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Structure of the ATP synthase from chloroplasts studied by electron microscopy and image processing

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The structure of the hydrophilic part of the ATP synthase from chloroplasts (CF_1) has been investigated by electron microscopy of negatively stained samples. The staining conditions, which are generally critical for such small objects as CF_1, could be improved by mixing CF_1 samples with a much larger protein with better staining properties. A total of 3300 images was interactively selected from 21 digitized micrographs, and the individual molecular images were subjected to computer-image analysis. The images were aligned relative to different reference images and were then submitted to a multivariate statistical classification procedure. The analysis shows that all projections are of a hexagonal or flattened hexagonal type. The differences between these projection types are marginal and originate largely from an uneven stain distribution around parts of the molecules. Although the outer shapes of the hexagonal projections of CF_1 and mitochondrial (MF_1) molecules are very similar, the central mass of CF_1 appears more substantial than in MF_1 and is often divided into two parts.

Introduction

ATP synthesis/hydrolysis coupled with a transmembrane proton transport is carried out by a large membrane-bound enzyme (ATP synthase) in different types of organelles (chloroplasts, mitochondria, bacteria). It consists of a membrane-integrated part, F_0, which is supposed to act as a proton channel through the membrane, and a hydrophilic part, F_1, which contains the nucleotide binding sites. The ATP synthases from different sources are very similar (for reviews, see Ref. 1–4).

The hydrophilic part of the ATP-synthase from chloroplasts, CF_1, is composed of five different subunits: alpha (59 kDa), beta (56 kDa), gamma (37 kDa), delta (17.5 kDa) and epsilon (13 kDa). One CF_1 molecule contains three copies of alpha and beta subunits and one copy of gamma [4]. The number of copies of delta and epsilon is yet uncertain [4].

The structure of the soluble part of the ATP synthase system has been studied by X-ray diffraction, resulting in a low-resolution model [6] showing six density regions of approx. equal size. An arrangement of the larger subunits was proposed on the basis of electron microscopy of crystals and single molecules [7–12]. These results show that the F_1 ATP synthase is built up from two layers of large subunits alpha and beta in the form of a flattened trigonal antiprism with dimensions of about $11.5 \times 11.5 \times 8$ nm.

Computer image analysis of single molecules in

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combination with multivariate statistical techniques were developed for analyzing mixed populations of images [13–16]. The characteristic views, present in the population of images, are determined with an automatic classification scheme [15]; the resulting class averages represent noise-reduced projection images of the molecule under investigation. In an earlier study we applied these techniques to F_1 molecules from bovine heart mitochondria (MF_1) [12]. In the current study we investigate CF_1 using the same techniques.

Materials and Methods

CF_1 ATP synthase was isolated from spinach chloroplasts as described in Ref. 17 and then dialyzed against 10 mM sodium-Tricine (pH 8.0) giving a final concentration of 5 mg/ml. Specimens for electron microscopy were prepared by the droplet method or the Valentine method [18], using uranyl acetate as a negative stain. The original protein batch was diluted 100 times immediately before preparation with a 10 mM phosphate buffer (pH 7.0) containing 4 mM ATP and 2 mM EDTA. In order to improve the homogeneity of the negative stain distribution over the specimen, the ATP-synthase samples were mixed with giant erythrocruorin from the earthworm *Lumbricus terrestris* (a gift of Dr. W. Hendrickson). Electron microscopy was performed with a Philips EM 300 electron microscope. Micrographs used for the analysis were taken with 80 kV at 70 000 magnification. Care was taken not to preilluminate the part of the specimen to be recorded, in order to avoid excessive specimen damage.

