A Unique Resting Position of the ATP-synthase from Chloroplasts*

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The chloroplast ATP-synthase catalyzes ATP synthesis-coupled to transmembrane proton transport. The enzyme consists of two parts, a membrane-embedded F₀ part and an extrinsic F₁ part, which are linked by two connectors. One of these rotates during catalysis and the other remains static. Although the atomic structures of various sub-complexes and individual subunits have been reported, only limited structural information on the complex, as a whole, is available. In particular, information on the static connector is lacking. We contribute a three-dimensional map at about 20-Å resolution, derived from electron cryomicroscopy of enzymes embedded in vitrified buffer followed by single particle image analysis. In the three-dimensional map both connectors, between the F₁ part and the F₀ part, are clearly visible. The static connector is tightly attached to an α subunit and faces the side of the neighboring β subunit. The three-dimensional map provides a scaffold for fitting in the known atomic structures of various subunits and sub-complexes, and suggests that the oxidized, non-activated ATP-synthase from chloroplasts adopts a unique resting position.

F-type ATP-synthases are found in bacteria, chloroplasts, and mitochondria. They catalyze ATP synthesis/hydrolysis coupled to transmembrane proton transport. All ATP-synthases consist of two parts, a membrane-embedded F₀ part and a membrane extrinsic F₁ part, which are connected by a thinner connecting region. In the ATP-synthase from chloroplasts, the F₁ part is composed of five different subunits with the stoichiometry (αβ)₃γδε. The α and β subunits surround the central γ subunit, which, together with the ε subunit, forms the central stalk. The F₀ part is involved in proton translocation and consists of four different subunits I, II, III, and IV. Subunit III is the major component, 14 copies of the subunit III form a ring (1). A segment of this ring, together with subunit IV, forms the proton channel (2). Subunits I and II have an amphiphilic character, with a single transmembrane helix serving as a membrane anchor. The homologous β subunits in Escherichia coli form a dimer with inner-dimer contacts in the transmembrane N-terminal region (3) as well as between amino acids 53 and 122 (4). The b-dimer connects the F₀ part to the F₁ part by interacting with the δ subunit (5) at the top of F₁ (6) and forms a peripheral stalk.

According to a current functional model for F-type ATPases (2, 7), the subunit III-ring in the membrane and the γ and ε subunit in the stalk region form a rotor, which rotates during proton translocation. This rotation induces conformational changes in the catalytic nucleotide binding sites during ATP synthesis/hydrolysis. A second static connection (stator) formed by subunits I and II, prevents co-rotation of the (αβ)₃ core complex. The orientation of the rotor determines the occupancy of the three catalytic nucleotide binding sites. There are three equivalent orientations of the central rotor relative to the individual catalytic binding sites as shown by micro videograms (8). Because rotational catalysis requires functional equivalence of the catalytic binding sites, all three conformations should occur with the same probability. In the ATP-synthases from chloroplasts, this type of rotational multisite catalysis requires activation of the enzyme by a transmembrane potential difference of protons, Δψ₁ (9, 10). Up to now it is not understood if the inactivate chloroplast ATP-synthase also adopts each of the three conformations with the same probability or takes up a unique resting conformation.

Although various high resolution structures of sub-complexes from different F-type ATPases are known (for example, bovine MF₁ (αβ)₃γδε (11); chloroplast CF₁ (αβ)₃ (12); E. coli EF₁ (αβ)₃γ (15); yeast F₁c₁₀ (αβ)₃γδεc₁₀ (14)), none of these sub-complexes include homologues of the smaller subunits that form the stator in the chloroplast ATP-synthase. Because the small static subunits are missing, which are asymmetrically attached to the (αβ)₃ core, the individual nucleotide binding sites are only defined in respect to the rotor and not in respect to the stator, which is insufficient to distinguish between the three possible conformations. Information on this issue can be obtained by electron microscopy and image reconstruction of a complete ATP-synthase. Up to now, projection maps of the ATP-synthase from mitochondria (15), chloroplasts (16), and E. coli (17), which show rotor and stator in the connecting region, have been obtained. However, two-dimensional projection maps alone, without prior knowledge of the three-dimensional shape, are inadequate to discern between different conformational states and projections of the same object in different directions. Such a discrimination can only be done, if the three-dimensional volumes are also known. Nevertheless, three-dimensional maps at 30- to 35-Å resolution, of the negatively stained E. coli (18) and chloroplast enzymes (19), show the stator either weakly or not at all. Therefore, three-dimensional maps are still insufficient to decide whether or not the complex adopts different conformations. Here we present a three-dimensional map of the ATP-synthase from chloroplasts embedded in vitrified buffer, in which the stator and rotor are clearly visible and only a single conformation, as expected for a unique resting position, is observed.

