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STRUCTURAL ANALYSIS OF THE ISOLATED CHLOROPLAST COUPLING FACTOR AND THE *N,N'*-DICYCLOHEXYLCARBODIIMIDE BINDING PROTEOLIPID

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Summary

Negative staining of purified spinach dicyclohexylcarbodiimide (DCCD) sensitive ATPase revealed a population of 110 Å subunits attached by stalks to short string-like aggregates. The interpretation of these data is that 110 Å CF_1 are attached by stalks to an aggregate of CF_0.

The CF_1-CF_0 complex was incorporated into phospholipid vesicles; freeze-fracture analysis of this preparation revealed a homogeneous population of particles spanning the lipid bilayer; these averaged 96 Å in diameter. The DCCD binding proteolipid (apparent molecular weight 7500), an integral component of CF_0, was isolated from membranes by butanol extraction and was incorporated into phospholipid vesicles. Freeze-fracture analysis of the DCCD-binding proteolipid/vesicle preparation revealed a population of particles averaging 83 Å in diameter suggesting that the DCCD-binding proteolipid self-associates in lipid to form a stable complex. This complex may be required for proton transport across chloroplast membranes in vivo. The size difference between CF_0 and DCCD-proteolipid freeze-fracture particles may be related to differences in polypeptide composition of the two complexes.

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Abbreviations: CF_1, hydrophilic component of the ATPase; CF_0, hydrophobic component of the ATPase; DCCD, *N,N'*-dicyclohexylcarbodiimide; EF and PF face, inner and outer half-membrane leaflets, respectively, revealed during freeze-fracture of chloroplast thylakoid membranes; SDS, sodium dodecyl sulfate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

Freeze-fracture analysis of thylakoid membranes reveals 100—180 Å particles on the EF fracture face and 80—115 Å particles on the PF fracture face [1]. It has been suggested that the ratio and composition of these particles is (EF : PF; 1 : 3) where EF particles correspond to Photosystem II and PF particles correspond to Photosystem I, cytochrome $b_6 f$ complexes and the hydrophobic portion of the coupling factor identified as CF_0 [1]. Several lines of evidence are available supporting the correlation between EF particles and Photosystem II [2—4]. In contrast, identification of PF particles is more difficult due to the lack of specific structural markers.

One approach which can be used to identify the PF particles involves purification of each tenative PF complex, and reconstitution in lipid vesicles with subsequent freeze-fracture analysis. Recently, this approach has been used to examine the structure of Photosystem I [5]. This analysis revealed particles averaging 107 Å in diameter, consistent with the location of Photosystem I on the PF fracture face. In this paper, freeze-fracture of lipid vesicles containing a second tentative PF particle, CF_0, is reported.

DCCD-sensitive ATPase isolated from a variety of sources may be functionally reconstituted in lipid vesicle preparations [6—8]. These complexes are comprised of a hydrophilic component (CF_1) which catalyzes the hydrolysis of ATP, and a hydrophobic component (CF_0) which serves as a proton channel and is the site of DCCD inhibition. Pick and Racker [9] have reported the isolation of an ATPase complex which contained the five-subunit CF_1, the DCCD-binding proteolipid, plus three additional polypeptides of 15.5, 17.5 and 13.5 kdaltons. The identity of the 15.5, 17.5 and 13.5 kdalton polypeptides was not determined although it was pointed out that CF_0 found in yeast and *Neurospora* mitochondria has been reported to consist of one to two polypeptides in addition to the inhibitor-binding proteolipid [10].

In the present work, a comparison of the structure of isolated DCCD-binding proteolipid and purified CF_1-CF_0 complexes was conducted in order to gain insight into the structural organization of these complexes and to relate their organization to protein complexes observed in intact chloroplast thylakoid membranes.