Selected micrographs were digitized with a Datacopy Model 610 F electronic digitizing camera (Datacopy Corporation, Long Beach, U.S.A.) mounted under a standard 6×9 cm enlarger equipped with a direct-current driven halogen lamp. The scanning step used was 32 μm, corresponding to a pixel (image element) size of 0.46 nm at the specimen level. Image analysis was carried out on a VAX 11/780 computer (Digital Equipment Corporation) using the IMAGIC software package [19]. Particles were selected interactively from the micrographs using a raster-scan image display system. A total of 3300 molecular images was extracted from 21 different micrographs. They were pretreated by bandpass filtering with a Gaussian width of $1/e$ to suppress the very low [$> (12\ \text{nm})^{-1}$] and very high [$< (1.2\ \text{nm})^{-1}$] spatial frequencies, which do not contain useful information but influence the alignment procedures [16]. Unnecessary background of the images was masked out by a circular mask. The average density inside the mask was set to zero and the variance of densities was normalized to an arbitrary value of 100 [16]. The next steps in the analysis – alignment of the images, the eigenvector-eigenvalue data compression procedure ('correspondence analysis') and the automatic classification – were carried out as described previously [13,15,16].

Results and Discussion

Negative staining – the contrast enhancement technique of surrounding macromolecules by heavy metal salts – was introduced almost thirty years ago [20]. Many refinements and modifications, cf. Ref. 18, have been proposed to optimize the staining conditions, which are critical and depend on such factors as the surface qualities of the supporting foil and air humidity. It is, however, still difficult to obtain a good, homogeneous, staining environment for small (< 500 kDa) particles: either the stain layer thickness is satisfactory but the particle concentration too high, or, at the apparent optimal particle concentration on the carbon support film, the stain layer becomes too thin. In contrast, large particles are much more often surrounded by the correct amount of stain. Therefore we tried to improve the staining of CF_1 particles by mixing them with an object eight times its mass: erythrocruorin (= earthworm hemoglobin), which shows no interaction with CF_1. In this way better distribution of the negative stain could be obtained easily. The best results were obtained with the Valentine method [18]; in comparison with the droplet method no flattening of the particles was caused. A typical micrograph showing molecules with a clear subunit structure is shown in Fig. 1. A total of 21 such negatives were used for image processing of CF_1 molecular projections.

In an earlier study on the F_1 part of the ATP-synthase from bovine heart mitochondria (MF_1)

