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Identification of a proteolipid oligomer as a constituent part of CF_0, the proton channel of the chloroplast ATP synthase

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We studied the action of the photophosphorylation inhibitor, *N,N'*-dicyclohexylcarbodiimide (DCCD) on the channel portion (CF_0) of the chloroplast ATP synthase (CF_0CF_1). We found that the target for binding of [^{14}C]DCCD was an oligomer of the small proteolipid-subunit (subunit III) of CF_0. We treated thylakoids with low concentrations of DCCD, sufficient to inhibit photophosphorylation. The ^{14}C-labelled inhibitor was found on polyacrylamide gels in a position corresponding to an apparent molecular mass of 50 kDa. This band comprised a homooligomer of proteolipid subunits of CF_0. At higher concentrations of DCCD, it fell apart into proteolipid monomers. This dissociation was prevented by the presence of venturicidin, another CF_0 inhibitor acting on the proteolipid subunit, during the incubation with DCCD. The existence of such an oligomeric substructure in CF_0 is discussed in the light of diverging structural models for the proton channel of F_0F_1-type ATPases.

Photosynthesis; Photophosphorylation; Proteolipid; ATP synthase; Proton channel; CF_0CF_1

1. INTRODUCTION

In photophosphorylation and oxidative phosphorylation, ATP is formed at the expense of the free energy stored in a transmembrane potential difference of the proton [1]. Enzymes of the F_0F_1 type, which translocate protons and generate ATP from ADP and P_i, have been highly conserved during evolution. They consist of two parts, F_1, a membrane extrinsic protein, and the membrane-spanning F_0. Whereas F_1 contains the catalytic sites of ATP synthesis and hydrolysis, F_0 is a proton-conducting device (reviews [2,3]).

In bacterial F_0, three different subunits have been identified and named a–c in order of decreasing molecular mass. Their amino acid sequences are known and predictions of their secondary and tertiary structures have been attempted [4]. A molar ratio of a : b : c of 1 : 2 : 10 has been proposed for EF_0 from *E. coli* [5,6]. Due to its hydrophobicity, the small subunit c (molecular mass around 8 kDa depending on the source [2]) has been classified as a proteolipid. MF_0 from mitochondria and CF_0 from chloroplasts contain a homologous proteolipid which is named subunit III in CF_0. In CF_0 and MF_0, however, the stoichiometry of the proteolipid has been reported to be six copies per F_0 rather than ten [7,8]. The proper correspondence of the other subunits of CF_0 to those in EF_0 has been debated. Recently, Henning and Herrmann [9] and Cozens et al. [10] from genetic evidence have proposed the existence of a fourth subunit in CF_0, homologous to the bacterial subunit a. Fromme et al. [11] established this gene product as a constituent part of CF_0, subunit IV. Subunit I in CF_0 is assumed to be related to subunit b in EF_0, and subunit II of CF_0 apparently has no counterpart in the *E. coli* enzyme.

We studied the action of an inhibitor of photo-

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Abbreviations: CF_1, chloroplast ATP synthase, soluble portion; CF_0, chloroplast ATP synthase, membrane portion; DCCD, *N,N'*-dicyclohexylcarbodiimide; PMS, phenazine-methosulfate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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phosphorylation, DCCD, on CF_0. On SDS-PAGE, a proteolipid oligomer was identified as the target of inhibitor binding. This oligomer was labelled by [^{14}C]DCCD at concentrations corresponding to those which inhibited ATP synthesis by CF_0CF_1. Higher concentrations of DCCD resulted in the disintegration of this oligomer on SDS-PAGE and enhanced the occurrence of proteolipid monomers.