Materials and Methods

ATPase was isolated from spinach as previously described except that phospholipids were not included in the sucrose gradients during separation of the ATPase complexes [9]. ATPase complexes were incorporated into soybean phospholipid vesicles (Type II-S, Sigma Chemical Co.) as previously described [9]. Partial separation of CF_1 and CF_0 was obtained by treatment of 2 ml (3 mg protein) of the isolated ATPase complex with 0.75 mM EDTA, pH 7.8, 25 mM dithiothreitol for 4 h. The preparation was then loaded on an 18 ml 0.1—1.0 M sucrose gradient containing 25 mM dithiothreitol, 10 mM Tricine-NaOH, pH 7.8. The gradients were centrifuged for 5 h at 100 000 × g, at 4° C, in a Beckman SW-27 swinging-bucket rotor. After centrifugation, the gradients were divided into 2-ml fractions and analyzed for protein by measuring the

absorbance at 280 nm and by negative staining.

The DCCD-binding proteolipid was isolated according to the method of Nelson et al. [11]. The precipitated proteolipid was dried under a stream of N_2 until all traces of butanol were removed. The proteolipid was incorporated into phospholipid vesicles by a freeze-thaw technique as described for the ATPase complex [9]. Negative staining was performed for 1 min in 2% phosphotungstic acid followed by three washes with H_2O.

Freeze-fracture methods [12] and gel electrophoresis techniques [5] have been described previously.

Results and Discussion

A. Polypeptides of CF_1-CF_0

A DCCD-sensitive ATPase complex and the DCCD-binding proteolipid were isolated in order to examine their polypeptide composition and structural characteristics in the absence of other integral chloroplast membrane complexes. Evidence for the purity of the preparations used in these studies is presented in Fig. 1. Based on earlier analyses, our preparation consisted of five polypeptides (58, 55, 37, 18 and 13.5 kdaltons) associated with CF_1 [9], the DCCD-proteolipid of 7.5 kdaltons [11] and two polypeptides of unknown function (16 and 12.5 kdaltons). The polypeptide composition of CF_1-CF_0 shown in Fig. 1 is identical to that reported by Pick and Racker [9] with the exception of the polypeptides of 12.5 kdaltons which we observe in our gel separations. This polypeptide may have comigrated with that of 13.5 kdaltons in the previous report [9]. The 7.5 kdalton polypeptide in the ATPase complex comigrated with purified DCCD-binding proteolipid. This polypeptide stained poorly in our gels and was only observed in overloaded gels. The purified DCCD-binding proteolipid ran as a single polypeptide band of 7.5 kdaltons as shown in Fig. 1.

B. Structure of the ATPase complex

Negatively stained ATPase preparations, when examined by electron microscopy, contained a mixture of free subunits (see inset for details) and branched, string-like structures (Fig. 2). The free 110 Å subunits correspond in size and shape (hollow centers) to previously described, purified CF_1 [13—15]. The string-like structures (60—100 Å in width) appeared to be the basal attachment unit of other 100—120 Å subunits of size and shape identical to those of the free 'CF_1'; at high magnification stalks (20—30 Å in length) appear to link the 110 Å subunits to the string-like structures; similar structures have been observed to link CF_1 to thylakoid membranes [16].

To clarify the nature of the 'strings' which form the site of CF_1 attachment, the ATPase complexes were reconstituted into phospholipid vesicles and subsequently examined by thin-section electron microscopy. The sectioned preparations were stained with phosphotungstic acid and uranyl acetate [14] to enhance visualization of CF_1 subunits. Surface-exposed subunits observed in the thin-sectioned material were similar in size and shape to previously reported CF_1 complexes of intact membranes [13]. To analyze the hydrophobic portion of the ATPase complex, freeze-fracture analysis of the ATPase/vesicle prepara-