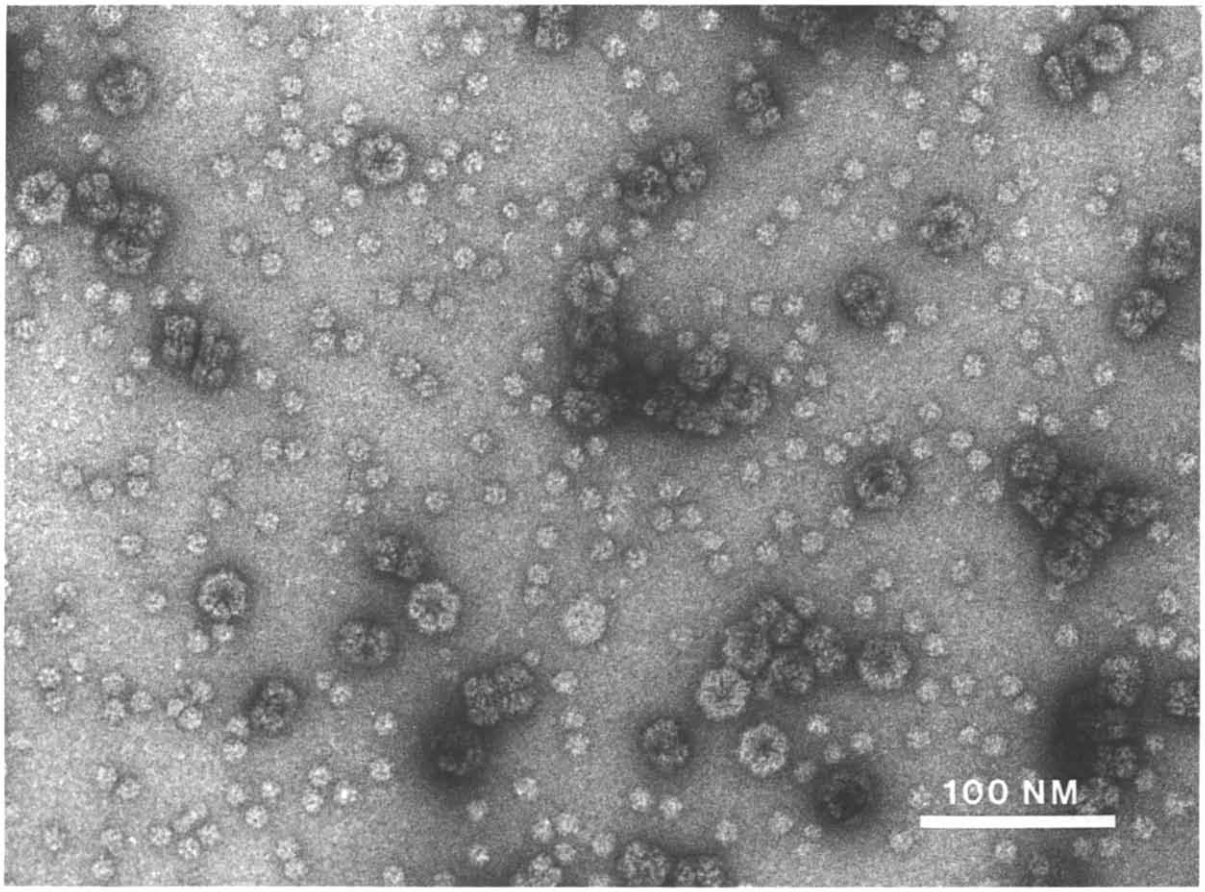

Fig. 1. Part of an original electron micrograph showing molecules of ATP synthase from chloroplasts used for the analysis of single particles. The specimen was mixed with erythrocruorin molecules just before preparation, and negatively stained with a 1% solution of uranyl acetate.

[12], we reported the problem that single ('noisy') images used as a starting reference bias the analysis. The total sum of a set of images aligned relative to a single reference image will normally resemble that reference image. Even if automatic classification schemes are used, the subset sums obtained may still reflect bias imposed by the reference, because these objective classification schemes cannot 'look beyond' a given alignment. Therefore a careful strategy in the choice of the first references is necessary. We started with a six-fold symmetrized reference (Fig. 2A) and a mirror-symmetrized reference (Fig. 2B) derived from initial alignments and classifications. From the classification five good class averages were selected as the next references used in a multireference alignment scheme. A total of 3300 images was partitioned into 20 classes, each containing between 100 and 160 members. During the classification, 20% of the images were rejected automatically based on their large contribution to the variance of the data set. Furthermore, four classes were rejected which showed the highest internal variance: an indication for poor class quality. The remaining 16 classes are shown in Fig. 3. These classes contain 65% of the images in the data set and show hexagonal projections. The classes N–P, however, appear smaller in one direction. Some (1–3) outer subunits appear slightly smaller and this is accompanied by a slight displacement of the central mass in the direction of the smaller appearing outer subunits. The band-pass filter im-

