Purification of the Chloroplast-Membrane Dicyclohexylcarbodi-imide-Binding Proteolipid by Ion-Exchange Chromatography

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An efficient, mild and rapid procedure is reported for the separation of the dicyclohexylcarbodi-imide-binding protein of chloroplast membranes from endogenous lipid components. By the use of ion-exchange chromatography the chloroplast proteolipid can be successfully separated from the major part of chlorophyll and other membrane lipids while being retained in a butan-1-ol milieu.

Current investigations have pointed to the possible involvement of small hydrophobic proteins as natural ionophores in varied membrane systems (Racker & Eytan, 1975; Nelson *et al.*, 1977; Criddle *et al.*, 1977). We have reported a method for isolating proteolipids from biological membranes by use of a single-phase butan-1-ol extraction technique (Sigrist *et al.*, 1977). By using this isolation procedure we were further able to demonstrate proteolipid enhancement of proton permeability in a liposomal system composed of the dicyclohexylcarbodi-imide-binding proteolipid and endogenous lipids of the chloroplast membrane (Nelson *et al.*, 1977). Although proton measurements are feasible in a system containing large amounts of chromophores such as chlorophyll, spectroscopic measurements involving fluorescent probes, optical absorption and spin labels become difficult, if not impossible. It therefore became imperative to separate the proteolipid from the chromophores while retaining functional activity. An efficient, easy and mild separation procedure during which the proteolipid is maintained in a butanol medium is reported here.

Methods and Materials

Labelling of membranes

Lettuce (*Lactuca scarida* var. *longifolia*) chloroplast membranes, isolated as previously described (Nelson *et al.*, 1977), were incubated with *NN'*-dicyclohexyl[¹⁴C]carbodi-imide (45 mCi/mmol, 30 nmol/mg of protein) for 2 h at 25°C. The membranes were recovered by centrifugation and washed twice with 10 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, pH 8. Chloroplast membranes were then suspended in water and injected into 20 vol. of butan-1-ol. After removal of the precipitate by centrifugation the butanol, containing the proteolipid and lipid, was concentrated in a rotary evaporator with the condenser cooled to -20°C under high vacuum. Published procedures were

followed for the determination of chlorophyll (Arnon, 1959) and protein (Lowry *et al.*, 1951). Bovine serum albumin served as standard for the protein determination.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out as described by Weber & Osborn (1969). The electrophoresis was performed in 0.1% dodecyl sulphate/50 mM-sodium phosphate buffer, pH 7.2, in 10% (w/v) polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue and destained with 7% (v/v) acetic acid. For molecular-weight determinations, bovine serum albumin (mol. wt. 68 000), ovalbumin (43 000), egg-white lysozyme (14 300) and bovine pancreatic insulin (5 700) served as standards. Two methods were used to ascertain the electrophoretic specificity of the *NN'*-dicyclohexyl[¹⁴C]carbodi-imide labelling. In one case, when *NN'*-dicyclohexyl[¹⁴C]carbodi-imide-labelled samples were electrophoresed, identical samples were applied to separate gels. One gel was stained; the duplicate was not stained but was immediately cut into 1 mm slices. The individual gel slices were extracted with 10 mM-Triton X-100 overnight. Scintillation fluid (Nelson *et al.*, 1977) was added and the slices were counted for radioactivity. The extraction of radioactivity from Coomassie Blue-stained gels was also carried out. After staining and destaining procedures the gels were cut into 1 mm slices and the following mixture was added: Lipoluma/Lumasolve/water (50:5:1, by vol.). The gel slices were extracted either overnight at room temperature (20°C) or for 5 h at 40-50°C. The slices were then counted for radioactivity directly.

Preparation of DEAE-cellulose

DEAE-cellulose (Cellex D; Bio-Rad, Bromley, Kent, U.K.) was treated essentially as described by Rouser *et al.* (1969). The resin was washed with

Fig. 1. Incorporation of *NN'*-dicyclohexyl[^{14}C]carbodi-imide into chloroplast membranes

Duplicate samples ($60\mu\text{g}$ of protein) of dicyclohexyl[^{14}C]carbodi-imide-labelled chloroplast membranes were applied to sodium dodecyl sulphate/polyacrylamide gels. After fixation the stained gels were scanned at 600nm. Distribution of radioactivity is shown below the densitometric trace. The cathode (–) and anode (+) are indicated. After electrophoresis the gel was immediately sliced and radioactivity was extracted. (b) The polyacrylamide gel was subjected to staining and destaining procedures before extraction of radioactivity.

