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F_1F_0-ATP Synthase—Stalking Mind and Imagination

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Electron microscopy together with image analysis has been used to study the structure of the intact F_1F_0-ATPsynthase from *Escherichia coli*. A procedure has been developed which allows preparation of detergent-free enzyme. Aside from the well known two-domain structure, images of F_1F_0 prepared by this procedure show a number of additional features, including a second stalk, which can be seen extending all the way from the F_0 to the top of the F_1 in some images, and a small protein on the very top of the F_1, which has been identified as the δ subunit by decoration with a monoclonal antibody. In light of these results, a refined model of the subunit arrangement of the complex is presented.

KEY WORDS: F_1F_0-ATPsynthase; δ subunit; second stalk; electron microscopy.

INTRODUCTION

F_1F_0-ATP synthase is found in the mitochondrial inner membrane, the inner membrane of bacteria, and the thylakoid membrane of chloroplasts, where it functions to synthesize ATP from ADP and inorganic phosphate (Senior, 1988). This process is driven by a proton gradient generated by the enzymes of the respiratory chain or photosynthesis during electron transport.

The bacterial ATPsynthase contains eight different subunits αβγδε*abc* in a ratio of 3:3:1:1:1:1:2:10–14. The enzyme can be divided into two structural and functional domains, a water-soluble, membrane-extrinsic F_1 composed of subunits αβγδε and a membrane-bound F_0 made of *abc* (subunit nomenclature of the *E. coli* enzyme). The F_1 and F_0 are joined by a central stalk, which rotates during enzyme functioning to couple events taking place in the three catalytic sites on the F_1β subunits to the single proton channel formed by the F_0 *a* and *c* subunits (Boyer, 1997; Junge *et. al.*, 1997). A second or peripheral stalk, which is made of the δ and the *b* subunits, keeps the F_1 and the proton channel in the correct spatial orientation for energy coupling to occur (Ogilvie *et al.*, 1998; Dunn *et al.*, 2000).

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Electron microscopy (EM) has long been used to study the structure of the ATP synthase and its subcomplexes. Over the years, the technique has provided a large amount of valuable information with respect to the structure and function of the enzyme. The data include the first direct evidence for an alternating arrangement of the α and β subunits in the F_1 (Lünsdorf *et al.*, 1984), the presence of a central stalk (Soper *et al.*, 1979; Gogol *et al.*, 1987), evidence for rotational catalysis (Gogol *et al.*, 1990) and mobility of the ε subunit (Wilkens and Capaldi, 1994), the first three-dimensional (3D) structure of the F_1 (Gogol et al., 1989) and, more recently, direct evidence for the second stalk (Wilkens and Capaldi, 1998a,b; Böttcher *et al.*, 1998; Karrasch and Walker, 1999), the binding site of the δ subunit (Wilkens *et al.*, 2000), and the first 3D model of the entire complex (Böttcher *et al.*, 2000). In this minireview, the more recent electron microscopic studies of the ATPsynthase structure are summarized and a refined model of the complex is presented.

DETERGENT-FREE ENZYME PREPARATION

The F_1F_0-ATP synthase is a membrane-bound protein, which requires the presence of detergent dur-

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ing the isolation process, to prevent aggregation. However, it has been known for some time that detergents can have a significant influence on the activity and structural integrity of the complex. Most importantly, detergents have the ability to uncouple ATP hydrolysis from proton pumping. This uncoupling can be observed in the enzyme, which is inactivated by a modification of *c*Asp61 by dicyclohexylcarbodiimide (DCCD). Lauryl dimethylamine oxide (LDAO) is best known for restoring ATPase activity after DCCD inhibition (Lötscher *et al.*, 1984). However, there are other detergents, most notably lauryl maltoside (LM), which can have a similar effect (Tsunoda *et al.*, 2000). LDAO has been shown to release the δ subunit from F_1 (Lötscher *et al.*, 1984), but the *c*-subunit ring and/or its interface to the *a* subunits is also sensitive to low CMC detergents, such as LDAO, LM, and Triton X-100.