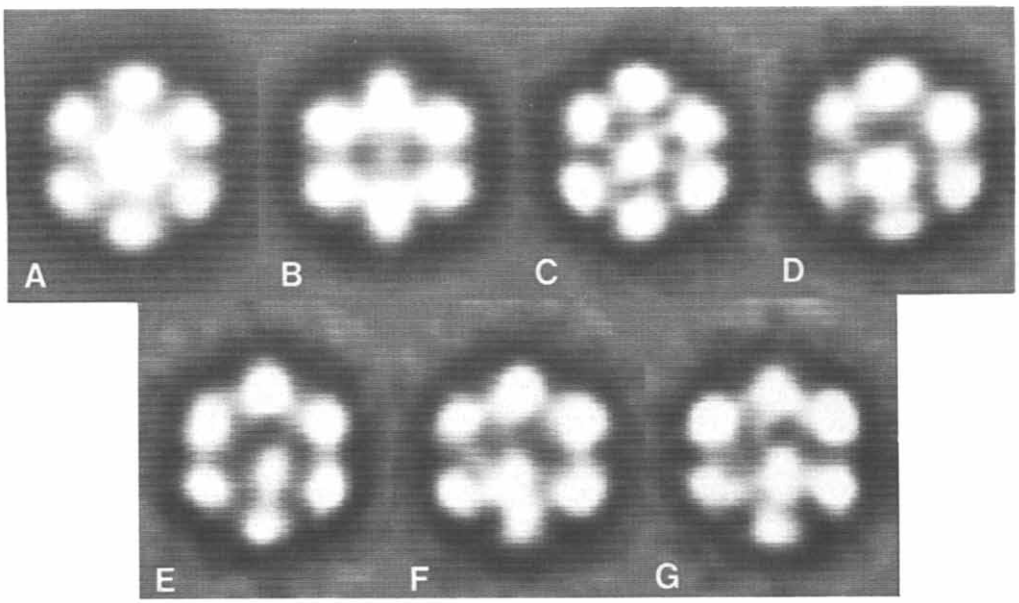

Fig. 2. References used for alignment of the data set. A and B are artificial references made from two classes with each 100 particles from a first classification of 600 projections. On a, six-fold symmetry was imposed; on B, horizontal and vertical mirror symmetry. References C–G were made from five classes taken from a second classification of the 600 projections and used for the multi-reference alignment of the whole data set.

posed on the images prior to the analysis suppresses the very low image frequencies such as are caused by a staining gradient in the neighbourhood of the particles. This filter can be undone by an inverse filtering operation, applied to the final class averages. In the case of the 'flattened hexagonal view' of Fig. 4C and D, it can be seen that the outer subunits are stained differently: the subunits appearing 'smaller' are more embedded in the negative stain. This observation explains in a large part the odd shape of the flattened hexagonal projections. It is, however, also possible that the flattening is (partly) caused by a specific tilting of molecules, due to a rudimentary stalk extending from the structure. In that case the distances in projection between large subunits would change in a specific way, since these subunits are on different levels within the CF_1 structure. Surprisingly, this was not observed and therefore it is concluded that the flattened hexagonal projections originate principally from an uneven stain distribution around the molecules. Flattened hexagonal projections were also found in our MF_1 analysis [12] and seem to be present in the work of others as well. The gallery of Fig. 3 in Ref. 21 shows a few images of F_1 projection views which are very similar to classes of Fig. 3N–P. The presence of both flattened and normal hexagonal projections could perhaps also explain the closer similarity of Fig. 3D in Tsuprun et al. [8] (rotated over 90°) to our Fig. 3N–P than to 3A–D.

The classes of Fig. 3 show the variation present in the hexagonal view. There are representatives of high (C, D) and low (I, K, M) stain accumulation in the central part. Classes F, G and H are similar; but since their asymmetric central features are mirror-related to those of C and D, we might interpret them as being projections from molecules oriented face-up and face-down on the carbon support. The views of A and B clearly show the central mass split into a smaller and a larger part. The reliability of this feature was verified by re-alignment of the members of this class; all showed the same 'double mass' even if a reference with only one asymmetric central mass was used. In fact, classes C–H and L also show the extra mass in the centre, although not as clearly separated as in B.