1M-HCl, water and with 0.1M-KOH, again followed by water. The procedure was repeated three times. The resin was then further washed with 3 vol. of acetic acid and 3 vol. of methanol, after which it was dried. After washing the DEAE-cellulose was converted into the acetate form by suspension in acetic acid and the column packed. The column was then equilibrated with butan-1-ol and was ready for use.

Thin-layer chromatography

Merck (Darmstadt, Germany) thin-layer silica-gel plates, without fluorescent indicator, were used. The solvent system was chloroform/methanol/water (65:25:4, by vol.). Plates were exposed to iodine and then either sprayed with ninhydrin to localize amino-positive lipids and protein or with naphthalenediol for sugar identification. The naphthalenediol spray was composed of 100mg of naphthalene-1,3-diol dissolved in 49ml of ethanol, with 1ml of conc. H_2SO_4 added. When the determination of the distribution of *NN'*-dicyclohexyl[^{14}C]carbodi-imide radioactivity was desired, the thin-layer plates were exposed to iodine and the radioactivity content of the lipid-positive spots was determined.

Lipoluma and Lumasolve were obtained from Fakola AG, Basel, Switzerland. *NN'*-dicyclohexyl[^{14}C]carbodi-imide was a generous gift of Dr. H. R. Kaback, Roche Institute of Molecular Biology, Nutley, NJ, U.S.A. All other chemicals and reagents were of the highest purity commercially available. Monogalactosyl-, digalactosyl- and sulphoquinovosyl-acylglycerol were kindly supplied by Dr. W. Eichenberger, University of Bern, Bern, Switzerland.

Results

The proteolipid of chloroplast membranes was most conveniently followed by the presence of covalently bound *NN'*-dicyclohexyl[^{14}C]carbodi-imide. It was found, however, that most of the radioactivity incorporated into the chloroplast membranes was due to non-covalent partitioning of *NN'*-dicyclohexyl[^{14}C]carbodi-imide or by-products into the membranes. Fig. 1 shows densitometer traces of sodium dodecyl sulphate/polyacrylamide gels of the *NN'*-dicyclohexyl[^{14}C]carbodi-imide-labelled chloroplast membranes. In Fig. 1(a) the gel was sliced immediately after electrophoresis and the radioactivity extracted, while a duplicate gel was stained. In contrast, the polyacrylamide gel shown in Fig. 1(b) was stained first with Coomassie Blue and then destained with acetic acid before extraction procedures were carried out. The difference in the labelling pattern between the two gels is immediately evident. Four peaks of radioactivity are present in the gel in Fig. 1(a), whereas only one smaller peak of radioactivity remains after staining and destaining procedures (Fig. 1b).

Dodecyl sulphate/polyacrylamide gels of the various fractions obtained during proteolipid isolation from *NN'*-dicyclohexyl[^{14}C]carbodi-imide-labelled chloroplast membranes are shown in Fig. 2. Distribution of radioactivity is indicated underneath each trace. Gels were sliced, and radioactivity was extracted immediately after termination of electrophoresis. Chloroplast-membrane protein that precipitated on addition of butan-1-ol (Fig. 2a) appears to give a protein pattern identical with that shown in Fig. 1. However, only one much diminished peak of radioactivity is present at a relative mobility identical with that in Fig. 1(b). The major peaks of radioactivity present in gels of the butanol supernatant (results not shown) remain soluble in ether (Fig. 2b). The smaller peak of radioactivity is precipitated by addition of ether and corresponds directly with the Coomassie Blue-staining proteolipid band whose apparent molecular weight is 8000 (Fig. 2c). Purity and homogeneity of the *NN'*-dicyclohexylcarbodi-imide-binding proteolipid have been demonstrated by high-voltage electrophoresis and *N*-terminal amino acid determination (Sigrist-Nelson *et al.*, 1977; K. Sigrist-Nelson, H. Sigrist & A. Azzi, unpublished work).

