Isolation and Characterization of a Supramolecular Complex of Subunit III of the ATP-Synthase from Chloroplasts

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Dedicated to Konrad G. Weil on the occasion of his 60th birthday

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In SDS gels of purified, highly active ATP-synthase from chloroplasts, CF_0F_1, a protein band was detected at an apparent molecular weight of 100 kDa. This protein was isolated on a preparative SDS gel. The 100 kDa protein can be dissociated at increased temperature or increased incubation time into an 8 kDa protein, which is identical with the subunit III of CF_0 (DCCD-binding protein or proteolipid). This implies that the 100 kDa band is a stable supramolecular complex containing at least 12 copies of subunit III. Electron micrographs reveal a diameter of 6.3 nm and a membrane spanning length of 6.1 nm. We assume that this supramolecular complex represents a stable native substructure of CF_0F_1.

Introduction

The membrane-bound ATP-synthase from chloroplasts, CF_0F_1, catalyzes ATP synthesis/hydrolysis coupled with a transmembrane proton transport. Like other ATP-synthases of the F_0F_1 type, it has a hydrophilic part, CF_1, containing the nucleotide-binding sites and a hydrophobic part, CF_0, which is supposed to act as a proton channel. CF_0 contains four different subunits with the apparent molecular weights of 18 kDa (I), 16 kDa (II), 8 kDa (III), and 20 kDa (IV) [1–6].

During the last years CF_0F_1 was isolated, purified and reconstituted into asolectin liposomes; these preparations show high rates of ATP synthesis (200 s^{-1} [7]) and ATP hydrolysis (20 s^{-1} [8]); *i.e.*, the enzyme has practically the same activity as in the thylakoid membrane.

In this work we have improved the purification of CF_0F_1 by a modification of the sucrose-density gradient centrifugation step. A supramolecular complex of the subunit III with a molecular weight of 100 kDa has been isolated from CF_0F_1, and we assume that this oligomeric structure represents a part of the native CF_0 moiety of the thylakoid membrane.

Abbreviations: CF_0F_1, proton translocating ATP-synthase from chloroplasts; SDS, sodium dodecylsulfate; DCCD, dicyclohexylcarbodiimide; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase.

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Materials and Methods

CF_0F_1 was isolated following the protocol in ref. [9] up to the ammonium sulfate precipitation step. Since the final product always contained some Rubisco, the density gradient centrifugation step of the purification procedure was modified as follows: The ammonium sulfate precipitation (35–45% saturation) was dissolved in 0.2 M sucrose, 20 mM Tricine-NaOH (pH = 8.0), 5 mM $MgCl_2$, 0.2 mM ATP and diluted threefold with the gradient medium before layered on top of the sucrose density gradient. After optimization, a discontinuous gradient with five steps was used with 12%, 18%, 24%, 30% and 40% (w/v) sucrose (0.9 ml each) in the gradient medium containing 30 mM Tris-succinate (pH = 6.5), 0.5 mM EDTA, 0.2% (w/v) Triton X-100, 0.2 mM ATP and 1 mg/ml asolectin. In both solutions ATP can be omitted without changing the enzyme activity. Centrifugation was carried out in a SW 55 rotor at 45,000 rpm (150,000 × *g*) in an ultracentrifuge (L 80, Beckman) for 16 h at 4 °C. After centrifugation, 300 µl fractions were taken from the top of the tube and analyzed for protein concentration and polypeptide composition.

For SDS-gel electrophoresis samples of 20 µg protein were preincubated in an SDS sample buffer containing 62.5 mM Tris-Cl (pH = 7.5), 2% (w/v) SDS,

5% (v/v) glycerol and 10% mercaptoethanol (SDS/protein ratio is 40:1 (w:w)).

SDS-gel electrophoresis of CF_0F_1 was carried out basically as described in ref. [10] and [11]. The stacking gel contains 3.75% acrylamide, the separation gel 15% acrylamid. Staining with Coomassie blue R250 and silver staining were carried out as described in ref. [6]. Protein concentrations were determined according to ref. [12].