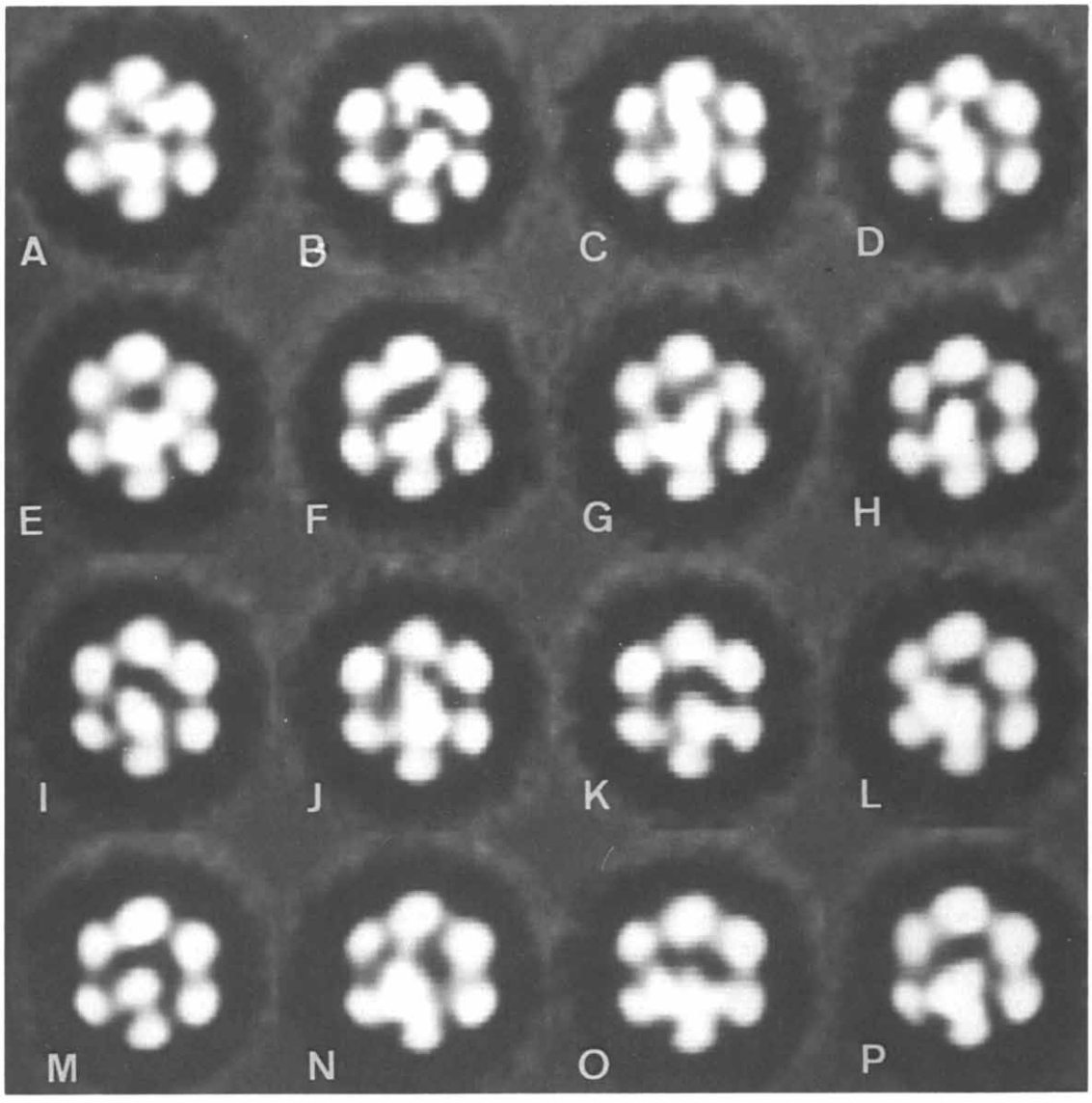

Fig. 3. Molecular projections of CF_1 ATP synthase as determined by multi-reference alignments and classification of 3300 molecules; the 16 classes show the image variation present in the data set. Number of class members: 125 (A); 129 (B); 112 (C); 104 (D); 151 (E); 152 (F); 119 (G); 125 (H); 143 (I); 120 (J); 150 (K); 136 (L); 166 (M); 118 (N); 127 (O); 166 (P). Note: the images are shown with imposed band-pass filter which is necessary for the suppression of unwanted noise during analysis. This filter allows a better analysis of the image features, but has the disadvantage of suppressing the central density (see also Fig. 4).

During the activation of the ATP synthase and during the catalytic cycle of ATP synthesis the enzyme occurs in different conformations [28,29]. It is possible that the different classes obtained in the multi-reference alignment represent different 'frozen' conformational states of the enzyme. However, presently it seems to us more plausible that these classes reflect differences in the stain distributions in the enzyme.