Based on these observations, it became clear early on that for structural studies of the intact ATP synthase, the presence of detergents had to be avoided all together, except maybe for the early steps during enzyme purification. Amphipols, a novel class of amphipathic polymers, have been described as being able to keep membrane proteins soluble in aqueous solution in absence of detergents (Tribet *et al.*, 1996). To make use of this class of compounds, a new enzyme isolation procedure was designed. For enzyme purification and detergent removal, a 6XHis tag was introduced into the N-terminus of the F_0 *a* subunit by site-directed mutagenesis (Wilkens *et al.*, 1998a,b, 2000, and in preparation). Briefly, F_1F_0 is extracted from washed inner membranes with conventional detergent (taurodeoxycholate) and subsequently bound to NiNTA-Sepharose. Detergent is then replaced on the Ni-column by Amphipol A8-75 (A8-75; synthesized according to Tribet *et al.*, 1996) and the protein is eluted with imidazole in presence of 0.1% A8-75. ATP synthase isolated by this way shows virtually no ATPase activity, which is probably due to delipidation and binding of the negatively charged A8-75 to the hydrophobic F_0. The basal ATPase activity of the enzyme (= activity in absence of detergent) eluted from the Ni-column in A8-75 containing buffer is stimulated 50–100fold (up to about 120 U/mg when measured in an ATP regenerating system) by addition of LDAO to the assay mix. Reconstitution of the protein into egg phosphatidylcholine bilayers leads to an increase of the basal activity to about 20 U/mg, a value typical for enzyme isolated *via* the conventional sucrose gradient procedure. The lipid reconstituted activity shows good sensitivity to DCCD (about 70%) and the resulting inhibition can be reversed by addition of LDAO.

To summarize, the activity data show that the low activity of the enzyme in presence of A8-75 is not due to an irreversible damage to the protein, but rather to the removal of lipids and the interaction with A8-75. Reversible inhibition due to enzyme preparation in absence of phospholipids had been observed earlier (Foster and Fillingame, 1979). Whether the Amphipol-containing enzyme still responds to different nucleotide conditions is currently under investigation.

ELECTRON MICROSCOPY OF THE ATP SYNTHASE

Electron microscopy of detergent-free *E. coli* ATP synthase has shown the protein to be highly monodisperse (Wilkens and Capaldi, 1998a;b; Wilkens *et al.*, 2000), a prerequisite for a computer-assisted analysis of electron microscopic images of single enzyme molecules. During specimen preparation, the majority of the molecules are oriented on the carbon film used for EM to produce the so called “side-view” projection, in which the molecule is seen perpendicular to its long axis or parallel to the membrane surface. Figure 1a shows some of the projection classes obtained from an analysis of a data set of ~3150 ATPase molecules.

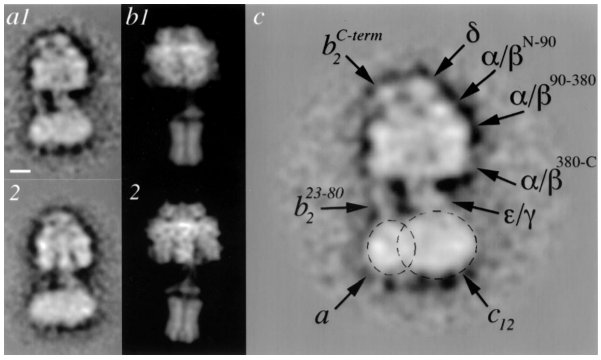

Fig. 1. Image analysis of detergent-free ATP synthase from *Escherichia coli*. A data set of 3152 F_1F_0 molecules, negatively stained with uranyl acetate, has been analyzed by alignment and classification procedures (for details see Wilkens *et al.*, 2000). (a) The bi- and trilobed projections of the enzyme. For details see text. Bar = 5 nm. (b) projection images of the 4.5 Å crystal structure (Stock *et al*, 1999; PDB code 1QO1) showing the bi- and trilobed views of the F_1. The projections have been generated in RasMol and Adobe Photoshop. (c) Summary of the structural features seen in the EM projections.

In these images, the molecule is rotated in such a way so that the F_1 is at the top and the F_0 is at the bottom. The overall dimensions of the complex are 22 × 12 × 12 nm, including the 10 × 12 nm F_1, the 12 × 6 nm F_0, and the 4 nm stalk domain. Some of the structural features visible in the projections are discussed below.

Structure of the F_1

The F_1 portion of the molecule shows the characteristic "bilobed" (image a1) and "trilobed" (image a2) views, in which either each three or two of the α and β subunits, respectively, overlap in projection. For comparison, Fig. 1b shows filtered images of the F_1–c_{10} "crystal structure" (PDB 1QO1), rotated and projected to produce similar projections as the ones seen in the EM images. The three domains of the large subunits (N-terminal, nucleotide binding, and C-terminal domain) can be clearly seen in the EM pictures of the ATP synthase (see arrows in Fig. 1c). The similarity of the EM and crystal structure projections can be taken as good evidence that the overall structure of the ATP synthase is well preserved under the conditions used for electron microscopy.