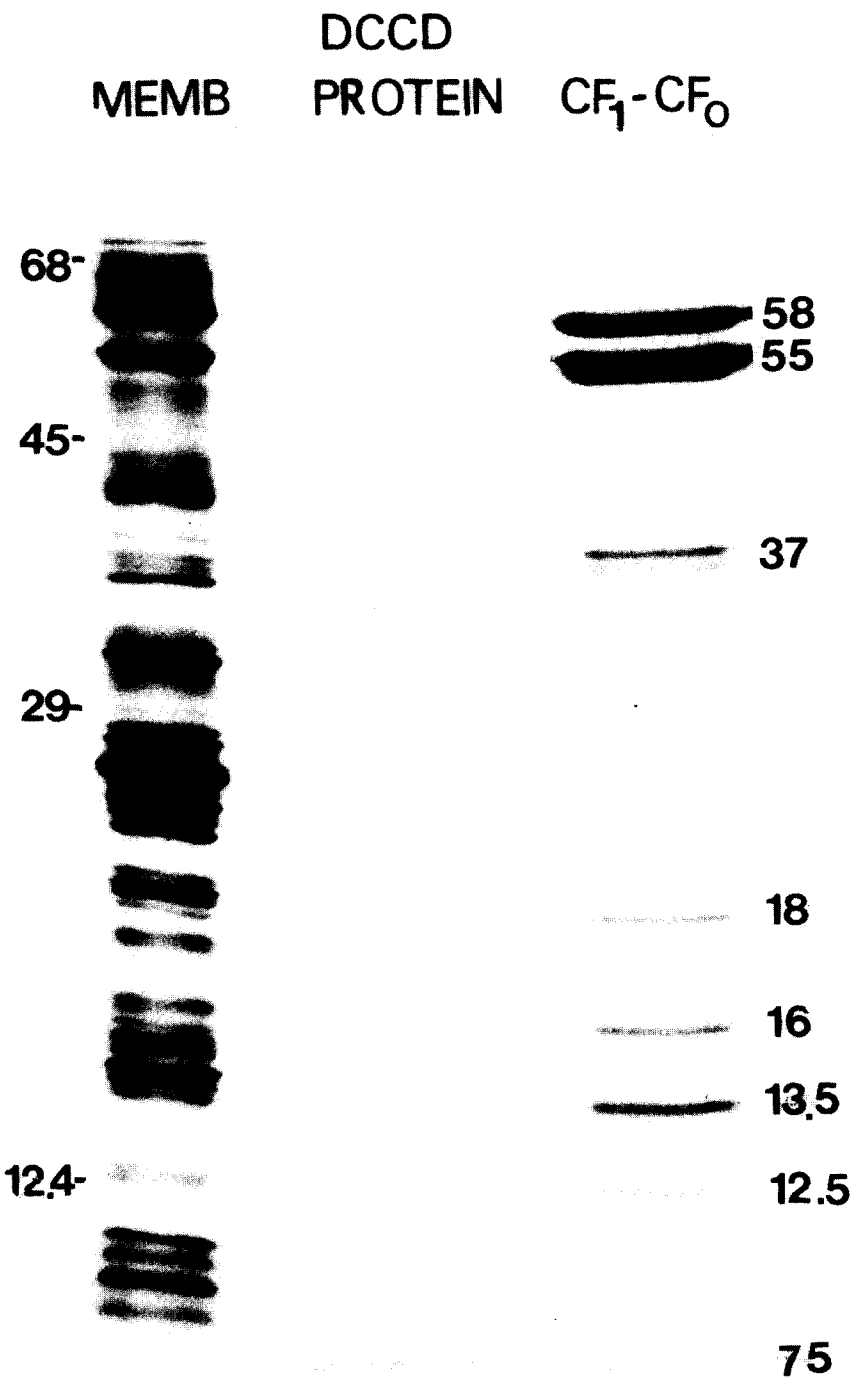

Fig. 1. SDS-polyacrylamide slab gel (7.5—15%) separation of spinach thylakoid membrane polypeptides (MEMB), the DCCD-binding proteolipid (DCCD PROTEIN), and the ATPase complex (CF_1-CF_0). Apparent molecular weights (in parentheses) were determined using the following standards: bovine serum albumin (68 000), ovalbumin (45 000) carbonic anhydrase (29 000), and cytochrome *c* (12 400). The figures next to the gel profiles refer to molecular mass in kdaltons.

