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Isolation of a chloroplast *N,N'*-dicyclohexylcarbodiimide-binding proteolipid, active in proton translocation

(1-butanol extraction/reconstitution of liposomes/dicyclohexylcarbodiimide-sensitive proton channel)

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ABSTRACT **The *N,N'*-dicyclohexylcarbodiimide-binding proteolipid from lettuce chloroplast membranes has been purified by a novel, rapid technique involving 1-butanol extraction and ether precipitation. Reconstitution of this proteolipid into liposomes composed of chloroplast lipids and subsequent incorporation of bacteriorhodopsin resulted in the formation of liposomes exhibiting a light-dependent accumulation of protons. This accumulation was significantly enhanced upon addition of *N,N'*-dicyclohexylcarbodiimide at concentrations similar to those that inhibit chloroplast adenosinetriphosphatase activity. Radioactively labeled *N,N'*-dicyclohexylcarbodiimide was found to be incorporated essentially into the proteolipid of the reconstituted liposomes. These results suggest that the functional unit responsible for proton channeling in the chloroplast membrane has been isolated and reconstituted in the native state.**

The energy-transducing ATPase complex of chloroplasts, mitochondria, and bacterial membranes has been found to be composed of at least two operationally and functionally distinct entities (for reviews on the subject see refs. 1–4). F_1, a sector of the ATPase complex exterior to the membrane, contains ATPase activity and behaves as an extrinsic protein in that it is readily dissociated from the membrane by relatively mild treatment. Second, the F_0 or membrane sector of the ATPase behaves as an intrinsic hydrophobic complex which can be isolated only by procedures based on the use of detergents or nonaqueous solvents. The F_0 has been identified indirectly by its capacity to bind certain ATPase inhibitors including *N,N'*-dicyclohexylcarbodiimide (DCCD). This reagent inhibits the ATPase activity of mitochondrial and chloroplast membrane vesicles but not that of the isolated F_1. Removal of F_1 from F_0 or modification of their interaction results in an enhanced proton permeability of the ATPase-containing membranes. Such an enhanced flux is blocked by incubation of the membrane with DCCD, suggesting that the reagents' mode of action is to block a site or sites in the F_0 that participate in proton translocation (5–7).

DCCD has been found to bind specifically and covalently to a small polypeptide that can be extracted in the form of a proteolipid complex (8–11). To date, however, it has not been possible to isolate a purified, defined proteolipid fraction in a state allowing reconstitution of proton-translocating activity (12). The present study reports the isolation of a proteolipid from lettuce chloroplasts membranes and its reconstitution into liposomes. Evidence is presented indicating that its capacity to function as a DCCD-sensitive proton channel is retained during the isolation and reconstitution procedures.

Abbreviations: ATPase, adenosinetriphosphatase; DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, N-[tris(hydroxymethyl)methyl] glycine.

MATERIALS AND METHODS

Chloroplasts from lettuce were prepared as previously described (13) with the following exceptions: bovine serum albumin was omitted; an extra wash with 0.15 M NaCl was introduced; and chloroplasts were suspended in 0.01 M *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) (pH 8) at a chlorophyll concentration of 3–4 mg/ml.

Bacteriorhodopsin was prepared from *Halobacterium halobium* according to Oesterhelt and Stoeckenius (14). The bacteria were grown in 2-liter Erlenmeyer flasks, containing 1.5 liters of medium, at 37° with gentle shaking for 5 days under light (10^3 ergs/cm^2 per sec) (1 erg = 10^{-7} J). Growth medium was prepared by the addition of 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 3 g of sodium citrate, 2 g of KCl, and 5 g of yeast extract (Difco 0127-01), into 1 liter of distilled water.

Preparation of Liposomes and Proton-Uptake Measurements. The chloroplast lipids, containing the proteolipid, were sonicated in a bath-type sonicator (15) in 0.15 M NaCl for approximately 15 min. The liposomes (0.4 ml) were then combined with 0.1 ml of bacteriorhodopsin (3 mg of protein per ml) and sonicated for an additional 10 min. The liposomes (0.05 ml) were assayed for light-induced proton uptake in a final volume of 1 ml of 0.15 M NaCl, adjusted to pH 6.7 with 0.1 M HCl, under a light intensity of 10^6 ergs/cm^2 per sec at 23°. Proton uptake was monitored by measuring the external pH.