For the preparative SDS-gel electrophoresis 3 mg of CF_0F_1 were layered on top of the gel, and electrophoresis was run for 5 h at a constant voltage of 150 V. The protein bands were made visible by incubation of the gel for 10 min in 4 M sodium acetate. The band at 100 kDa was cut out and dialyzed for 12 h against (62 mM Tris-Cl (pH 7.8), 10% (v/v) glycerol and 0.1% (w/v) mercaptoethanol as described in ref. [13]. Electroelution was carried out for 18 h at 20 °C and 7.5 mA. The protein was dialyzed against 1 l of 0.1% (v/w) SDS for 48 h with 5 changes of buffer. The solution was freeze-dried in aliquots. For all dialysis steps and electroelution, benzylated cellulose tubing (cut-off mol. weight 2.0 kDa, Sigma D 7884) was used. The membrane was heated to 100 °C in 0.1 M EDTA for 1 h before use.

For electron microscopy 30 µg of the freeze-dried protein was dissolved in 200 µl water. Specimens were prepared by the droplet method or the Valentine method [14] using 1% uranyl acetate solution as negative stain. Electron microscopy was carried out on a Philips EM 300 microscope at 70,000 magnification.

Results

Fig. 1 shows the result of the sucrose-density gradient centrifugation. On the top, the protein concentration is depicted as a function of the gradient volume. A maximum in the protein concentration is observed in the 30% (w/v) sucrose step, a small peak appears in the 18% step. The lower part of Fig. 1 shows the fractions in the 24%, 30%, and 40% (w/v) sucrose steps after SDS-gel electrophoresis. Before electrophoresis, aliquots of the fractions have been incubated for 10 min at 20 °C in SDS sample buffer.

Nearly all CF_0F_1 is found in the 30% sucrose step, whereas the Rubisco is found only in the 40% sucrose step. For CF_0F_1 the following subunits are observed (see Fig. 1, bottom fractions no. 13, 14 and 15): α, β, γ, δ, ε, I, II and IV [6] and a band at

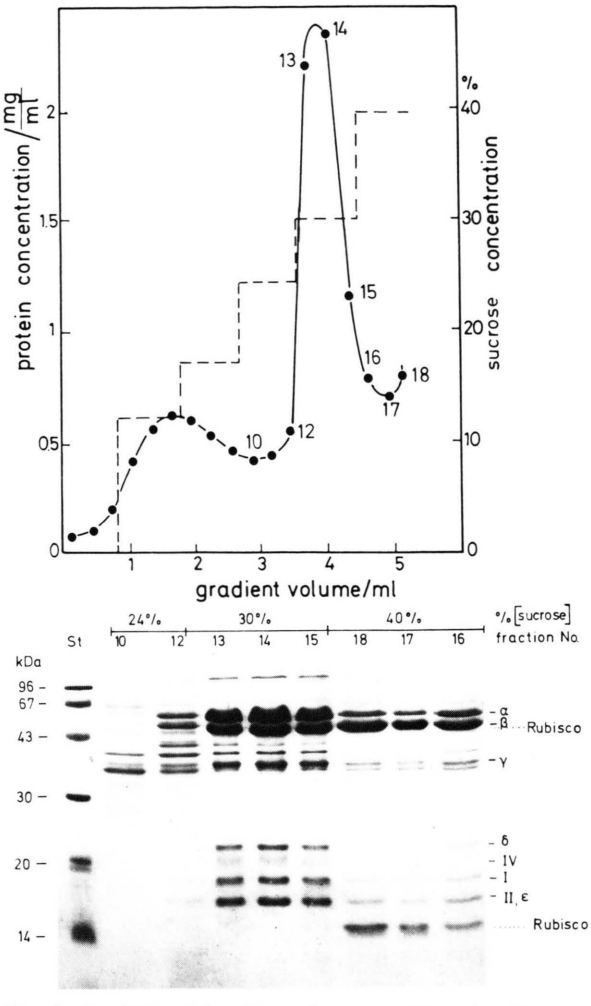

Fig. 1. Analysis of the discontinuous sucrose density gradient centrifugation step of the CF_0F_1 isolation procedure. Top: protein concentration after ultracentrifugation (——), sucrose concentration (– – –). Bottom: SDS-gel electrophoresis of different fractions in the 24%, 30% and 40% sucrose steps; 20 µg protein/fraction. The left lane shows the protein standards.