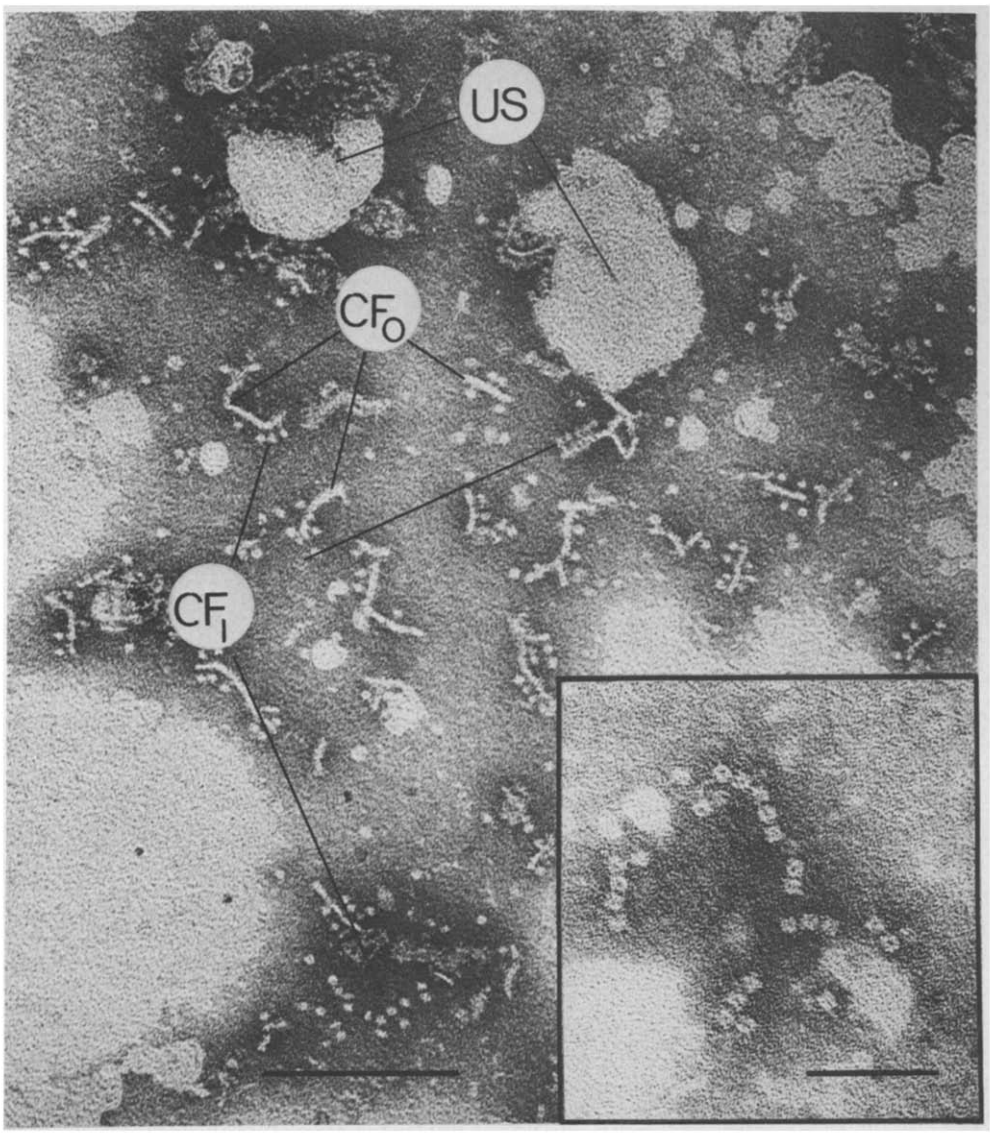

Fig. 2. Electron micrograph of the isolated ATPase revealed by negative staining. Bar = 2.0 μM. The following structures are labeled: CF_0, basal lipophilic portion of the ATPase (string-like aggregates of CF_0). CF_1, hydrophilic subunit of the ATPase observed attached to CF_0 aggregates or in a separated state (see inset). US, unidentified negative staining structure. An enlarged view of the CF_1 subunits in a separated state is shown in the inset. The hollow nature of these 110 Å structures is apparent. Inset bar = 0.1 μM.

tion was conducted. The data (Fig. 3) revealed a population of hydrophobic particles, 96 Å in diameter, which were randomly dispersed in the lipid bilayer. The intramembrane complexes shown in Fig. 3 are interpreted as arising from a hydrophobic subunit which is present in the ATPase complex but not part of CF_1. This interpretation is based on studies which show removal of CF_1 from thylakoid membranes without disruption of the lipid bilayer [17].

Based on the results presented above, it is proposed that the isolated ATPase

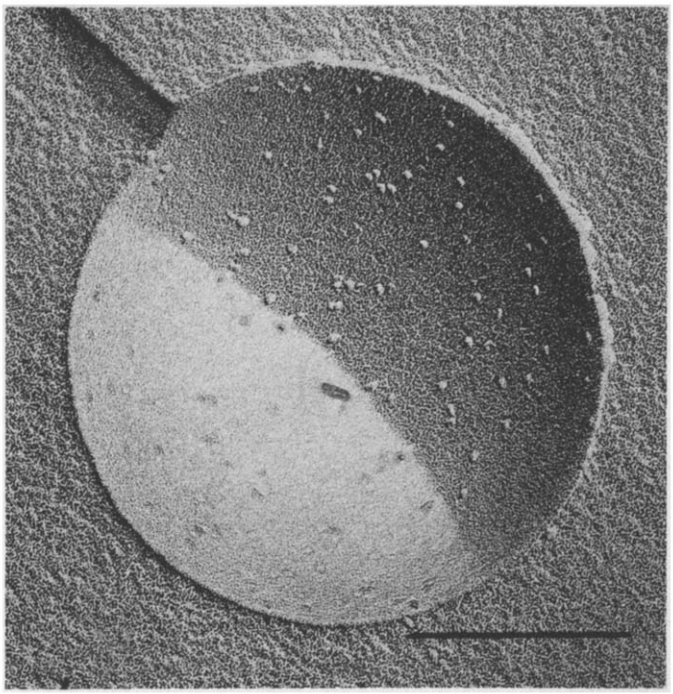

Fig. 3 Electron micrograph of a freeze-fractured ATPase/phospholipid vesicle preparation. ATPase particles averaged 96 Å in diameter. Bar = 0.25 μM.

is composed of CF_1 attached by a stalk to a string-like aggregate of CF_0. The CF_0 probably aggregates when extracted from native membranes due to interaction between hydrophobic regions found on each CF_0 subunit. Incorporation of the string-like aggregates into a lipid bilayer may reduce hydrophobic bonding between the subunits, thus allowing them to disperse randomly in the vesicle bilayer.

The freeze-fracture results shown in Fig. 3 also provide direct structural evidence for the presence of CF_0, in addition to CF_1, in the ATPase preparations. The size and freeze-fracture characteristics of the putative CF_0 particles indicate that these complexes span the lipid bilayer. This result is consistent with the proton translocation function of CF_0 and its capacity to bind CF_1 on the external thylakoid membrane surface. The ATPase preparation examined in this study has previously been reported to contain functional activity associated with both CF_1 and CF_0 [9].