EXPERIMENTAL PROCEDURES

Preparation of the Protein for Electron Microscopy—The ATP-synthase was purified according to a previous study (19), which yields a complex where the regulatory disulfide bond of the γ subunit is oxidized. The buffer was changed by passage through a Sephadex G-50 spin-column equilibrated in 0.1 mM dodecyl maltoside, 10 mM MgCl₂, 10
The scaled three-dimensional Fourier transform was transformed to real space to give the final three-dimensional map (Fig. 5), which showed the same gross features as the raw map but smaller holes and reduced dark fringes, which facilitated fitting of the high resolution structures. In this approach, the absolute threshold for binarization was not too critical, however, the absolute size and shape of the binarized reference map were crucial. Therefore, scaling the electron microscopic reconstructions with atomic models of smaller sub-complexes, or models lacking the detergent micelle, yields poorer results than using the binarized reconstruction for scaling (data not shown).

To demonstrate the effect of the scaling approach, a model experiment was performed: from the yeast F1c10 sub-complex (Protein Data Bank 1QO1 (14)) a density map at 20Å resolution was calculated (Fig. 1A). This map was convoluted with the contrast transfer function as calculated for a defocus of 3000 nm using a Philips CM-200-FEG operating at 200 kV. The resulting map showed dark fringes and holes at places where holes were not observed in the original map. After correcting phases for the contrast transfer function, the holes were observed at the correct positions (Fig. 1C). However, they were darker and larger than in the original data. In addition, dark fringes surrounded the particle. Only little improvement was observed, when various reconstructions were calculated using different defocus values and had phases corrected for the contrast transfer functions were combined (Fig. 1D). We assume, that the dark fringes and holes are predominantly effects of uncorrected amplitudes at low spatial frequencies. In the "true" reconstruction, we do not expect any negative values (dark fringes and dark holes). This situation was approximated by a binarized map (Fig. 1E), the threshold of which was chosen so the total number of voxels set to 1 accounted for the expected molecular mass of the ATP-synthase surrounded by the detergent micelle (∼750 kDa). Raw map and binarized map were Fourier-transformed. For each band of spatial frequencies (\( R_1 \leq R < R_2 \)) the ratio of the average amplitudes \( A \) was calculated,

\[
\text{ratio (band)} = \frac{\sum_{R<R_2} A_{\text{raw}}(R)}{\sum_{R<R_1} A_{\text{raw}}(R)}
\]

The ratio varied at low spatial frequencies \( R \) and reached constant values for a small band of frequencies at about \( RR = 1/45 \) Å⁻¹. At frequencies below this band, the ratio was used as a scaling factor and at frequencies above this band the constant ratio in the band was used for scaling of the amplitudes \( A_{\text{raw}} \) in the raw map as follows. For \( band < RR \),

\[
A_{\text{bin}}(R) = A_{\text{raw}}(R) \times \text{ratio (band)}
\]

and for \( band \geq RR \),

\[
A_{\text{bin}}(R) = A_{\text{raw}}(R) \times \text{ratio (RR)}
\]

The abbreviations used are: AMP-PNP, adenosine 5’-(β,γ-imino)-triphosphate; PDB, Protein Data Bank.
The Connecting Region—The F1 and F0 part were joined by two connecting elements, a thin peripheral connector and one that was larger, and more centrally localized. The latter was formed by subunits γ and ε. In the past, two different conformations have been observed for the central connection. One was derived from crystals of a γε sub-complex of the E. coli enzyme (29) and the other from a mitochondrial F1 (11), and it has been argued that both conformations occur during catalysis (13). The conformations vary in the orientation of the ε subunit (or related δ subunit in mitochondria F1) relative to the γ subunit with the ε subunit rotating 81° and undergoing a net translation of 23 Å. We fitted both types of central connections into our map (not shown) and found the conformation observed in the mitochondrial F1 was the best match (Fig. 6).

In contrast to an earlier reconstruction of the negatively stained ATP-synthase from E. coli, where two peripheral connectors have been identified (18), only one peripheral connection was observed. Whether or not the two connections observed in the ATP-synthase of E. coli are a specialty of the bacterial enzyme or some artifact probably caused by the staining procedure remains open.