Published procedures were followed for the determination of chlorophyll (16), protein (17), and sodium dodecyl sulfate gel electrophoresis (18, 19).

RESULTS

Isolation of the Chloroplast Proteolipid. The butanol extraction procedure of Sigrist *et al.* (11) was employed to isolate a proteolipid fraction from chloroplast membranes. Two milliliters of a chloroplast suspension was injected into 100 ml of 1-butanol at 0° under vigorous stirring. Following agitation for 30 min, the suspension was centrifuged twice at 20,000 × *g* for 10 min. Diethyl ether (500 ml) was added to the butanol supernatant. After incubation for 30 min at 0° the proteolipid was precipitated by centrifugation (10,000 × *g*, 10 min). The pellets were suspended in 1 ml of distilled water or desired aqueous solution. A yield of approximately 300 μg of proteolipid protein was routinely obtained.

The purification procedure was monitored by analyzing sodium dodecyl sulfate electrophoretic patterns of protein and ^{14}C incorporation after membrane labeling with [^{14}C]DCCD. The radioactivity was found to be incorporated predominantly into a low-molecular-weight protein (approximately 8000, Fig. 1). To a lesser extent radioactivity also appeared to be associated

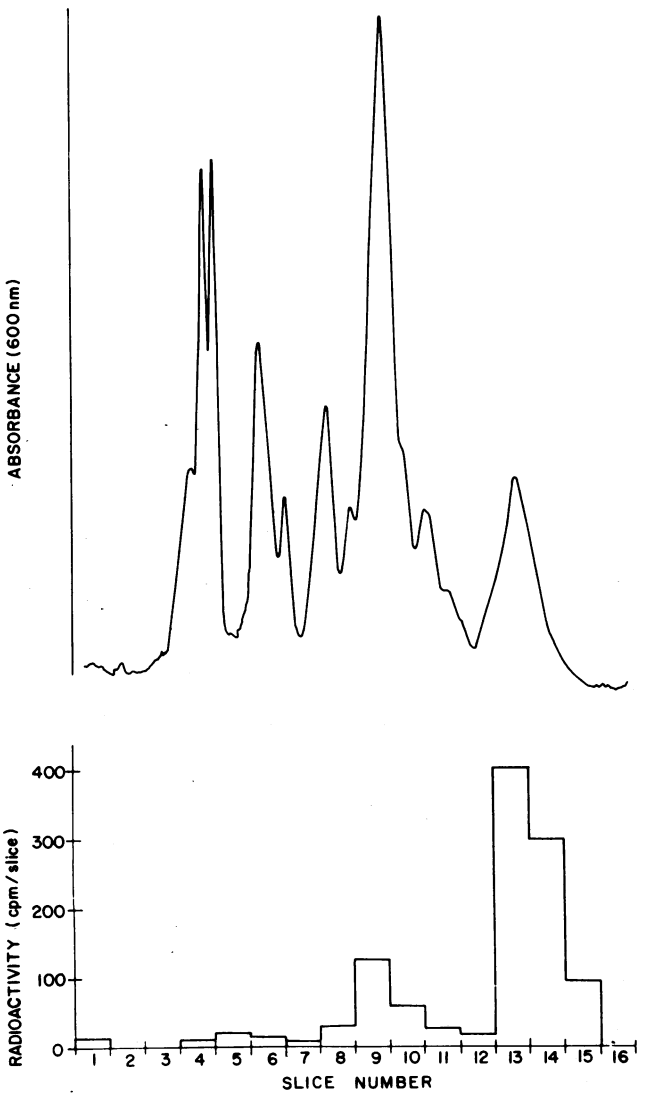

FIG. 1. Incorporation of [^{14}C]DCCD into chloroplast membranes. Chloroplast membranes (3.5 mg of chlorophyll in 1 ml) were incubated for 1 hr at room temperature with 100 nmol of [^{14}C]DCCD (45 mCi/mmol in ethanol). A 0.05-ml aliquot was diluted with 0.2 ml of 0.4 M sucrose/10 mM Tricine at pH 8 and solubilized at room temperature with 2% sodium dodecyl sulfate containing 2% (vol/vol) mercaptoethanol. Samples containing approximately 70 μg of membrane protein were analyzed by sodium dodecyl sulfate gel electrophoresis as previously described (19). After