In order to gain additional evidence for the identification of the CF_1 and CF_0 structures, a separation between CF_1 and the proposed CF_0 aggregates was attempted by means of a standard technique used to separate CF_1 from chloroplast membranes [9,16]. After treatment, the preparation was centrifuged on sucrose gradients and aliquots were examined for protein content and by negative staining. As can be seen in Fig. 4, fractions which were enriched in CF_1 (Fig. 4A) (top three fractions of the gradients) contained the free structural complexes of 120 Å diameter. In contrast, fraction 8 contained string-like aggregates identified as CF_0 (Fig. 4B). We conclude that the CF_0 aggregates,

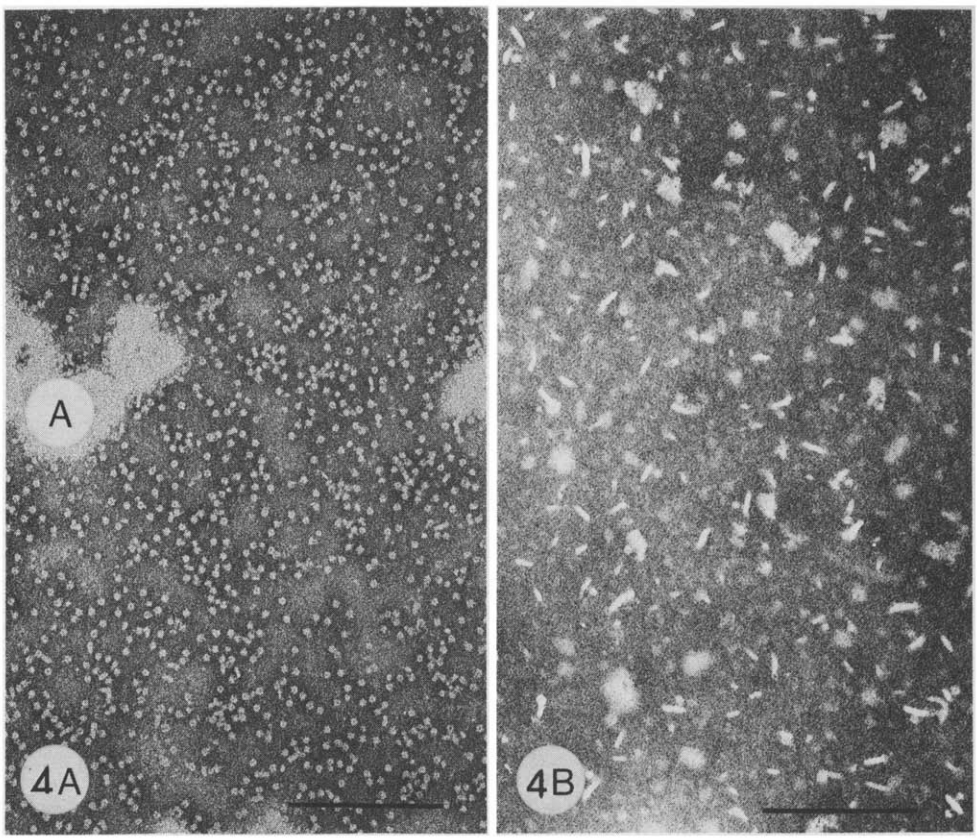

Fig. 4. (A) Electron micrograph of negatively stained CF_1 which was separated from the ATPase complex. The 110 Å structures were occasionally clumped to large aggregates (labelled A). (B) Electron micrograph of negatively stained CF_0 string-like aggregates derived from the ATPase complex. Bars = 0.2 μM.

which bind CF_1 reversibly, form strings when isolated due to an edge-to-edge hydrophobic interaction, but separate into discrete structural subunits in a lipid bilayer.