F_1 ATP synthase is built up from two layers of large subunits arranged in a trigonal antiprism [7,8]. Immuno-electron microscopy has shown that the main view shows particles in a hexagonal projection in which the alpha and beta subunits

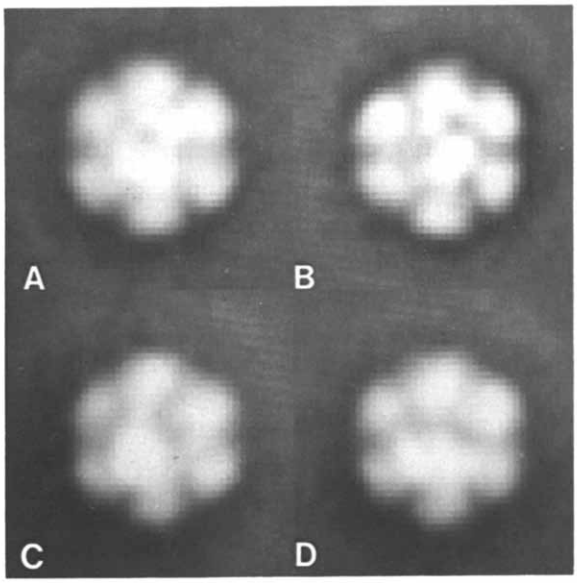

Fig. 4. Images of classes of Fig. 3A, B, N and O (A, B, C, D, respectively) displayed without band-pass filter; this facilitates the examination of the stain distribution around the molecules.

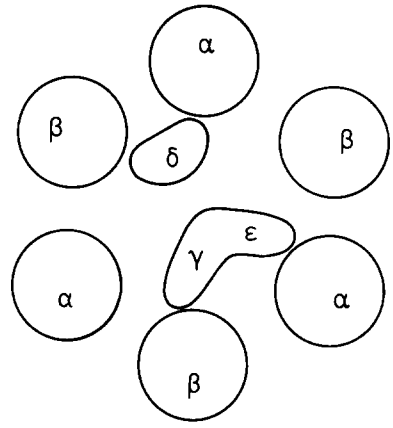

Fig. 5. Scheme of the subunit arrangement in the hexagonal view of chloroplast F_1 ATP-synthase.

are in an alternating arrangement [10,11]. The investigations of Tsuprun et al. [8] indicate that the large subunits are fairly spherical in shape and that the trigonal antiprism is slightly flattened. The results from our analysis show that only hexagonal projections are present in the data set. The very characteristic side views in Ref. 8 are so rare in our data set that no reliable class resulted, although 3300 molecular projections were processed. However, when CF_1 was prepared with a different preparation technique, more side views similar to the ones in Ref. 8 could be observed (results not shown). From these additional observations we conclude that projections from molecules that could give rise to side views with 1:2 or 2:1 alpha/beta subunits attached to the carbon support are missing.

The features of the hexagonal view of CF_1 can be compared with those of the hexagonal view of MF_1 from a previous study [12]. In both cases the arrangement of the large (alpha and beta) subunits is practically the same and the hexagonal views differ mainly in the center.

(1) The central mass of CF_1 is larger than the one of MF_1. The small subunits appear as single copies in F_1 ATP synthase from mitochondria [22]. In the case of CF_1 ATP synthase, the stoichiometry of the smaller subunits has not been unambiguously determined [4]. Between 1 and 3 delta subunits have been reported [25,26] and 1 to 2 epsilon subunits have been found [25,27]. Our electron microscopy results could, therefore, support a higher stoichiometry.

(2) The central mass in CF_1 is frequently split into two parts, a feature that was never observed in the different MF_1 classes [12]. The central mass with the shape of the letter V, that is placed asymmetrically in the center of the enzyme, is more or less similar in both cases. The second smaller part of the central mass was detected in CF_1 only. The MF_1 preparation analyzed in Ref. 12 lacks OSCP (oligomycin sensitivity-conferring protein), which is supposed to be functionally equivalent to delta of CF_1 [30,31]. Therefore, the V-shaped structure observed in both cases is possibly the gamma and epsilon subunit and the second mass the CF_1 delta subunit. Investigations with OSPC-containing MF_1 are in progress. The scheme in Fig. 5 shows a hypothetical assignment based on these considerations and on the results of Refs. 10 and 11. This scheme is supported by recent electron microscopic studies on MF_1 using a ferritin label [32], from which it was concluded that gamma and/or epsilon subunits are asymmetrically oriented.

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