fixation the stained gels were scanned at 600 nm and subsequently cut into 0.5-cm slices. For radioactivity measurements, the slices were dissolved (2 hr, 70°) with 0.5 ml of Soluene 350 (Packard). Upon bleaching of the Coomassie blue stain, 10 ml of scintillation fluid was added before radioactivity was measured.

with higher-molecular-weight proteins. Addition of the DCCD-labeled chloroplast membranes to butanol resulted in the precipitation of all membrane proteins except the DCCD-binding proteolipid. The electrophoretic polypeptide pattern and the distribution of radioactivity observed in the precipitate fraction is shown in Fig. 2. Nearly quantitative removal of the approximately 8000 dalton band from the butanol precipitate occurred. Subsequent addition of ether to the butanol fraction resulted in the precipitation of the proteolipid. The electrophoretic pattern of the ether precipitate fraction showed a single Coomassie blue staining band containing all the radioactivity (Fig. 3); without NH_2-terminal amino acid analysis it is not as yet possible to establish whether a single polypeptide was obtained. It must be noted that the radioactivity does not appear to coincide exactly with the Coomassie blue staining band. A fraction of firmly bound lipids may, however, be present in the proteolipid fraction and could lead to electrophoretic heterogeneity within the protein band (20). No definite conclusions are yet possible.

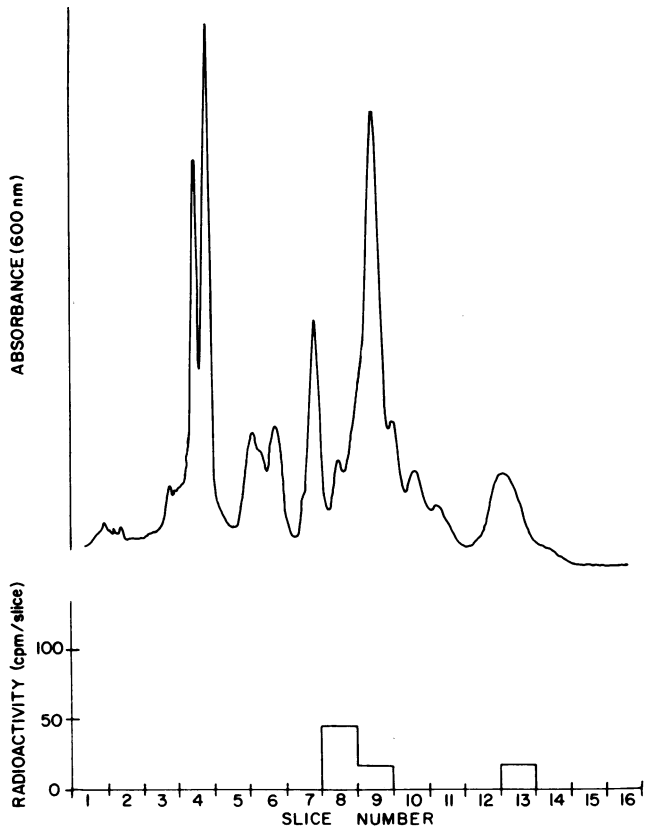

FIG. 2. Protein and radioactivity patterns of the butanol precipitate from [^{14}C]DCCD-treated chloroplast membranes. [^{14}C]-DCCD-labeled chloroplast membranes (see legend of Fig. 1) were treated with 1-butanol. The precipitate was dried under nitrogen and solubilized as described in Fig. 1. Samples containing approximately 50 μg of protein were electrophoresed.