Fig. 3 shows the elution pattern obtained when the concentrated butanol supernatant of *NN'*-dicyclohexyl[^{14}C]carbodi-imide-labelled membranes (see the Methods and Materials section: 'Labelling of membranes') was applied to the DEAE-cellulose column; Butan-1-ol was the eluent. One large peak of radioactivity was eluted, accompanied by an

Fig. 3. DEAE-cellulose chromatography of the butanol-extracted dicyclohexylcarbodi-imide-binding proteolipid. Chloroplast membranes (12mg) were labelled with dicyclohexyl[^{14}C]carbodi-imide and the proteolipid was extracted with butan-1-ol as described in the Methods and Materials section. Butanol supernatant was applied to the column (column volume 19 ml) and eluted with butan-1-ol. Samples of column fractions were taken for A_{645} (Δ) and radioactivity ($\bullet$) measurements. A denotes the material eluted with butan-1-ol. After at least 2 column volumes the eluent was changed. B denotes the peak eluted with butan-1-ol/formic acid (50:1, v/v).

Fig. 2. Isolation of the dicyclohexyl[^{14}C]carbodi-imide-binding protein from chloroplast membranes: distribution of protein and radioactivity in sodium dodecyl sulphate/polyacrylamide gels

(a) Butanol precipitate (60 μg of protein); (b) ether supernatant (equivalent of 10 μg of protein); (c) ether precipitate (10 μg of protein). The cathode (–) and anode (+) are indicated.

intense green colour. The column was further washed with at least 2 column volumes of butan-1-ol. No additional radioactivity or increase in absorbance was noted. The column was then eluted with butan-1-ol/formic acid (50:1, v/v). A second peak of radioactivity and a corresponding small increase in material absorbing at 645 nm was eluted. Again, further washing of the column did not elute additional material.

Polyacrylamide gels were run of the fractions eluted from the DEAE-cellulose column. Fraction A of Fig. 3 was complexed with sodium dodecyl sulphate and run on dodecyl sulphate/polyacrylamide gels. Although a green chlorophyll band was visible, a Coomassie Blue-staining band was not detectable (Fig. 4a). Radioactivity was present, however, in the form of three large peaks when the gels were sliced and extracted directly after electrophoresis. The peaks of radioactivity disappeared when the gels were first stained and destained before extraction of radioactivity, indicating that the radioactivity was not covalently associated with protein. Electrophoretic analysis of fraction B of Fig. 3 showed a single peak of radioactivity whose mobility corresponded to a diffuse Coomassie Blue-staining band (Fig. 4b); concomitant with the blue-staining band was the presence of a faint green band. The apparent molecular weight of the band ranged from 12000 to 24000. Additionally, the two peaks of radioactivity, A and B, were separately combined with 5 vol. of diethyl ether. The mixture was left for 1 h at -20°C , after which a precipitate was recovered by centrifugation. Neither radioactivity nor protein

was precipitated when fraction A was combined with ether. When the second peak, fraction B, was combined with ether a precipitate containing 1% of the total membrane protein and corresponding radioactivity was obtained. The electrophoretic mobility of the protein is shown in Fig. 4(c). Its apparent molecular weight is 8000.

The butanol supernatant and fractions eluted from the DEAE-cellulose column were analysed by t.l.c. For this purpose NN' -dicyclohexyl[^{14}C]carbodi-imide-labelled chloroplast membranes were used. The distribution of radioactivity and of lipid are shown in Fig. 5. Ninhydrin-positive and sugar-containing lipids are indicated. The pattern of lipid and chlorophyll distribution is similar in the butanol supernatant and the first fraction eluted from the ion-exchange column (fraction A). A radioactively labelled ninhydrin-positive spot is, however, present at the origin in the butanol supernatant. Fraction B, the second peak of Fig. 3, which was eluted with butanol/formic acid (50:1, v/v), also shows a radioactive ninhydrin-positive spot at the origin. Radioactivity present in the chlorophyll region of the butanol supernatant and fraction A is missing from fraction B. Indeed, in fraction B all the radioactivity is associated with the ninhydrin-positive spot at the origin. Radioautography confirmed this observation. To calibrate the thin-layer system two phospholipids (phosphatidylcholine and phosphatidylethanolamine) and three glycolipids (monogalacto-, digalacto- and sulphonolipid) were utilized. The fraction eluted by butanol/formic acid is composed of two differently migrating glycolipids, one whose mobility is similar

Fig. 4. Electrophoretic analysis of fractions eluted from the DEAE-cellulose column

Chloroplast membranes were incubated with dicyclohexyl[^{14}C]carbodi-imide, extracted with butan-1-ol and applied to the ion-exchange column as indicated in Fig. 3. Radioactivity was extracted immediately after electrophoresis; duplicate gels were stained. Distribution of radioactivity is shown underneath each densitometric trace. The cathode (–) and anode (+) are indicated. (a) represents the peak obtained (A, Fig. 3) with butan-1-ol as eluent; (b) shows the second peak (B, Fig. 3) obtained with butanol/formic acid (50:1, v/v) (15 μg of protein); (c) shows the ether-precipitated fraction of the peak B (Fig. 3) (10 μg of protein). See the text for details.