C. Structure of purified DCCD proteolipid

The existence of a hydrophobic proton channel, which spans the lipid bilayer and functions in vivo to bind CF_1, has been proposed on the basis of activity and inhibitor studies [6—11]. These studies have provided evidence that a 7.5 kdalton proteolipid forms a DCCD-sensitive proton channel when reconstituted into lipid vesicles [11]. In order to examine the structure of the proposed proton channel, the DCCD-binding proteolipid was isolated, reconstituted into lipid vesicles and examined by freeze-fracture techniques. The reconstituted DCCD-binding proteolipid vesicle preparation, shown in Fig. 5, was found to contain a homogeneous population of particles averaging 83 Å in diameter. The existence of 83 Å particles in this preparation suggests that the DCCD-binding proteolipid self-associated to form hydrophobic complexes. We should note that examination of control phospholipid vesicles, with no protein

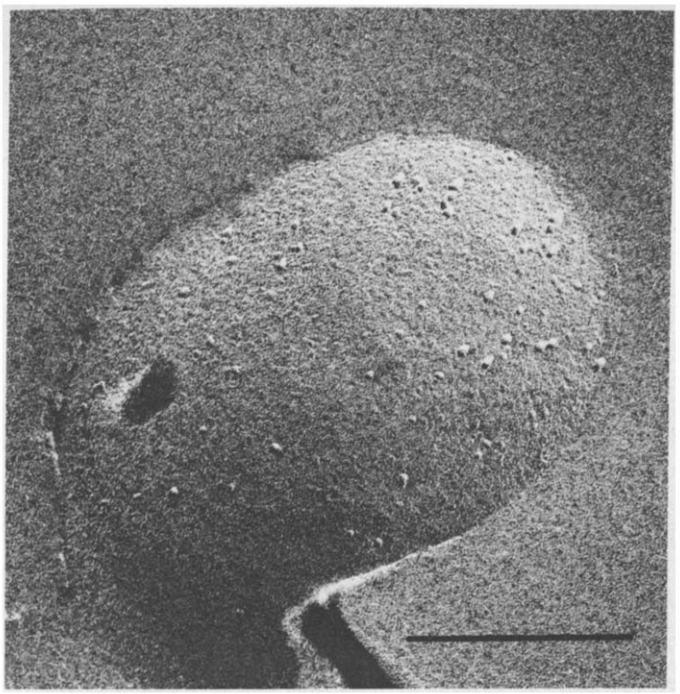

Fig. 5. Electron micrograph of a freeze-fractured DCCD-binding proteolipid/vesicle preparation. Particles observed in this preparation averaged 83 Å in diameter. Bar = 0.25 μM.

reconstituted, revealed smooth fracture faces. The exact number of polypeptides per DCCD-binding proteolipid complex may not be determined from the structural evidence provided herein. However, based on standard protein density values (1.37 [19]), measured particle size (83—60 Å diameter; the 60 Å value is a lower limit to correct for possible size overestimation due to platinum replication [20]) and assuming the particle spans a 60 Å lipid bilayer, it can be calculated that four to seven DCCD-binding proteolipids may be associated in each complex. In chloroplasts, five DCCD-binding proteolipids have been reported per CF_1 [16]. It is therefore possible that the DCCD-binding proteolipid freeze-fracture particles represent the hydrophobic core of CF_0. The difference in size between CF_0 particles found in the ATPase complex (Fig. 3) and proteolipid particles (Fig. 5) may be due to compositional differences between CF_0 and the DCCD-binding proteolipid complex; the former is thought to contain two additional polypeptides (see section A).

D. Relationship of structural analysis of CF_0 to chloroplast membrane structure

We have demonstrated that lipid vesicles containing either the self-associated DCCD-binding proteolipid or reconstituted ATPase contain particulate substructures (protein aggregates) of approx. 80—95 Å diameter. These are of the same size class as particles observed on the PF face of freeze-fractured chloroplast membranes. The data are consistent with an earlier membrane model [1] in which it was indicated that a portion of the subunits of the PF face are the hydrophobic portion of the coupling factor.

Acknowledgements

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