100 kDa. The two bands between β and γ must be considered to be impurities; they are almost absent if the tubes are punctured from the side so that the 30% sucrose fraction can be removed with a syringe with less contamination from the lighter fractions (*e.g.*, Fig. 3).

The 100 kDa band is present in all our preparations and, therefore, this band has been investigated in detail. Fig. 2 shows densitometer scans of SDS gels after Coomassie Blue staining. For scan no. 1 the sample has been incubated for 20 min in SDS

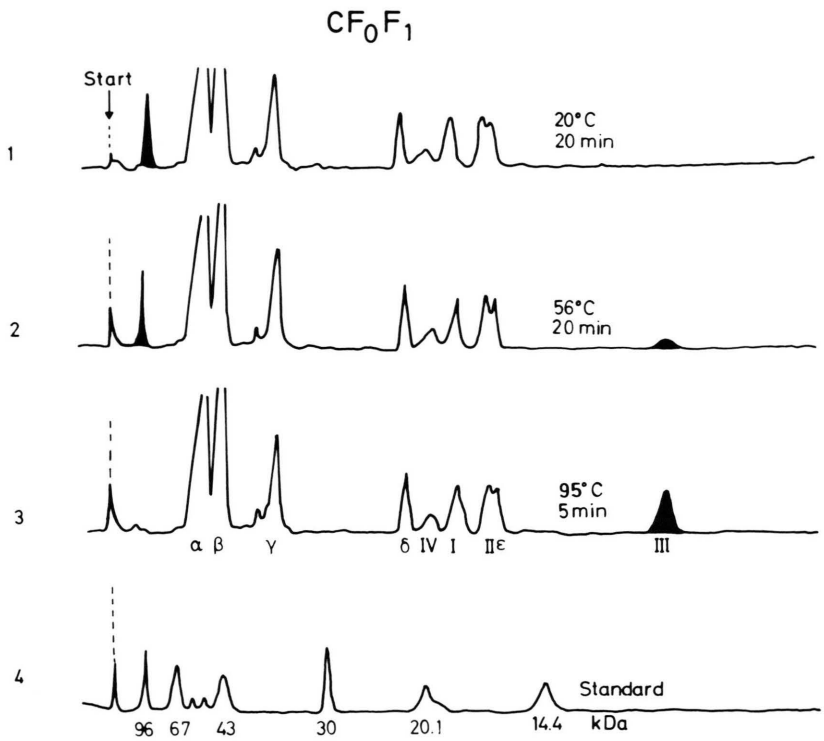

Fig. 2. Densitometer scans of a Coomassie Blue stained SDS gel: CF_0F_1 was preincubated in SDS buffer before electrophoresis: (1) 20 min at 25 °C; (2) 20 min at 56 °C; (3) 5 min at 95 °C; (4) protein standards. The 100 kDa band and the 8 kDa band (subunit III) are marked in black.

sample buffer at room temperature. The 100 kDa band is clearly visible, but subunit III does not show up at its 8 kDa position. If the sample is incubated for 20 min at 56 °C in SDS sample buffer, the 100 kDa band becomes weaker, and a band at 8 kDa appears (scan no. 2). For scan no. 3, the sample was incubated for 5 min at 95 °C in SDS sample buffer. The band at 100 kDa has disappeared completely and the band at 8 kDa has become stronger. Obviously, incubation in SDS buffer at higher temperatures leads to a dissociation of the 100 kDa protein giving rise to monomeric subunit III. However, these results do not exclude that the 100 kDa band contains also other subunits of CF_0F_1 besides subunit III.