2. MATERIALS AND METHODS

Broken pea chloroplasts (thylakoids) were prepared from 10–14-day-old plants [12]. SDS-PAGE was carried out with a discontinuous buffer system and 10% acrylamide in the separating gel as in [13] and gels were silver stained according to [14]. Western blot analysis was done after Howe and Hershey [15], with rabbit anti-CF_1-antiserum as first antibody and peroxidase-coupled anti rabbit IgG as second antibody. The ATp synthase activity of thylakoids was measured as described [16]. DCCD was added from ethanolic stock solution and incubated for 10 min in the dark prior to measurement. The ethanol concentration was always held below 0.5% in order to avoid unspecific damage of thylakoid membranes. DCCD was obtained from Sigma, venturicidin from BDH, and [^{14}C]DCCD from Amersham. The radioactive compound was delivered in toluene, which was evaporated and exchanged for ethanol prior to use. The luciferin/luciferase assay was obtained from LKB. All other chemicals were from either Merck or Sigma and of the highest grade available.

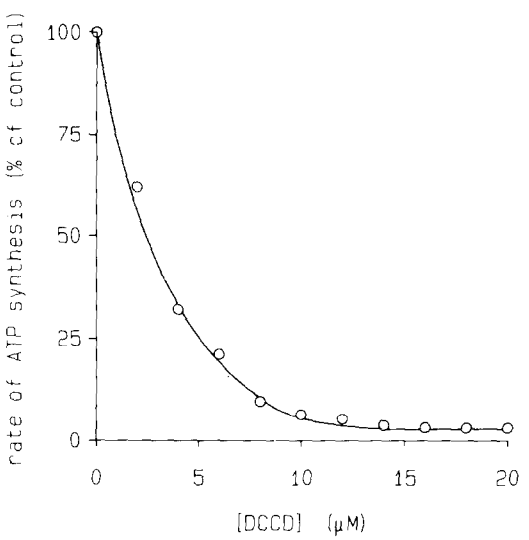

Fig.1. Inhibition of photophosphorylation by DCCD. Thylakoids were incubated for 10 min in the dark and in the presence of the indicated concentrations of DCCD. ATP synthesis was measured as in [16].

3. RESULTS

Fig.1 shows the concentration dependence of the inhibition of photophosphorylation by DCCD. With 10 μM chlorophyll, ATP synthesis was almost completely abolished by 10 μM DCCD. Thylakoids which had been treated with saturating concentrations of DCCD (20 μM were run on SDS-PAGE and compared with untreated samples (fig.2, lanes

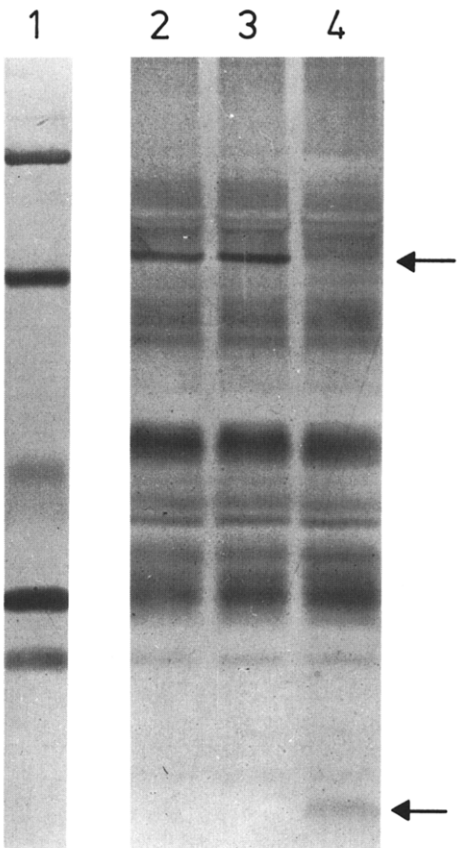

Fig.2. SDS-PAGE of DCCD-treated thylakoids. In lane 1, 1 μg each of bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), myoglobin (17.8 kDa), and cytochrome (12.3 kDa) were run as molecular mass markers. Samples in lanes 2–4 were incubated for 10 min in the dark in 10 ml of 1 mM Tricine-NaOH, pH 7.5, and 10 mM NaCl, with the following additions: (lane 2) 20 μM DCCD, 40 ng/ml venturicidin; (3) control, without additions; (4) 20 μM DCCD. After 20 min centrifugation, thylakoids equivalent to 4 μg chlorophyll were diluted in 100 μl sample buffer. After 2 min heating at 100°C, samples were applied to the gel and electrophoresis was run overnight at 30 V. The arrows indicate protein bands running at 50 and 8 kDa, respectively.

