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Atomic force microscopy imaging of the membrane part CF_0 and the subunit III complex of chloroplast ATP-synthase

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Introduction

The membrane part CF_0 of H^+-ATPase from chloroplasts (CF_0F_1) is composed of the subunits I, II, III and IV [Pick and Racker, 1979],[Fromme et al., 1987a]. Only subunit III is present in more than one copy, these form an oligomeric subcomplex which is stable during SDS-PAGE with an apparent mass in the range of 100 kDa. This complex was isolated from the SDS gel and shown to contain only subunit III. Regarding the apparent molecular mass, it was concluded that 12 monomeric subunits III at 8 kDA each form the complex. Transmission electron microscopy revealed that the complex forms string-like structures, the membrane spanning length being 6.1 nm and the diameter in the membrane 6.2 nm [Fromme at al., 1987 b].
After reconstitution of CF_0F_1 into liposomes and removal of the CF_1-part, the CF_0-part was obtained in a functionally active state in the liposomes. A proton conductivity of 16 H^+ per CF_0 per s was observed, based on the assumption that all complexes are functionally active. The CF_0–part in the liposome membrane was found to be unstable after removal of CF_1. It slowly loses its ability to conduct protons, presumably by dissociation of the complex [Grotjohann and Gräber,1990].
Recently, the structure of the subunit III complex was investigated by atomic force microscopy. In high resolution images a ring-like structure containing 14 copies of subunit III has been observed [Seelert et al., 2000]. In current rotation models of coupling between proton translocation and ATP-synthesis, the ratio of proton translocating subunits to nucleotide binding subunits plays an important role for the H^+ to ATP stoichiometry [Engelbrecht and Junge, 1997]. Therefore, we investigated the subunit III complex and CF_0 with transmission electron microscopy and atomic force microscopy.

Material and Methods

We isolated the membrane part F_0 from *Spinacia oleracea* chloroplasts using a new protocol. CF_0F_1 was isolated as described [Fromme et al., 1987b]. CF_1 was removed by treatment with Na-thiocyanate, F_0 was then purified by a sucrose density gradient centrifugation followed by native preparative electrophoresis. Due to the mild purification conditions all four F_0 subunits I, II, III and IV were found in the resulting complex. Heat treatment of this complex leaves intact only the subunit III complex. Since the protein was obtained in dodecylmaltoside-lipid-micelles, two dimensional crystallisation was initiated by dialysis in presence of additional lipid. The subunit III

complex forms two different kinds of two dimensional crystals, while F_0 builds large two dimensional sheets of F_0 aggregated in an unordered way. These sheets and crystals were imaged in two ways: they were scanned on mica surface by liquid contact mode atomic force microscopy (AFM) and, after negative staining with methylamino-tungstate, they were imaged in transmission electron microscopy (TEM). Both forms of resulting images were processed with the program IMAGIC-5. This included particle selection, alignment as single particles, summing to average pictures, classification and iterative multi-reference-alignment.

Results and discussion

TEM data from subunit III complex show a concentric double ring structure (outer diameter of the outer ring 7.8 nm, inner ring 3.9 nm) which is symmetrical and structured along the ring. In CF_0 images, a double ring can be seen as well, though it is less symmetric (outer diameter of outer ring 7.1 nm). In most picture classes, the outer ring structure is opened up at one side, with higher protein density neighbouring this gap, which might indicate the location of subunit I and II. The inner ring is smaller and less uniform.
The same samples were also scanned by atomic force microscopy. AFM delivers three dimensional surface profiles while TEM pictures show a two dimensional projection of the total protein density. Contrast is usually stronger in AFM, noise is lower and resolution can be higher. The AFM data for subunit III complex show crystals in two different lattice forms, in both of which two alternating orientations of the complex are displaying either wide, empty ring structures or small, filled ones. Fig.1. is an unprocessed image detail and shows examples of both structures. The outer diameter of the wide ring is 6.4 nm, the width of the ring is 1.9 nm and the outer diameter of the narrow ring is 6.0 nm. Whereas the wide ring seems to be empty, the small ring is filled with material which might be either protein or lipid.

Only in the most immaculate rings, the number of subunits can be counted directly. We calculated the rotational power spectra of the wide and small rings to analyse periodicity and found predominantly 14- and 15-fold symmetries, which is partly in accordance with the data of [Seelert et al, 2000].