In the chloroplast ATP-synthase the thinner, peripheral connection was attached at the periphery of the α subunit of the F1 part (Fig. 3B, element 1). The connection was wider at the sites of interaction with the F0 and F1 part and thinner between these points. It remains uncertain if this slimming is a genuine feature of the connection or only appears in our model due to increased flexibility in this area. Whichever the case, we think that this peripheral link is the stator, formed by subunits I and II, in the ATP-synthase from chloroplasts. For the related

domain corresponded to the F1 part and the smaller domain to the F0 part, shielded by the detergent micelle.

The class averages were combined into a three-dimensional map by exact filtered back-projection reconstruction (25) (Fig. 3B). All class averages could be matched by projections of the three-dimensional map (Fig. 3C). The Euler angles of the class averages covered most of the asymmetric unit (Fig. 4), leaving only small areas unaccounted for. To estimate the overall resolution, we calculated the Fourier shell correlation between two maps, each representing half of the data. The correlation dropped to 0.5 at (1/21) Å⁻¹ and cut the three-times noise correlation curve at (1/18) Å⁻¹, indicating an overall resolution of about 20 Å.

The F1 Part—In our three-dimensional map the F1 part was formed by six elongated elements arranged roughly hexagonally around a central rod (see map in Fig. 5B, labeled 1–6). These elements could also be recognized by projections of the surface representations (Fig. 3B) and most likely correspond to the α and β subunits. To decide which of the elements were α and which were β subunits, we compared this region of the map to the atomic structures of different F1 sub-complexes (PS3-(αβ)₃ (26); chloroplasts-(αβ)₃ (12); mitochondrial-(αβ)₃γ (27)). Although these sub-complexes had different nucleotide occupancies and varied significantly in the region nearest to the connecting region, the α subunits always extended further from the center than the β subunits. We observed a similar pattern in our map (Fig. 5B, 2), where the elements 1, 3, and 5 extended further from the center than 2, 4, and 6. For this reason, we conclude that the α subunits form elements 1, 3, and 5 and that the β subunits are represented by elements 2, 4, and 6. The αβ-dimers in our map were not related by strict 3-fold symmetry. Therefore, we fitted the asymmetric (αβ)₃ sub-complex of the mitochondrial F1 (PDB 1E79 (11)), rather than the structure of the symmetric (αβ)₃ sub-complex of the F1, from chloroplasts (PDB 1FX0 (12)), into our three-dimensional map (Fig. 6). The match between the mitochondrial F1 sub-complex and our map was best when the β subunit with the empty nucleotide binding site was superimposed to the elongated element 6 in our map.

We propose that the rod in the center of the six elongated elements (Fig. 5A, elements 6 and 7, and 5B, element 2) correspond to the two long helices of the γ subunit in the atomic structure of the mitochondrial F1 part.

Fitting the α and β subunits and the two long helices of the γ subunit to the map, as described above, accounted for most of the observed density in the F1 part. In the F1 part only a peanut-shaped density at the top and an elongated density, running parallel to the α subunit corresponding to element 1, was unaccounted. The peanut-shaped feature at the top of F1 part consisted of two domains (Fig. 5B, 1). One of the domains was located in the center of a crown-like region at the top of the α and β subunits, formed by the β-barrels, as seen in the x-ray structure (PDB 1E79 (11)). The other domain created a bridge to the α subunit in element 1. We assume that most of the peanut-shaped feature was occupied by subunit δ, because its central domain coincided with the area that is recognized by a monoclonal antibody against the C-terminal region of subunit δ in E. coli (6). Consequently, in our three-dimensional map the N-terminal domain of subunit δ must occupy the remaining peripheral domain into which the structure of the N-terminal domain of E. coli δ subunit (28) fitted nicely (Fig. 6).
b-subunits of *E. coli*, secondary structure determination indicates a helix content of 80% (30), suggesting that the observed stator might contain only a little more than two extended helices, which is also supported by x-ray analysis (31). In the F₁ part, the stator does not account for an individual feature and is presumably tightly attached to the \( \gamma \)-subunit (element 1), pointing toward the \( \delta \)-subunit (element 6).

To follow the path of the stator through the F₁ structure, we calculated the difference between the volume occupied by the manually fitted x-ray structures and the volume occupied by our three-dimensional map (Fig. 7 A). In the F₁ part and in the connecting region, the fitted x-ray structures did not account for subunits I and II, the C-terminal domain of the \( \gamma \)-subunit, and the disordered N-terminal residues of the \( \alpha \)- and \( \beta \)-subunits. According to the difference, the stator contacted the \( \alpha \)-subunit (element 1), involved in forming the tight catalytic nucleotide binding site in the center, at the “bottom.” Then the stator lined the side of the \( \alpha \)-subunit, which faced the \( \beta \)-subunit that forms part of the empty catalytic nucleotide binding site (Fig. 5 B, element 6). At the top of the F₁ part, the stator ended with a contact to the \( \delta \)-subunit at the uppermost part of the N-terminal domain. The observed position of the stator is in agreement with earlier cross-linking data for the stator-forming \( \beta \) and \( \alpha \)-subunits in *E. coli* (32, 33).