Therefore, the 100 kDa band was isolated by preparative SDS gel electrophoresis as described in Materials and Methods. Fig. 3 shows the silver stained SDS gel of the isolated 100 kDa band: lane 4 shows the protein incubated in SDS sample buffer at 20 °C for 15 min. As it can be seen from the silver

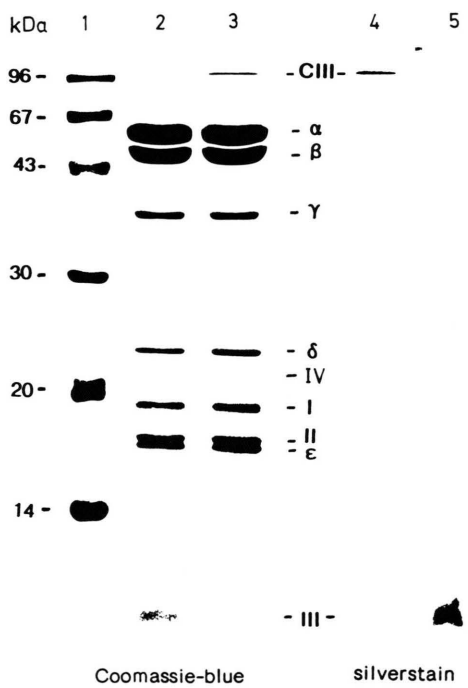

Fig. 3. SDS gel of CF_0F_1 and the isolated 100 kDa protein after different pretreatments in SDS buffer: (1) protein standards, (2) CF_0F_1 incubated 5 min at 95 °C, (3) CF_0F_1 incubated for 20 min at 25 °C, 25 μg protein in lanes 2 and 3, all stained with Coomassie Blue, (4) the isolated 100 kDa protein incubated for 20 min at 25 °C, (5) the isolated 100 kDa protein incubated for 5 min at 95 °C, 8 μg protein in lanes 4 and 5, stained with silver.

stained gel the isolated protein is completely free from impurities. Lane 5 shows the same protein, treated with SDS sample buffer for 5 min at 95 °C before electrophoresis. The protein is dissociated into the 8 kDa band of the subunit III only. For comparison, in lanes 2 and 3 the band pattern of the Coomassie stained SDS gel of CF_0F_1 subjected to the same treatment as the isolated 100 kDa band is presented.

For an unequivocal identification, the isolated 100 kDa protein was subjected to a partial amino acid sequence analysis from the N-terminus similar to the one described for subunit IV of CF_0 [6]. The first 5 amino acid residues determined are identical with the ones for subunit III of CF_0 reported in [15, 16].

Fig. 4 shows the results of electron microscopy of the isolated 100 kDa subunit III complex. The com-