DCCD-Sensitive Proton Translocation. The previously detailed isolation procedure readily allowed the incorporation of the proteolipid into liposomes composed of endogenous chloroplast lipids. Liposomes were formed by evaporation of butanol from the butanol supernatant and sonication, following addition of an aqueous solution. Further sonication of the liposomes in the presence of bacteriorhodopsin led to the formation of lipsomes showing light-induced proton uptake (Fig. 4). DCCD addition (20 μM) to these liposomes resulted in a progressive increases in the light-induced proton uptake. Fifteen minutes after addition of DCCD the proton uptake was found to be twice that measured in the absence of added DCCD. Comparison of the rates of proton uptake and release show that DCCD altered only the total magnitude of the uptake and not the observed rates. Addition of DCCD to liposomes identical to those discussed in Fig. 4, but not containing proteolipid, effected no change in light-induced proton uptake or release in the dark even after prolonged incubation (data not shown).

Incorporation of [^{14}C]DCCD into the Reconstituted Proteolipid. Addition of [^{14}C]DCCD to liposomes containing the proteolipid, chloroplast lipids, and bacteriorhodopsin resulted in the majority of the radioactivity being associated with the proteolipid band (Fig. 5). In contrast, the bacteriorhodopsin protein band failed to show any enhancement in the binding

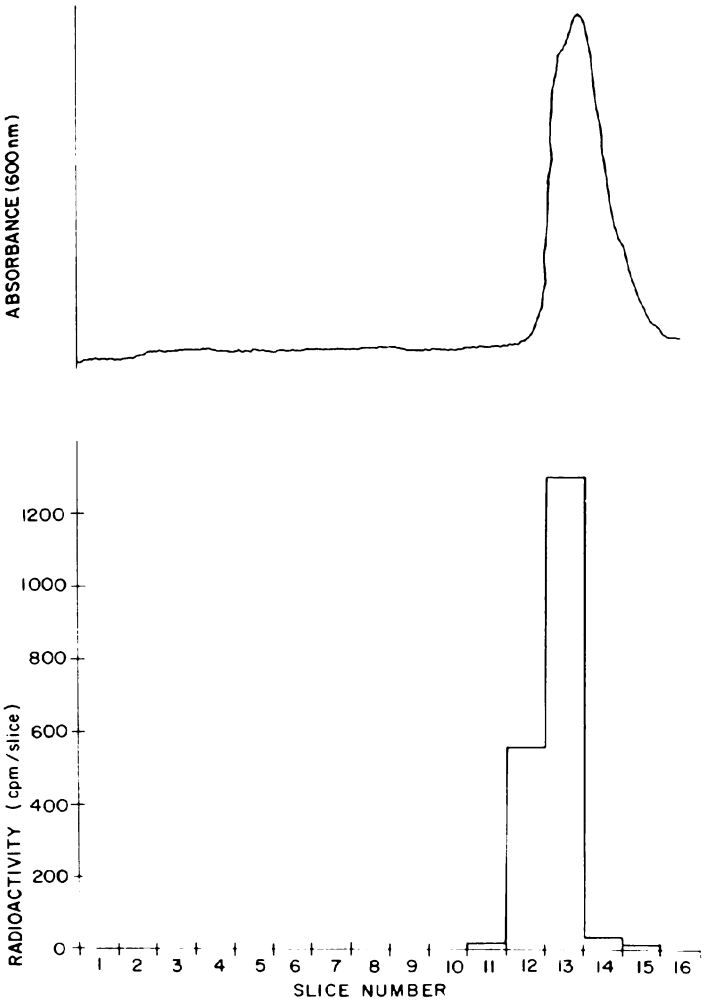

FIG. 3. Incorporation of [^{14}C]DCCD into the proteolipid fraction. The proteolipid fraction was isolated as reported in *Results*. Samples, pretreated as in Fig. 1 and containing 10 μg of protein, were applied for electrophoresis.

of DCCD over background level. Some smearing of radioactivity was observed in the gel of Fig. 5 as contrasted with that of Fig. 3. This may be due to the presence of reaction products such as dicyclohexylurea that were formed during the incubation process and were not removed from the labeled liposomes prior to electrophoresis. Hydrophobic byproducts of the DCCD labeling procedure may tend to smear somewhat due to the high lipid-to-protein ratio of the sample.