Structure of the F_0

The F_0 has an asymmetric appearance, consistent with the idea that the *a* subunit is attached on the outside of a more or less symmetric ring of *c* subunits. This is the generally accepted model for the F_0 subunit arrangement, which had been proposed earlier on the basis of EM pictures of antibody-decorated *E. coli* F_0 (Birkenhäger *et al.*, 1995) and atomic force microscopic (AFM) images of the membrane-bound proton channel of the same species (Singh *et al.*, 1996). The dimensions of the F_0 in the EM images are in the order 12 × 8 × 6 nm. In comparison, the ring of 10 *c* subunits seen in the crystal structure of the yeast enzyme is about 5.2 nm wide and 6 nm long (Stock *et al.*, 1999). The larger width of the subunit *c* oligomer in the EM structure (about 8 nm for the c_{12} part) can be explained by the presence of a layer of A8–75 covering the hydrophobic, membrane-exposed parts of the *c* subunits.

The Central Stalk

Not much detail can be seen in the central stalk region. The molecular weight of the F_1 extrinsic portion of γ together with ε is only about 35 kDa and therefore at the limit of what can be resolved by the EM technique. Nevertheless, the bulk of the mass (see arrow pointing to γ/ε in Fig. 1c) in the central stalk seems to be closer to the F_0 consistent with cross-linking data, which indicate that the β sandwich domain of ε and some part of γ interact with the *c*-subunit loops. What also might complicate things is that this region of the molecule probably exists in several (possibly three) equivalent orientations with respect to, e.g., the *a* subunit. Since it is a small feature to begin with, any detail might easily get lost in the eigenvector/eigenvalue data compression used for sorting the projections.

The Second Stalk

The necessity for a second stalk or stator in the ATP synthase was recognized after experimental evidence was mounting that rotation of the γ subunit was indeed part of the coupling mechanism in ATPsynthase, as had been foreseen by Boyer (1997). Around the same time, a variety of chemical (Lill *et al.*, 1996) and disulfide (Ogilvie *et al.*, 1997) cross-linking data suggested a position for at least part of the δ subunit near the top of the F_1 close to the N-termini of the α and β subunits. These data, taken together with evidence of a direct interaction between δ and the hydrophilic portion(s) of the *b* subunits (Rodgers *et al.*, 1997), clearly pointed to the existence of a second stalk in the ATP synthase. A second connection between ATPase domain and ion channel was first seen in EM pictures of a sodium transporting V-ATPase (Boekema *et al.*, 1997) and, subsequently, in images of the F-ATPases from *E. coli* (Wilkens and Capaldi, 1998a,b), chloroplasts (Böttcher *et al.*, 1998), and bovine heart mitochondria (Karrasch and Walker, 1999). Figure lc shows an enlarged view of one of the projection images shown in Fig. 1a, which summarizes the interpretable features in the EM projections. Not much can be seen of the second stalk while bound to the outside of the lower half of the F_1 (but see below). Dunn and co-workers recently determined some of the interaction sites of the *b* subunit(s) with the α and β subunit in the *E. coli* enzyme (McLachlin *et al.*, 2000a). According to these data, residues between positions 80 and 109 of the *b* subunit(s) can be crosslinked to residues in the lower two thirds of the α and β subunits of the F_1. This suggests that the stator is bound in an interface between an α and a β subunit, where it would be hard

to see in projection. The data also imply that the portion of the second stalk, which is visible between the F_1 and F_0 is probably formed by residues between positions 24–80 of the *b* subunits. This would correspond to a molecular weight of about 12 kDa for both *b* subunits, less than 2.5% of the mass of the entire complex. The second stalk emerges again in the upper half of the F_1 to form a "sausagelike" density visible in some projections of the enzyme (see Fig. 1a, top image). This region is probably the contact point between the C-termini of the δ and *b* subunits (for the position of the δ subunit, see below).

It has to be pointed out (see also Böttcher *et al.*, 2000) that seeing a feature such as the second stalk in the two-dimensional projections of the negatively stained ATP synthase is more surprising than expected, considering that the stator is probably formed by only two α helixes (from residues 24–80), which are probably folded as a coiled-coil or a four-helix bundle. In addition to the small mass, the stator might also be flexible, as suggested by experiments in which residues of the *b* subunits have been removed or additional ones introduced without affecting the function of the complex (Sorgen *et al.*, 1998). It has been speculated that this flexibility of the stator is an important feature of the energy-conversion mechanism of the ATP synthase (Cherepanov *et al.*, 1999).