3,4). The DCCD incubation resulted in the disappearance of a band of apparent molecular mass 50 kDa. Instead, a new band appeared at approx. 8 kDa (both marked by arrows in fig.2). This effect was remarkably reduced (lane 2) when the DCCD incubation was carried out in the presence of 40 μg venturicidin per μg chlorophyll (which was also saturating in the inhibition of photophosphorylation; not shown).

We incubated thylakoids with [^{14}C]DCCD over the concentration range which inhibited photophosphorylation (cf. fig.1). The samples were subjected to SDS-PAGE and the radioactivity was monitored by autoradiography. As demonstrated in fig.3 (left), besides several chlorophyll proteins in the 20 kDa range and little ^{14}C in α and β of CF_1, two bands at 50 and 8 kDa (cf. fig.2) were labelled by [^{14}C]DCCD. The incorporation of ^{14}C into the 50 kDa band increased at low DCCD concentrations. A quantitative evaluation of the counts incorporated into this band showed that the bound inhibitor molecules never exceeded a ratio of 1 per CF_0CF_1 (maximal 0.67 mol DCCD per mol CF_0CF_1 at 5 μM DCCD). At further increased concentrations of DCCD, the 50 kDa band disappeared as already seen on the silver-stained gel, and instead radioactivity accumulated in the 8 kDa band. After transfer onto nitrocellulose, a Western blot analysis with a polyclonal antiserum directed against spinach proteolipid showed that the two bands contained the proteolipid subunit of CF_0 (fig.3, right).

CF_0CF_1 was prepared according to Schmidt and Gräber [17]. The 50 kDa band was apparent in this preparation (fig.4, lane 1). This suggested that the proteolipid oligomer was a constituent part of the chloroplast ATP synthase. The respective region of the gel was excised, and the protein was electroeluted and re-electrophoresed. As shown in fig.4 (lane 3), the oligomeric structure remained intact during this procedure. After addition of 0.5 M urea to the electroelution buffer however, the 50 kDa band disintegrated and the 8 kDa band representing proteolipid monomers appeared on the gel