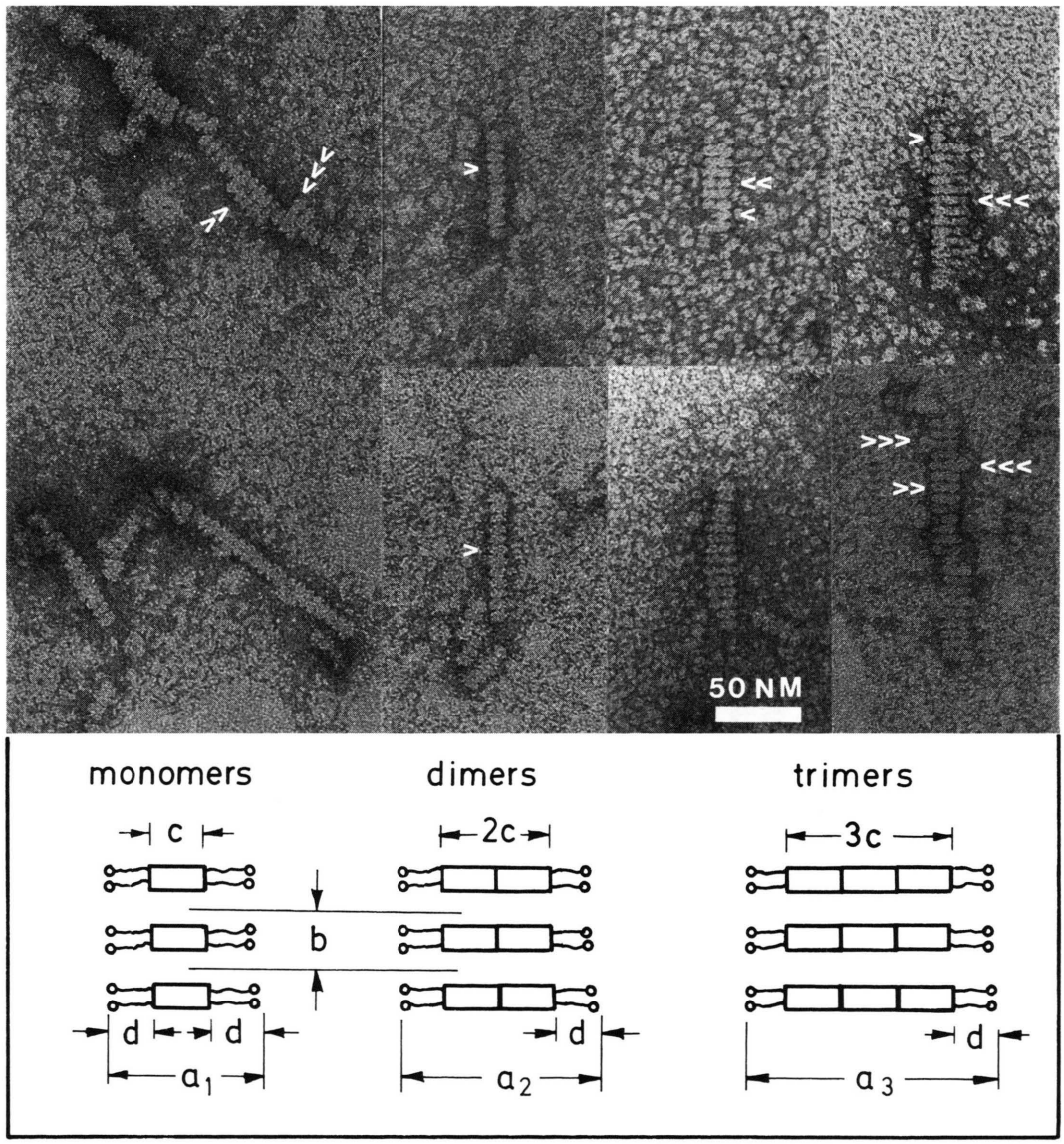

Fig. 4. Electron micrographs of the isolated 100 kDa complex of subunit III. Top: overview and gallery of monomers, dimers and trimers; the number of arrows indicates monomers, dimers and trimers, resp. Bottom: interpretation of arrangement of the complexes in the strings and definition of parameters.

plex forms string-like structures, with up to 35 units of the 100 kDa complex arranged along the long axis of the string. The complexes are lying on their side. Few single top views with a diameter of about 7 nm exist too, but are difficult to analyze at present since the preparations suffer from a high background of SDS micelles (Fig. 4). The diameter of the strings varies, indicating that one ("monomer"), two ("dimer") or three ("trimer") complexes can form one repeating unit of a string. Fig. 4 shows an overview (left) and a gallery of strings formed by monomers, dimers and trimers. If the repeating unit is a dimer, a small cleft between the two complexes can be seen frequently. At the bottom of Fig. 4 the measured parameters are defined: a_1, a_2, and a_3 are the diameters of the string units, b is the repeating distance along the string, c is the "real" diameter of one 100 kDa subunit III complex corrected for detergent, and d is the length of the detergent SDS. The measured and calculated dimensions are given in Table I. It is assumed that the detergent substitutes the lipid environment of CF_0; *i.e.*, it is located on the hydrophobic outer surfaces of the complex as indicated in Fig. 4. Moreover, we assume that no detergent intercalates between any neighboring complex molecules (see Discussion for an extended explanation). With these assumptions, the parameter c and the length of detergent can be calculated from the equations $a_1 = c + 2d$; $a_2 = 2c + 2d$; $a_3 = 3c + 2d$ (see Fig. 4). These data are also given in Table I. For the detergent it is found that $d = 2.2$ nm in accordance with the length of SDS. Since the detergent is the substitute for the lipid bilayer membrane, the parameter $c = 6.3$ nm gives the diameter of the 100 kDa complex, and the parameter $b = 6.1$ nm gives the length of the complex through the membrane.