Fig. 5. Thin-layer-chromatographic analysis of fractions derived from the DEAE-cellulose column

Dicyclohexyl[^{14}C]carbodi-imide-labelled chloroplast membranes were treated as described in Fig. 3. Samples of the butanol supernatant and the two fractions eluted from the ion-exchange column were dried, weighed and then dissolved in chloroform/methanol (2:1, v/v) for sample application. Vertical bars indicate ninhydrin-positive spots, and horizontal bars, naphthalenediol-positive spots. With a duplicate plate, lipid spots were localized with iodine, scraped off, and their radioactive contents determined. Samples: 1, 100 μg of butanol supernatant; 2, phosphatidylcholine; 3, 130 μg of peak A (fraction eluted with butan-1-ol, Fig. 3); 4, phosphatidylethanolamine; 5, 115 μg of peak B [fraction eluted with butanol/formic acid (50:1, v/v); Fig. 3]; 6, monogalactosyldiacylglycerol; 7, digalactosyldiacylglycerol; 8, sulphoquinovosyldiacylglycerol.

to that of monogalactosyldiacylglycerol, a lipid with a mobility similar to that of phosphatidylcholine, two chlorophylls and the dicyclohexylcarbodi-imide-binding proteolipid.

Discussion

A proteolipid-isolation procedure in which butan-1-ol was shown to solubilize selectively a dicyclohexylcarbodi-imide-binding protein has been reported (Sigrist *et al.*, 1977). A further purification of the dicyclohexylcarbodi-imide-reactive protein of chloroplast membranes by using ion-exchange chromatography in butan-1-ol is reported in the present paper. The column chromatography presents a mild, easy and rapid method for separating the proteolipid from large amounts of endogenous lipids which are co-solubilized in butanol.

When dicyclohexyl[^{14}C]carbodi-imide is allowed to react with chloroplast membranes, large amounts of radioactivity partition into the membranes. A small fraction of the radioactivity is specifically and

covalently associated with only one protein, the dicyclohexylcarbodi-imide-binding proteolipid. Over 90% of the radioactivity, however, is non-specifically solubilized in the membranes. On sodium dodecyl sulphate/polyacrylamide gels the non-covalently associated radioactivity appears as three peaks, which are eluted from the gels during destaining procedures. During the proteolipid isolation this radioactivity also becomes solubilized in butanol and is not precipitated by addition of ether. An additional advantage of ion-exchange chromatography is that the dicyclohexylcarbodi-imide-reactive proteolipid may be washed free of dicyclohexylcarbodi-imide that has not reacted and radioactive by-products while remaining solubilized in butan-1-ol. This may prove quite useful when other hydrophobic protein labels, such as photoactive iodonaphthalene azide (Sigrist-Nelson *et al.*, 1977), are used. Furthermore, the DEAE-cellulose chromatography allows for easy concentration of the proteolipid. In contrast with the ether-precipitation procedure, after which the proteolipid becomes insoluble in butanol and most other organic solvents, the proteolipid fraction eluted from the DEAE-cellulose column retains its initial solubility characteristics. This is important in that it greatly aids further manipulation.

A noteworthy finding is that a small amount of lipid is retained by the DEAE-cellulose column and eluted with the proteolipid fraction. Sodium dodecyl sulphate/polyacrylamide gels of this fraction produce a single diffuse Coomassie Blue-staining band with associated radioactivity and chlorophyll. The apparent molecular weight for this complex ranges from 24000 to 12000. In contrast, when the eluate of the acidified butanol is combined with ether, the proteolipid found in the precipitated fraction appears as a sharp Coomassie Blue-staining band with an apparent mol.wt. of 8000 and no co-migrating chlorophyll. It is tempting to speculate that some of the lipid may be specifically, although not covalently, associated with the dicyclohexylcarbodi-imide-reactive proteolipid, its presence perhaps being related to stabilization of proteolipid aggregates and retention of ionophoric activity in butan-1-ol (Nelson *et al.*, 1977). For the time being, however, such suppositions remain highly speculative and await further experimental evidence for verification.

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