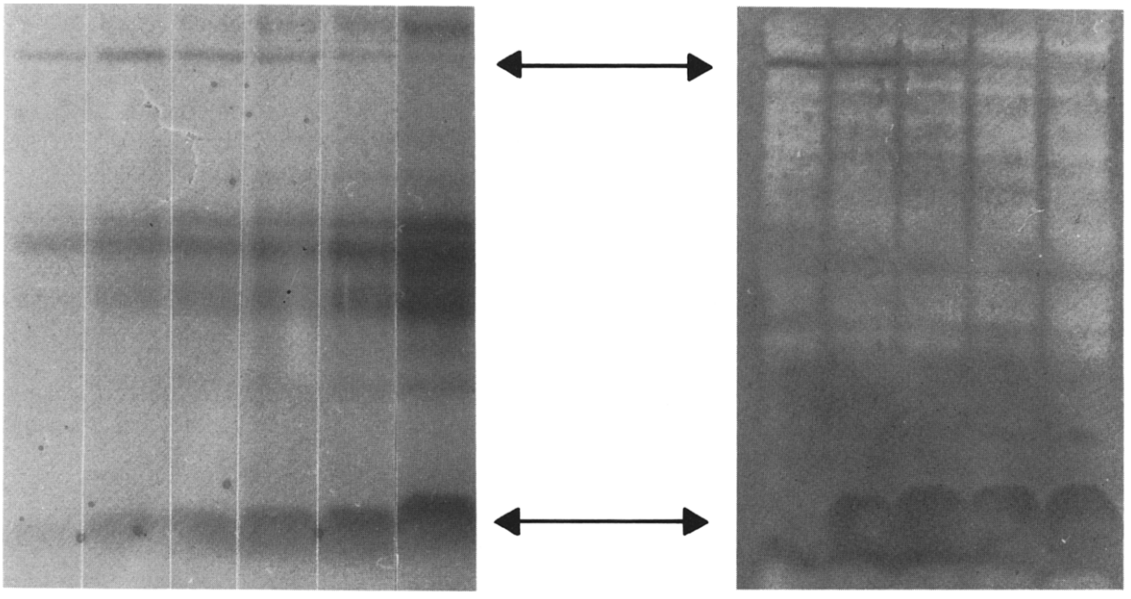

Fig.3. SDS-PAGE of [^{14}C]DCCD labelled thylakoids and Western blot analysis of the DCCD-binding protein bands. In the autoradiogram on the left, [^{14}C]DCCD with a specific activity of 54 mCi/mmol was used. Incubation and sample preparation was carried out as in fig.2 (DCCD concentration from left to right: 2.5, 5, 7.5, 10, 15, and 20 μM), except that 30 μg chlorophyll was loaded into each slot. After the run, the gel was fixed and stained with Coomassie brilliant blue and dried onto a sheet of filter paper. Autoradiography was carried out at room temperature for 72 h on Hyperfilm βmax from Amersham. For the Western blot on the right, 20 μg chlorophyll were loaded into each slot. Thylakoids were incubated with (from left to right) 0, 5, 10, 15, and 20 μM DCCD prior to electrophoresis. Arrows indicate positions corresponding to 50 and 8 kDa as in fig.2.

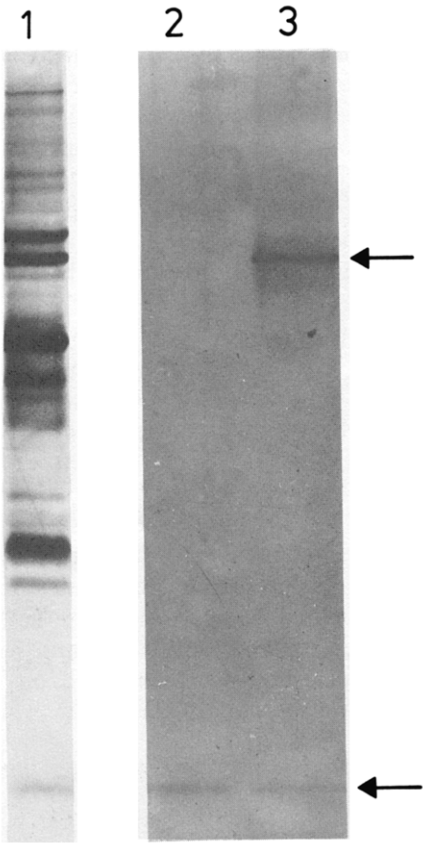

Fig.4. SDS-PAGE of purified CF_0CF_1 and of the electroeluted 50 kDa band. In lane 1, 5 μg CF_0CF_1 were run. Lanes 2,3 show the band at 50 kDa re-electrophoresed after electroelution from a gel identical to that one shown in fig.2, lane 2. The 50 kDa band was electroeluted into the cathode buffer of the electrophoresis system (0.1 M Tris, 0.1 M Tricine, and 0.1% (w/v) SDS), except that in lane 2, additional 0.5 M urea was present. 50 μl of the respective eluate was mixed with 100 μl sample buffer and electrophoresis was carried out as in fig.2.

(fig.4, lane 2). The absence of any other band in this lane suggested that the 50 kDa band was a homooligomeric assembly of proteolipid subunits.