Table I. Dimensions of the isolated 100 kDa complex of subunit III as measured from the electron micrographs. The parameters are defined in Fig. 4. Additionally, the standard error and the number of measurements are given.

	Monomers	Dimers	Trimers
No. of Measurements	57	37	9
a/nm	10.8 ± 0.6	16.8 ± 0.7	22.6 ± 0.9
b/nm	6.1 ± 0.1	6.1 ± 0.1	6.1 ± 0.1
c/nm	6.4	6.2	6.2
d/nm	2.2	2.2	2.2

Discussion

This work shows that incubation of CF_0F_1 in SDS buffer at room temperature leads to a dissociation into the subunits α, β, γ, δ, ε, I, II and IV. Under the same conditions, subunit III is detected as a supramolecular complex with the apparent mole mass of 100 kDa. The complex possibly contains some chloroplast lipids (about 5 sulpholipids (sulphoquinovosyl-diacylglycerol) per CF_0F_1 have been found [17]). With a mole mass of about 860 kDa per lipid, the mole mass of the protein component of the lipid-free complex amounts to approx. 95.7 kDa. Taking into account that molecular weights of hydrophobic proteins are frequently underestimated on SDS gels [18, 19] and using a mole mass of 8 kDa for subunit III, we would suggest a stoichiometry of at least 12 copies per complex. In earlier work, 6 copies of subunit III per CF_0F_1 were found using a ^{14}C-label technique for the aminoacids [20]. However, in the most recent report based on this technique [4] the original data show a stoichiometry between 10–14 copies of subunit III per CF_0F_1 in the thylakoid membrane. For CF_0F_1, isolated by Triton X-100 treatment and immunoprecipitation with antibodies against CF_1, only 4–5 copies of III per CF_0F_1 are found [4]. The stoichiometry of the other subunits of CF_0 is lower than 1 (I = 0.3), (II = 0.7) so that presumably some CF_0 is lost during isolation. If we assume that there is one subunit I in CF_0F_1, all CF_0 subunits must be corrected by a factor 3 and 2.1 subunits II and 12–15 subunits III are obtained.

The supramolecular complex of the subunit III can be dissociated in the presence of excess amounts of SDS either at increased temperature or increased incubation time. If the complex is an artificial aggregate of subunit III, as a result of the SDS incubation of CF_0F_1 an increase of the aggregation with increasing incubation time or increased temperature should be expected. Additionally, when the protein is not preincubated in SDS buffer, the same band pattern is seen as in Fig. 3, lane 3 (data not shown). Furthermore, no other aggregates of subunit III with different stoichiometries are found. We consider it highly improbable that distinct local, SDS-induced conformational changes of subunit III monomers, which then should equally well be evoked by 0.1% (without preincubation) and 2% (with preincubation) SDS, might be the cause of the oligomer formation. The resistance against dissociation during SDS incubation at room temperature implies a high stability of the

supramolecular complex, also in the absence of other F_0 subunits.

From these results we conclude that the isolated 100 kDa complex of subunit III is the native supramolecular structure of the subunit and that it represents a stable substructure of CF_0F_1 in the thylakoid membrane.

The 100 kDa band can also be seen in some of the SDS gels of CF_0F_1 published earlier (*e.g.*, ref. [21]). However, its relation to subunit III has not been detected before. The proteolipid from yeast mitochondria (which corresponds to subunit III) was detected on SDS gels as a hexamer and treatment with organic solvents leads to a dissociation to the monomeric form [22, 23]. At present it is not clear whether in mitochondria the native F_0 contains a hexamer of the proteolipid or whether in this case the dodekamer is dissociated during SDS incubation. It should be mentioned that the stoichiometry of subunit c in F_0 from *E. coli* (which is homologous to subunit III) is about 10–12 [24, 25].

In beef heart mitochondria monomeric and dimeric forms have been observed [37].

CF_0 contains the subunits I, II, III and IV with the molecular weights of 18 kDa, 16 kDa, 8 kDa and 20–25 kDa [1–6, 26, 27]. According to the considerations above, the stoichiometry of these subunits in CF_0 is I, II_2, III_{12}, IV. This results in a mole mass of 171 kDa for CF_0.