DISCUSSION

The present report contributes strong supporting evidence for the role of the DCCD-binding proteolipid in proton-translocation activity (5–7). We have isolated this fraction from chloroplast membranes and shown it to be the main DCCD-binding component therein. This extends similar reports in other membrane systems (8–11, 21).

The isolation was based on the observation that the proteolipid is the only polypeptide component of chloroplast membranes soluble in butanol/2% water. This allows readily for its solubilization together with the majority of the membrane lipids. Subsequently it can be selectively precipitated from the butanol by cold ether. The total procedure and especially the butanol solubilization can be performed in a very short time period, minimizing the exposure of the polypeptide to possible unfavorable conditions. The detailed characterization of this entity is still incomplete. However, from the available results

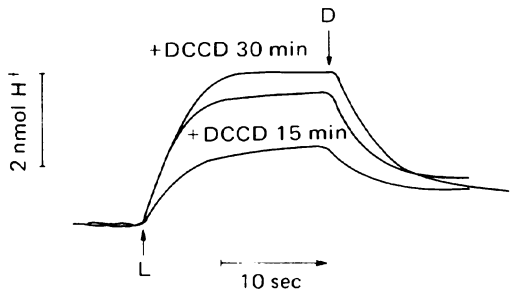

FIG. 4. Enhancement of light-induced proton uptake by DCCD in proteolipid–bacteriorhodopsin vesicles. After butanol extraction of 2 ml of chloroplast membranes (equivalent to 7.0 mg of chlorophyll) by the procedure described in *Results* (omitting the ether precipitation step), 100 ml of the butanol supernatant was dried at low temperature and redissolved in 2 ml of 1-butanol. Aliquots (0.5 ml) were dried under nitrogen. Procedures for liposome formation and proton uptake measurements are described in *Materials and Methods*. DCCD, when present, was 20 μM. pH measurements were performed at 5-min intervals. L and D represent light on and off, respectively.

the DCCD-binding component appears to be a small protein having a fraction of tightly bound lipids. Its molecular weight (approximately 8300) is similar to that of equivalent fractions isolated from other sources (8–11). On the basis of yield of proteolipid and assuming complete extraction, it may be calculated that there are some four to six proteolipid molecules per F_1 molecule in chloroplast membranes.

The isolated proteolipid fraction, when reincorporated into a liposomal system, appears to retain some of the functions ascribed to it *in situ*. Namely, it mediates proton translocation in the reconstituted systems, the H^+ leak being sensitive to DCCD. It should be emphasized that the system under study does not allow the direct measurement of the proton-translocating capacity of the proteolipid. Its implication in such a role is based on the increased accumulation of protons observed in the presence of DCCD and on the fact that this DCCD effect is due to its binding preferentially to the proteolipid. It is conceivable that the mere addition of protein to a liposome enhances its permeability to protons. Thus, addition of bacteriorhodopsin to liposomes leads, as expected, to accumulation of protons within the liposomes in the light, but a rapid leak ensues in the dark. However, such a leak is completely insensitive to added DCCD. Furthermore, as seen in Fig. 5, there is little, if any, binding of DCCD to the bacteriorhodopsin moiety.

Even though, as noted, DCCD increased significantly the proton accumulation in the proteolipid-containing liposomes, it did not appear to modify the rates of translocation either in the light or in the dark (Fig. 4). The most plausible explanation is that a mixture of liposomes is present. Some, without appreciable functional proteolipid, lead to the uptake observed in the absence of DCCD. The rest, containing the proteolipid, show no net uptake in the absence of DCCD, due to the proteolipid-induced leak. Upon addition of DCCD, the proteolipid would cease to function as a proton translocator and under these conditions all liposomes would behave as if no proteolipid were present.‡ Results not presented indicate that liposomes containing only bacteriorhodopsin show a proton accumulation and release similar to that depicted in Fig. 4 for liposomes in the presence of the DCCD-inhibited proteolipid.