4. DISCUSSION

We examined the action of DCCD, a photophosphorylation inhibitor, on CF_0, the proton channel of the chloroplast ATP synthase. SDS gel electrophoresis of thylakoid membranes treated with DCCD in the concentration range which inhibited ATP synthesis showed that a 50 kDa oligomer of the proteolipid subunit (subunit III) of the channel was the target for DCCD binding. DCCD was introduced for the quantification of carboxylic acid groups in proteins [18]. It reacts covalently with a single glutamic acid residue in the proteolipid of CF_0 [7], thereby blocking the proton channel. Upon reaction with more than one molecule of DCCD the 50 kDa band disappeared from the gels in favour of the appearance of proteolipid monomers. The dissociation required covalent binding of DCCD, since it did not occur with other CF_0-directed photophosphorylation inhibitors, namely venturicidin and triphenyltinchloride (not shown; see [19] for a description of the inhibitors). On the other hand, inhibition of ATP synthesis did not require the destruction of the oligomer, since it occurred at DCCD concentrations which only labelled the oligomer instead of destroying it. Moreover, other inhibitors like venturicidin did not exhibit any similar effect. On the contrary, venturicidin protected the oligomer from DCCD-induced disintegration. In the proteolipid subunit c of *E. coli*, the binding site for venturicidin overlaps with that for DCCD, as shown by genetical mapping of resistant mutants [20]. Probably, the sheltering effect of venturicidin was indicative of a similar topography for the respective binding sites in the chloroplast enzyme.

The 50 kDa band contained exclusively proteolipid subunits of CF_0, as indicated by re-electrophoresis of the electroeluted and urea-denatured protein, which produced only one band at 8 kDa. Its apparent molecular mass of 50 kDa suggested a stoichiometry of six proteolipid subunits per oligomer, each contributing approx. 8 kDa. This would be in line with the results of Sigrist-Nelson et al. [7] who found that 1 mol DCCD per 6 mol proteolipid abolished the light-induced ATPase activity of chloroplasts and concluded that six copies of the proteolipid were present in CF_0. Also, in MF_0, six copies of the proteolipid have been reported [8]. These appear to form a similar oligomer [21], which was recently studied by use of monoclonal antibodies directed against the mitochondrial proteolipid [22]. For the sodium-translocating ATP synthase of *P. modestum*, Laubinger and Dimroth [29] found a homooligomer of the proteolipid with an apparent molecular mass of 45 kDa, possibly a hexamer. A larger oligomer was only reported by Fromme et al. [23] who proposed that an aggregate of 12 proteolipid monomers was present in pre-

parations of CF_0CF_1 in solution. We also found that the oligomer revealed higher apparent molecular mass when the electroeluted band was run on gels prepared after Laemmli [24] as in [23] instead of a run over the discontinuous gel system of Schägger and Von Jagow [13] which was used in this work. Therefore, we cannot exclude the possibility of a misinterpretation of its molecular mass. That the oligomer was generated during electrophoresis seems highly unlikely. This was expected to generate aggregates of various sizes which resulted in several bands in SDS-PAGE. Moreover, the urea-treated and dissociated sample used in fig.4 did not reaggregate on the gel.

The proteolipid oligomer was stable even in the presence of SDS. This is highly interesting in the light of current concepts of the structure and function of F_0-type proton channels (see, e.g. [25–27]). In recent models, the rotation of a ring of proteolipid molecules was assumed relative to one or more of the other subunits of F_0. The proton-conducting pathway would then exist at the interface between one proteolipid and another subunit of CF_0. As the DCCD-binding residue seems to be intimately involved in the conduction process (review [2]), its physical blockage by the binding of DCCD could interrupt the proton-conducting pathway [28]. Another reasonable possibility would be the restriction by sterical hindrance of the rotational movement of the oligomer after the binding of an inhibitor molecule.

The cited references disagree as to whether the proteolipids enwrap the transmembrane helices of the other subunits (e.g. [25]), or form an aggregate by themselves which interacts side by side with other subunits [26]. In this respect, the number of proteolipid monomers in the oligomeric structure (6 or 12) is of great importance. It is also important to ascertain whether the disintegration of the oligomeric structure upon binding of DCCD already occurs in the membrane. Initial results from our laboratory point towards a more peripheral attachment of the proteolipid oligomer to the entire ATP synthase complex. Labelling by eosin isothiocyanate of purified CF_0CF_1 incorporated into asolectin vesicles [17] produced proteolipid monomers which probably diffused freely in the membrane (Wagner, R. and Apley, E., personal communication). However, by immunoelectrophoresis [12] we found that the disintegration of the oligomer by eosin isothiocyanate binding did not lead to any loss of CF_1 from thylakoid membranes (not shown).

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