POSITION OF THE δ SUBUNIT

Comparison of projection images, such as the ones shown in Fig. 1a, with the crystal structure of the F_1 revealed the presence of a 3×4 nm density on the very top of the F_1 which is not present in the crystal structure (Fig. 1b). A very similar density can be seen on top of the ATP synthase in cryo images of lipid-bound enzyme (Gogol *et al.*, 1987), indicating that the density seen in the detergent-free complex is not an artifact due to negative staining. Originally, this density had been interpreted as the very N-termini of the α and β subunits (Wilkens and Capaldi, 1998a,b), which are more or less disordered in the F_1 crystal structure (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998). However, adding up the N-termini not present in the coordinate file gives only 88 residues or about 10 kDa. Because the observed density looked more like 20–25 kDa, something else had to be there, binding on top of the F_1. An obvious candidate for the density was the δ subunit, which was known to be near the top of the F_1, close to the αβ N-termini (see above). Therefore, we decided to determine the position of the δ subunit by electron microscopy. Dunn and Tozer (1986) had described a monoclonal antibody (δ-2) that was able to bind to the intact enzyme in *E. coli* inner membranes (Dunn and Tozer, 1986). Electron microscopic images of detergent-free *E. coli* ATP synthase decorated with δ-2 showed the antibody binding on the very top of the F_1, thus confirming that the density seen on the top of the F_1 is indeed, at least in part, constituted by the δ subunit (Wilkens *et al.*, 2000). Figure 2 summarizes image analysis of the *E. coli* ATP synthase decorated with δ-2 mAb.

As can be seen from Fig. 2, very similar projections for the δ-2 labeled complex are obtained as for the unlabeled enzyme shown in Fig. 1a. The main difference is the presence of additional density on top of the F_1 as a result from the bound antibody (see arrow in image a1). Immunoblotting of various fragments of the δ subunit with δ-2 showed that the epitope of the antibody is located in the C-terminal 42 residues of the subunit (Wilkens *et al.*, 2000). These results show that the δ C-terminus is part of the density seen on top of the F_1. The δ and *b* subunits have been shown to interact via their C-terminal residues (McLachlin *et al.*, 1998). Taking these data together implies that the *b* subunit(s) have to go all the way from the membrane

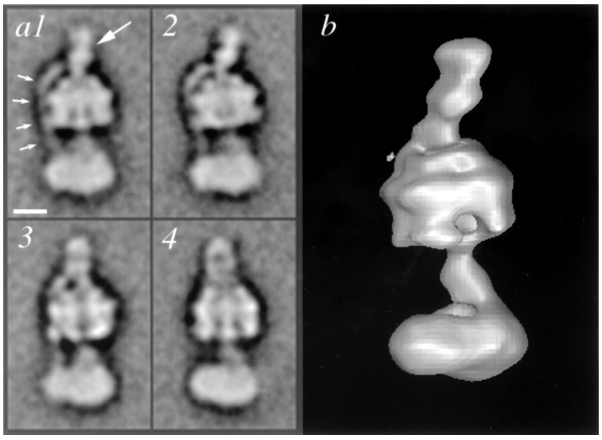

Fig. 2. Image analysis of the *E. coli* ATP synthase decorated with a monoclonal antibody directed against the δ subunit. A data set of 3746 molecules has been analyzed. (a) The images show the same features as the projections shown in Fig. 1, except the additional density on top of the F_1 from the δ-2 monoclonal antibody. Bar = 5 nm. (b) Three dimensional model calculated from two-dimensional projections, such as the ones shown in (a) (S. Wilkens, unpublished). The antibody can be clearly seen binding in the center top of the F_1. The second stalk is not visible at the contour level chosen.

surface to the top of the F_1 to meet the δ subunit C-terminus.

Interestingly, when looking at the averages of the δ-2 decorated enzyme, it appears that the second stalk is now visible all the way from the membrane surface to the very top of the F_1 (see small white arrows in Fig. 2, image *1*). It is possible that the antibody-labeled enzyme adopts slightly different orientations than the unlabeled complex, which might lead to the more prominent appearance of the stator in these images. It also should be noted here that the appearance of the second stalk in image *1* of Fig. 2 is virtually identical to the appearance of (one of) the second stalk(s) of the related V-ATPase, including the sausagelike density (Wilkens *et al.*, 1999, image *5* of Fig. 3). This striking similarity is further evidence for the structural and functional conservation of the F- and V-ATPases.