The reported findings suggest that the native state of the proteolipid is preserved during the isolation and reconstitution experiments. The available evidence indicates that, in the

‡ Preliminary experiments (N. Nelson, unpublished results) indicate that the two sets of vesicles indeed exist and can be separated by density centrifugation.

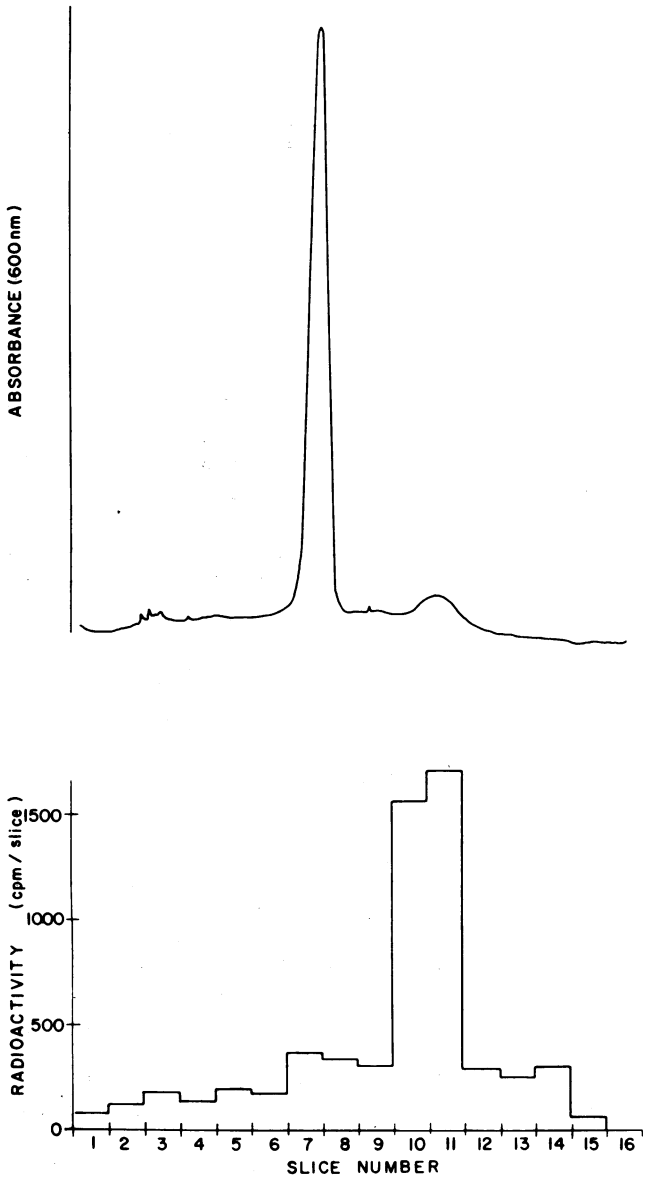

FIG. 5. [^{14}C]DCCD labeling of the proteolipid-containing liposomes. Liposome formation and bacteriorhodopsin incorporation were performed as described in *Materials and Methods*, with the butanol supernatant containing approximately 50 μg of proteolipid protein. Upon incubation (room temperature, 1 hr) with [^{14}C]DCCD (40 μM), the liposome suspension was solubilized (2% sodium dodecyl sulfate, 2% mercaptoethanol) and electrophoresed as described in Fig. 1.

chloroplast, the proteolipid resides within the membrane bilayer. Therefore, its native conformation might depend on its being surrounded by an apolar environment. Thus, during isolation, exposure to polar solvents should be minimized. Butanol is a weakly protic solvent that might accomplish this requirement. This is in contrast to the usual water/methanol/chloroform previously utilized for proteolipid isolation. Furthermore, the procedure utilized is rapid and therefore minimizes the time of exposure to the solvents.

One important question that is not resolved is the role of the other polypeptides reported to be a part of F_0 (1–4). Also it is not known how the F_0 is coupled to the F_1 not only structurally but in its role of synthesizing ATP. One possible approach to examine such coupling would be to use the fluorescence enhancements of bound ethidium (22), which appears to interact selectively with the DCCD-binding proteolipid (20).

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