolate proteolipids from cat grey matter and white matter to the isolation of ox heart mitochondrial proteolipid, and show that a significant proportion of the radioactivity from [¹⁴C]DCCD-treated mitochondria is bound to several proteolipid fractions. Proteolipid is the name given to protein-lipid complexes that are insoluble in water but soluble in organic solvents (Folch-Pi, 1963).

Methods. Ox heart mitochondria were prepared by the method of Sanadi & Fluharty (1963). Submitochondrial particles were prepared by sonicating (MSE 100 W Ultrasonicator; 2 min) 40–50 ml portions of mitochondria (40 mg of protein/ml) suspended in medium containing (final concentrations) 250 mM-sucrose, 1 mM-ATP, 1 mM-succinate, 5 mM-MgSO₄ and 10 mM-tris-sulphate buffer, pH 7.6. The submitochondrial particles were sedimented by centrifugation at 105 000g for 30 min and were washed once with the above solution. The submitochondrial particles were resuspended in this solution and incubated for 20 h at 0°C with 1.5 nmol of [¹⁴C]DCCD/mg of protein. On re-isolation the particles contained 1.31 nmol of [¹⁴C]DCCD/mg of protein. Their ability to catalyse

*Abbreviation: DCCD, dicyclohexylcarbodi-imide.

the ATP-driven reduction of NAD⁺ by succinate was completely inhibited.

A crude mitochondrial proteolipid fraction was prepared by using a slight modification of the method described by Soto *et al.* (1969). [¹⁴C]DCCD-treated submitochondrial particles were sedimented by centrifugation at 105 000g for 30 min and the pellet was suspended in 40 times its volume of chloroform-methanol (2:1, v/v) at room temperature. After being stirred for 14 h the suspension was filtered through a sintered-glass funnel and the filtrate was washed with one-fifth its volume of water. The chloroform-methanol phase was cooled on ice, and 5 vol. of ice-cold diethyl ether was added slowly as the solution was stirred. After at least 12 h the precipitate was collected by centrifugation at –10°C in closed glass centrifuge tubes at 2000g for 30 min. The white residue readily dissolved in a small volume of chloroform-methanol (2:1, v/v) at room temperature to give a clear solution.

A column (2 cm × 20 cm) of Sephadex LH 20 was prepared by using the procedures described by Soto *et al.* (1969). When packed the column was equilibrated with chloroform.

Phospholipids were chromatographed on silica-gel t.l.c. plates with chloroform-methanol-aq. 32.5% (w/v) ammonia (17:7:1, by vol.) as the developing solvent (Cuzner & Davison, 1967). Molybdenum Blue reagent (Dittmer & Lester, 1964) and iodine vapour were used to detect phospholipids.

Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrophotometer, with Bray's (1960) solution that contained Cab-O-Sil (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) to keep solid material in suspension. Protein was determined in samples of submitochondrial particles by the biuret method of Gornall, Bardawill & David (1949) and in samples from organic solvents by the Lowry method as modified by Hess & Lewin (1965). Phosphorus was measured by the method of Chen, Toribara & Warner (1956).

Results and discussion. Chloroform-methanol

extraction of washed submitochondrial particles containing 1.31 nmol of DCCD/mg of protein resulted in the removal of 73.5% of the radioactivity into the crude lipid extracts. The proteolipid fraction obtained by ether precipitation of the washed chloroform-methanol extracts contained 52% of the radioactivity and 5.3% of the protein originally present in the submitochondrial particles; thus 13.5 nmol of DCCD was present/mg of protein. A portion (containing 13.75 mg of protein) of the redissolved ether precipitate was placed on the Sephadex LH 20 column at room temperature and eluted with the following sequence of solvents: 80 ml of chloroform, 20 ml of chloroform-methanol (15:1, v/v), 20 ml of chloroform-methanol (10:1, v/v), 20 ml of chloroform-methanol (8:1, v/v), 20 ml of chloroform-methanol (6:1, v/v), 20 ml of chloroform-methanol (4:1, v/v), 20 ml of chloroform-methanol (2:1, v/v), 50 ml of chloroform-methanol (1:1, v/v). Fractions (4–5 ml) of the eluate were collected in graduated tubes and the protein, phosphorus and radioactive contents of each fraction were determined: 85.4% of the protein and 88% of the radioactivity were recovered from the column. The elution profiles of the three components analysed are shown in Fig. 1, which indicates that the protein was separated into a considerable number of fractions. It is also apparent that the bulk of the radioactivity recovered was associated with protein peaks numbered 1 to 4, which contained 12.2, 27.8, 40 and 39.14 nmol of [^{14}C]DCCD/mg of protein respectively; most of this radioactivity was present in peaks 3 and 4. In contrast, the greater proportion of phosphorus-containing compounds, which were shown by t.l.c. to be phospholipids, were eluted with the first 60 ml of chloroform passed through the column. Consequently only small amounts of phosphorus were associated with

the protein peaks. In peaks 3 and 4 there were 0.094 and 0.135 μg -atom of phosphorus/mg of protein respectively.

If it is assumed (i) that 1 mol of protein in the proteolipid fractions has reacted with 1 mol of [^{14}C]DCCD, (ii) that all protein molecules present are combined with [^{14}C]DCCD and (iii) that the protein fractions are homogeneous, then an estimate of the molecular weights of the DCCD-binding proteins can be obtained. On this basis the molecular weights of the proteins in peaks 3 and 4 are both approx. 25 000. The phosphorus contents of these peaks are therefore 2.35 and 3.38 g-atoms/mol of protein respectively. The possibility exists that the phosphorus in these fractions is associated with tightly bound phospholipid and that the [^{14}C]DCCD is bound to phospholipid rather than protein. However, this is unlikely, since the radioactivity remained at the origin when samples of proteolipid from peaks 3 and 4 were subjected to t.l.c. with the methods designed for separating phospholipids.

Preliminary investigations on the proteolipid fractions have shown them to be insoluble in water and almost completely soluble in phenol-acetic acid-water (2:1:1, w/v/v) and 1% (w/v) sodium dodecyl sulphate. The u.v.-absorption spectra of the proteolipids show broad absorption bands around 280 nm.

It is apparent from the volume and nature of the solvents required to elute the proteolipids from the column that the separation obtained is not simply due to gel-filtration effects. As Soto *et al.* (1969) suggest, it is probable that the phospholipids and the protein eluted with chloroform are separated according to molecular size and the protein fractions eluted with chloroform-methanol are separated by partition chromatography.

The demonstration that at least 50% of the radio-

Fig. 1. Chromatography of a crude ox heart mitochondrial proteolipid fraction on Sephadex LH 20. Proteolipid containing 13.75 mg of protein was loaded on to the column and eluted with the sequence of solvents shown (the solvent change-over points indicated are approximate). Fractions (4–5 ml) of eluate were collected and each fraction was analysed for protein (—), phosphorus (----) and radioactivity (.....).

activity from [^{14}C]DCCD-treated submitochondrial particles is combined with at least four proteolipid fractions confirms the preliminary findings of Knight *et al.* (1968). In addition, the methods described are a rapid and reproducible means of preparing ox heart mitochondrial proteolipid. The problems remaining, however, concern the nature of the DCCD-binding site on the proteolipids, the relationship between the different proteolipid fractions, the significance of the phosphorus present in the proteolipids and the role that proteolipids play in mitochondrial function. The fact that they are the site of action of DCCD suggests that at least one of the proteolipid fractions may be either directly or indirectly involved in oxidative phosphorylation.

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