Recently, Böttcher and co-workers reported a 3D model of the ATP synthase calculated from two-dimensional sideview projections of the negatively stained *E. coli* enzyme (Böttcher *et al.*, 2000). The most dramatic conclusion of the analysis was that the density on top of the F_1 (interpreted in the paper as the N-termini of the large subunits) is undergoing a large conformational change in absence of nucleotide compared to enzyme saturated with AMP–PNP. This intriguing result would not necessarily have been expected based on a number of previous observations: First, the structure of the α and β N-terminal domains is essentially the same, no matter what nucleotide is bound to the corresponding subunit (Abrahams *et al.*, 1994; Shirakihara *et al.*, 1997; Bianchet *et al.*, 1998). Second, crosslinking of δ to α (Ogilvie *et al.*, 1997; 1998) or δ to *b* (McLachlin and Dunn, 2000b) has no effect on enzyme functioning. On the other hand, crosslinking of *b* to α does affect enzyme functioning (Rodgers and Capaldi, 1998), which indicates that some structural changes on the top of the F_1 might occur during catalysis. Further study will be required to understand the nature of these changes in this region of the molecule.

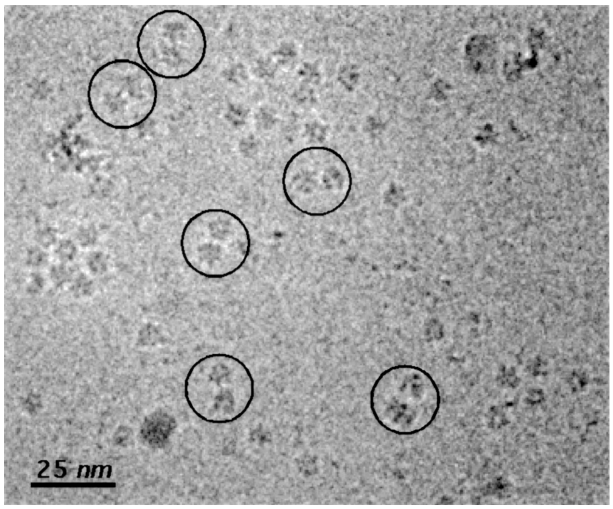

Fig. 3. Cryoelectron microscopy of the detergent-free ATPsynthase. A few projections, which could be interpreted as side views are circled.

CRYO ELECTRON MICROSCOPY OF THE ATP SYNTHASE

The image analysis, presented so far, has been performed with images of negatively stained protein molecules. The resolution in these images is limited by the grain size of the heavy metal salt (here uranyl acetate) and the nonnativeness of the stain environment. Particularly, small changes to the protein structure due to dehydration can not be ruled out although the above-described comparison of the F_1 crystal structure with the EM projections shows no evidence that this happens to a significant degree. Nevertheless, the preferred method for studying the structure of the solubilized ATP synthase should be cryoelectron microscopy ("cryo") in combination with image analysis. This emerging technique has been used successfully to study the 3D structure of large, water-soluble macromolecules in the 10–20 Å resolution range. Examples include the ribosome, viral capsids, and the *E. coli* F_1-ATPase. However, there are only few examples where detergent-solubilized membrane proteins have been analyzed by cryo EM. Detergents often lead to difficulties in obtaining good quality images and protein aggregation is frequently observed. Amphipols, on the other hand, do not share the adverse properties of detergents. Figure 3 shows cryo EM of the detergent-free *E. coli* ATP synthase.

The diminished contrast of the cryo images compared to images of stain-embedded protein, as well as the increased orientational freedom of the molecules, makes analysis of a large number of individual projections necessary. A detailed analysis of images, such as one shown in Fig. 3, is ongoing.