The results from electron microscopy show that the dimensions of the complex of subunit III are about 6.3 nm × 6.1 nm. There is, however, the question of how this complex is oriented in the membrane. We postulate that it is arranged in such a way that the membrane-spanning helices are surrounded by detergent and that the top and base of the complex, which are the more hydrophilic parts, stick together to form the strings (see Fig. 4). According to this interpretation, the complex would have a diameter in the membrane of 6.3 nm and a membrane-spanning dimension of 6.1 nm.

Electron microscopy on other chloroplast proteins strongly supports our interpretation of the orientation. Several proteins, like photosystems I and II from *Synechococcus* [28, 29] and the light-harvesting chlorophyll *a/b* protein complex [30], show particles stacked similarly as in the subunit III complex, if they are prepared in detergent. In all cases the repeating distance is about 6 nm and the narrow hydrophilic gap between the molecules is filled with stain.

The subunit III has been isolated in its monomeric form and reconstituted into phospholipid vesicles [3]. Electron micrographs from such vesicles using the freezefracture technique have shown particles with an average diameter of 8.3 nm [31]. If the possible size overestimation due to platinum replication is corrected, a diameter of 6.0 nm is obtained [31] in good agreement with our results. These results imply that the monomeric subunit III has a strong tendency for self-assembly in bilayer membranes; *i.e.*, if it is brought into phospholipid vesicles, stable dodecameric complexes are formed.

The amino acid sequence of subunit III is known and from the polarity profile of the sequence it has been concluded that the protein traverses the membrane in a hairpin-like structure with two α-helical segments [32]. Assuming a diameter of 0.9 nm for one α-helix, the 12 subunits III must be arranged in a structure with 6.3 nm diameter. Fig. 5 shows two examples of possible hypothetical structures, which have been chosen because of the highly symmetrical arrangement. In model (a) the subunits III are arranged in two non-identical positions. In the middle of the structure there is space for one α-helix. Therefore, most of the other CF_0 subunits must be arranged outside of this subunit III substructure. This arrangement would be in accordance with the conclusions derived from the labeling pattern of the F_0 subunits with 3-(trifluoromethyl)-3-(m[^{125}J]) iodophenyldiazirine in *E. coli*, *Neurospora crassa* and chloroplasts [33] and a model proposed by Sebald *et al.* [34]. Model (b) in Fig. 5 shows all subunits III arranged in identical positions in one ring.

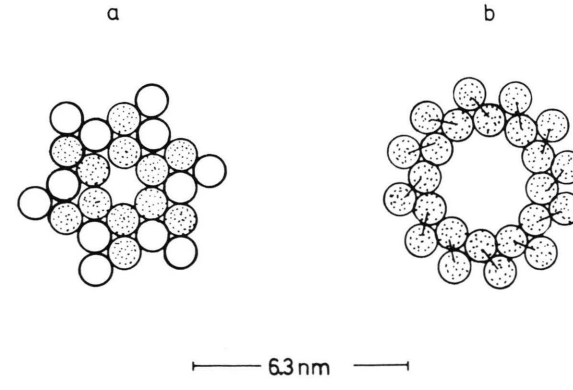

Fig. 5. Two hypothetical arrangements of helices in the complex of subunit III (top view) each pair of circles representing the two helices of a subunit III monomer.

In this case, the middle part of the structure could be filled with 7 additional α-helices. From hydrophobicity patterns it is expected that subunit IV has 4 transmembrane helices [26] and subunit I has 1 transmembrane helix [35]. For subunit II the amino acid sequence is not yet known. This means that all the other F_0 subunits can possibly be located inside the ring structure formed by subunit III. This model is similar to the one proposed by Cox *et al.* [36]. Presently, we cannot distinguish between these two and other possible structures on the basis of our data. However, now that a stable 100 kDa oligomeric subunit III substructure of CF_0 has been discovered and isolated, high resolution electron microscopy of top views might well be applied to determine the arrangement of the helices of this substructure and to elucidate its structural role in accommodating the other CF_0 subunits. This would give the structural basis for attempts to explain the very high conductivity of CF_0 [38, 39].

Acknowledgements

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