**The F₀ Part**—The F₀ part had an elliptical cross-section in the plane of the membrane (Fig. 5 B, 4). An outer oval belt, probably formed by detergent, surrounded an inner circular ring of similar diameter, as the subunit III complex observed by atomic force microscopy (1). Therefore, we conclude that the subunit III complex forms the inner ring. To get some impression of its space requirements in the context of our map, we generated a ring of 14 copies of the related *E. coli* c-subunit. There are two structures available from NMR measurements that vary in the deprotonation of Asp-61, which in *E. coli* has a \( pK_a \) value of 7.1 (34). Accordingly, we generated a ring of the protonated (35) and the deprotonated (36) conformation of the related c-subunit (36) using MolMol (37) and placed both in the observed density. The manual placement was guided by the hole in the center of the F₀ part in our map, which we superimposed on the central channel of the modeled ring. A belt of unaccounted density surrounded both modeled rings. This belt was smaller due to the modeled ring of the deprotonated form. However, at the level of observed detail, both fits were somewhat arbitrary. In Fig. 6 the modeled ring of the deprotonated form is shown, because at pH 7.2 this conformation should be adopted by the majority subunits. The ring was sealed from one side by the central stalk and from the other...
side by a plug. The nature of the plug was unclear, but it was probably formed by either detergent or by tightly bound lipids. The latter was observed in two-dimensional crystals of the related c complex of I. tartaricu (38). When the volume occupied by the modeled ring was subtracted from the observed density, a belt with a bulge was left at the side where the stator emerged. We conclude that the membrane domains of subunits I, II, and IV formed this bulge.

DISCUSSION

ATP-synthases work with a rotational mechanism, which requires the catalytic nucleotide binding sites to be functionally equivalent. Accordingly, there should be three equivalent conformations in which the catalytic nucleotide binding sites have the same overall occupancy, but vary in the occupancy of the individual binding sites as depicted in Fig. 8. In all three conformations, the core of (αβ)3γε IIIγε should have the same structural organization. Only the presence of the small peripheral subunits (I, II, IV, and δ) provides the means to distinguish between the three conformations. In the three conformations, the small peripheral subunits also have to adapt to spatially different environments, because the invariable core does not have a strict 3-fold symmetry. In our samples, we have the small static subunits present, and therefore, we are in principle, able to discern between the three conformations. Nevertheless, at the given resolution, our data does not show any indication of the presence of multiple conformational states. All observed projections can be matched by projections calculated from the same three-dimensional map, which is one indication that a conformationally homogeneous particle population was explored (19). We can also exclude the possibility that particle images representing different conformations were accidentally combined in the three-dimensional map for the following reason: if particles with the described variation in conformations would have been averaged in our three-dimensional map, we would either expect the occurrence of three weak peripheral connectors or of a central connector with a 3-fold symmetry axis approximately parallel to the long molecule axis and/or with a fuzzy outline. However, we observe only a single peripheral connector and a central stalk with a well-defined asymmetric shape. This stalk accommodates the atomic model of the mitochondrial central stalk in only one orientation. The two alternative orientations of the central stalk, which could be expected for the other two equivalent conformations (Fig. 8), do not match the observed density (not shown). Therefore, we conclude that the majority of the isolated, inactive ATP-synthases adopts a single unique resting position (equivalent to the one depicted in the center of Fig. 8). At present we do not know if this is a specialty of the ATP-synthase from chloroplasts, which requires activation by D. J. (2000) Nature 274, 418–419

FIG. 8. Schematic representations of the three-functionally equivalent conformations of the ATP-synthase. The ATP-synthase is depicted from the top, perpendicular to the plane of membrane. The conformationally invariant core is marked by a black outline and consists of α subunits (white), β subunits (medium grey), the γ subunit III ring (light grey), and the γ subunit (black). The subunits forming the catalytic binding sites are labeled according to the nomenclature introduced by Abrahams et al. (27) (E for empty, D for ADP, and T for ATP or ADP). This naming convention does not necessarily reflect different occupancy in the catalytic binding sites but is also used in other structures (40) where the identity of the sites is determined by the orientation of the γ subunit. At the periphery of F, the stator (outlined by the dashed lines) is formed by subunits I, II, and IV and adapts to the spatially different environments of the three conformations. The putative, unique resting position is equivalent to the conformation shown in the center.

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