MODEL OF THE SUBUNIT ARRANGEMENT IN THE ATP SYNTHASE

Figure 4 illustrates the current working model of the subunit arrangement in the bacterial F_1F_0-ATP

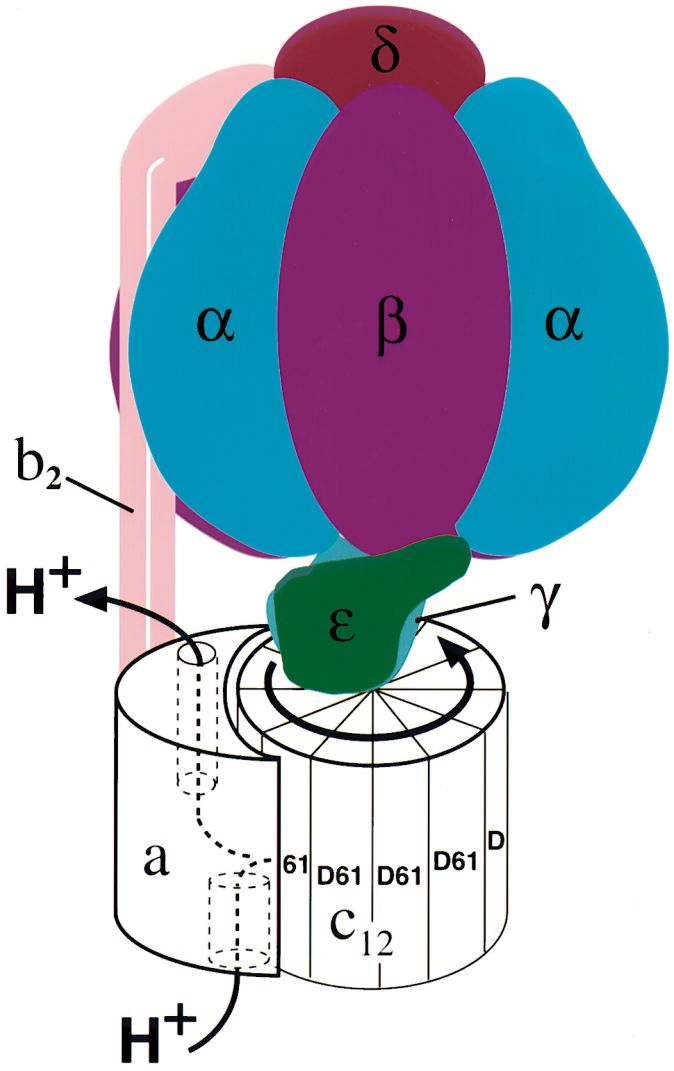

Fig. 4. Refined model of the subunit arrangement in the *E. coli* F_1F_0-ATPsynthase. For details, see text.

synthase. Positioning of the δ subunit together with the second stalk completes the overall subunit arrangement in the F_1-domain. The C-terminus of δ is poorly ordered in solution (Wilkens *et al.*, 1997), while some tertiary structure might exist in the *b* subunit C-terminus (Dunn *et al.*, 2000). In the model, the C-termini of both the *b* and δ subunits interact to form a tight connection between membrane domain and F_1.

Much less is known about the structure of the F_0. The structure of the *c* subunit has been solved by NMR (Girvin *et al.*, 1998) and based on genetic and cross-linking experiments, a detailed model of the F_0 is emerging (Fillingame *et al.*, 2000). Interestingly, recent structural and genetic/biochemical studies seem to indicate that the number of *c* subunits might not be the same for all species and/or growth conditions. Rings of 10 and 14 *c* subunits have been observed by X-ray analysis of a subcomplex of the yeast ATP synthase (Stock et al., 1999) and by AFM studies of 2D crystals of a chloroplast ATP synthase complex III oligomer (Seelert *et al.*, 2000), respectively. In the *E. coli* ATP synthase, a number of 12 *c* subunits per F_0 has been found by genetic fusion experiments (Jones and Fillingame, 1998), while other reports indicate that the number of *c* subunits in *E. coli* might be dependent on the conditions under which the organism is grown (Schemidt *et al.*, 1998).

Taken together, these data clearly show that *c* subunits (from whatever organism) are able to form oligomeric rings containing between 20 and 28 trans-

membrane α helixes. Further structural studies will be necessary to reveal the arrangement of the *c* subunits in their native environments in presence of all the subunits interacting with the proteolipid ring.

EPILOG

It would be highly preferred to explore the structure of the ATP synthase while the enzyme is bound in a lipid bilayer, in presence of an electrochemical gradient. Obviously, new methods have to be developed, or existing ones refined, to make this possible. This means that for the time being, we have to take the enzyme out of its native environment to be able to study its structure at high resolution. As yet, the ATP synthase has escaped a full structural analysis by X-ray crystallography, probably due to the presence of detergents and conformational heterogeneity. That means that for the time being, electron microscopy will continue to provide structural information which will lead to a more detailed understanding of the mechanism of the ATP synthase.

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