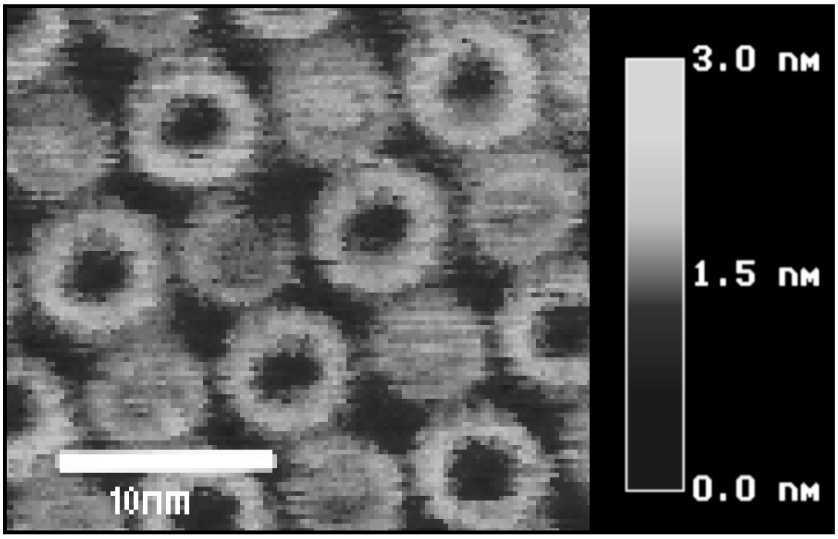

Fig.1. Detail of an original AFM image showing a twodimensional aggregate of subunit III complex. The different orientations of the complex are clearly distinguishable.

Though the raw data are detailed already, they can be digitally averaged, too. Fig. 2. shows averaged images of the wide rings (left) and the small rings (right).
As shown by two dimensional gelelectrophoresis (first dimension native electrophoresis, second dimension SDS-PAGE), the isolated CF_0 contains nothing but all the four subunits I, II, III and IV. We tried to get two dimensional crystals of CF_0 by dialysis removal of detergent. However, we only obtained large, flat two dimensional sheets of sideways aggregated F_0 lacking long range order. AFM on these sheets yields surface profiles of weak height contrast, the single protein complexes are barely visible in the raw data. Nevertheless, we tried single particle averaging. The result is shown in Fig.3. It was not possible to perform reasonable classification on the single particles, so it is likely, that images of both sides of the complex have contributed to this average image.

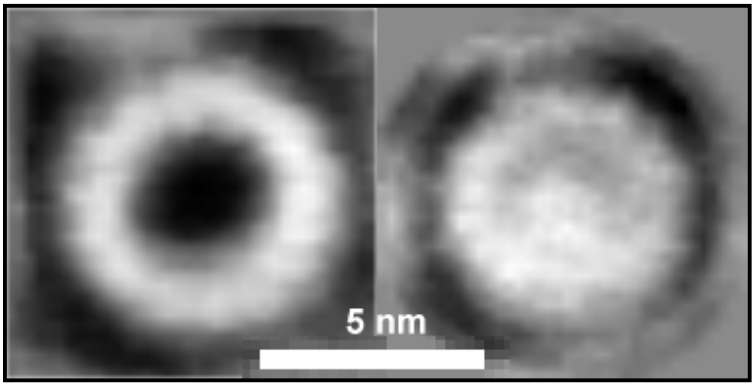

Fig.2. Averaged AFM images of subunit III complex, wide end (left) and small end (right). 16 resp. 13 single images were aligned and averaged.

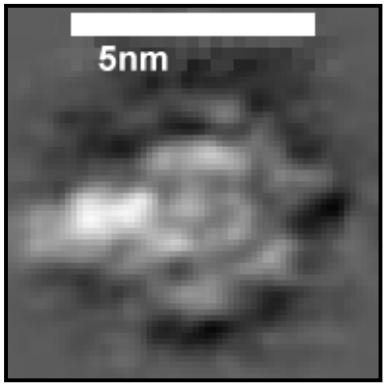

Fig.3. Averaged AFM image of CF_0. 146 single images were aligned and averaged.

Here, an inner and outer ring structure can be recognised (outer diameter of the whole complex ca. 5.0 nm). The outer ring is not as smooth as in the subunit III complex. At one side (left here) there is a conspicuous protein mass extending out of the two rings. It might reflect the position of subunits I and II (or IV).
Whereas the diameter of the ring structure is only slightly smaller than in the subunit III

complex, the distribution of protein mass is very different. CF_0 seems to have a very asymmetric structure.
The two different purification procedures for subunit III complex give similar stoichiometry. Surprisingly, CF_0 appears to be smaller, both in TEM and in AFM images. This suggests that stoichiometry in the subunit III complex could be smaller than in the whole CF_0. Existing evidence for yeast MF_0F_1 [Stock et al., 1999] and for *E.coli* EF_0F_1 [Jiang et al., 2001] indicate that the stoichiometry in the holoenzyme is ten equivalent subunits per complex. Still we believe, it is more likely, the subunit III complex widens up by a conformational change upon removal of subunits I, II and IV than that the complex increases in number of monomers.
Ordered CF_0 aggregates or average images generated from higher numbers of particles might allow stoichiometry determination in CF_0, too, and visualisation of the location of the subunits I, II and IV.

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