Ground State Structure of F$_1$-ATPase from Bovine Heart Mitochondria at 1.9 Å Resolution

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The structure of bovine F$_1$-ATPase, crystallized in the presence of AMP-PNP and ADP, but in the absence of azide, has been determined at 1.9 Å resolution. This structure has been compared with the previously described structure of bovine F$_1$-ATPase determined at 1.95 Å resolution with crystals grown under conditions identical to those used in the first structural analysis, azide was resolved in the DP$_i$-subunit. In the present structure, the nucleotide binding sites in the $\beta_{DP}$- and $\beta_{TP}$-subunits are both occupied by AMP-PNP, whereas in the earlier structure, the $\beta_{TP}$ site was occupied by AMP-PNP and the $\beta_{DP}$ site by ADP, where its binding is enhanced by a bound azide ion. Also, the conformation of the side chain of the catalytically important residue, $\alpha$Arg-373 differs in the $\beta_{DP}$- and $\beta_{TP}$-subunits. Thus, the structure with bound azide represents the ADP inhibited state of the enzyme, and the new structure represents a ground state intermediate in the active catalytic cycle of ATP hydrolysis.

Our current understanding of the molecular mechanism of F$_1$-ATPase is based on the structural analysis by x-ray crystallography of the enzyme from bovine heart mitochondria. The first high resolution structure (1), now known as the “reference” structure, determined at 2.8 Å resolution with crystals grown in the presence of both ADP and the nonhydrolyzable ATP analog AMP-PNP, showed that the three noncatalytic $\alpha$-subunits and the three catalytic $\beta$-subunits are arranged in alternation around an asymmetric $\alpha$-helical structure in the single $\gamma$-subunit. The $\alpha$- and $\beta$-subunits have similar folds consisting of an N-terminal domain with six $\beta$-strands, a central nucleotide binding domain made of both $\alpha$-helices and $\beta$-strands and an $\alpha$-helical C-terminal domain containing six $\alpha$-helices in $\beta$-subunits and seven in $\beta$-subunits. Because of the asymmetry of the $\gamma$-subunit, the catalytic $\beta$-subunits adopt different conformations with different nucleotide occupancies. Two of them have similar conformations, but one, designated as $\beta_{DP}$, contains bound ADP, and the second, $\beta_{TP}$, has bound AMP-PNP. The third has adopted a radically different conformation in which the nucleotide binding domain has been disrupted by an outward hinging movement of part of the domain and the attached C-terminal domain in response to the curvature of the central $\alpha$-helical structure of the $\gamma$-subunit. This $\beta$-subunit has no bound nucleotide, and so it is known as the “empty” or open state, designated as $\beta_{z}$. To explain the interconversion of catalytic sites through “tight,” “loose,” and “open” states required by a binding change mechanism of catalysis of ATP hydrolysis by F$_1$-ATPase (2), it was proposed that the interconversion of sites is effected by a mechanical rotation of the $\gamma$-subunit, each 360° rotation taking each $\beta$-subunit through the three states and thereby hydrolyzing three ATP molecules. It was shown subsequently that during ATP hydrolysis, either in an $\alpha\beta_2\gamma$ complex or in the intact F$_1$F$_{0}$-ATPase, that the direction of rotation is counterclockwise (as viewed from the membrane domain of the enzyme) during ATP hydrolysis and clockwise during ATP synthesis (3, 4). This first structure of bovine F$_1$-ATPase was interpreted as representing a state in the active catalytic cycle, the ADP-inhibited state of the enzyme, or both. The crystals used in this analysis were grown in the presence of azide, primarily to prevent microbial growth during the 2–3-month period of crystallogenesis. However, in the presence of ADP, azide is an inhibitor of F$_1$-ATPase (5), but because of the relatively modest resolution of the data, its presence was not detected in the structure.

Recently, in a related structure at 1.95 Å resolution, determined with crystals grown under conditions identical to those used in the first structural analysis, azide was resolved in the $\beta_{DP}$-subunit (6). It is associated via the partial positive charge of its two peripheral nitrogen atoms with $\beta$Lys-162 in the P-loop region of the nucleotide binding site and with the catalytically essential residue, $\alpha$Arg-373. Hence, azide appeared to enhance the binding of ADP to the $\beta_{DP}$ site and to stabilize the ADP-inhibited state of the enzyme. In the present work, crystals of bovine F$_1$-ATPase were grown in the presence of both ADP and AMP-PNP under conditions identical to those employed previously, except that azide was omitted. The structure of this complex determined at 1.9 Å resolution (see footnote $b$ in Table 1) shows that, despite the presence of both ADP and AMP-PNP, both the $\beta_{TP}$ and $\beta_{DP}$ nucleotide binding sites were occupied by AMP-PNP. As described below, this structure is the most accurate represen-
tation to date of an intermediate in the catalytic cycle of ATP hydrolysis by the enzyme.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Crystallization—Bovine mitochondrial F$_1$-ATPase was purified as described previously (7), except that the Sephacryl S-300 column was replaced by a HiLoad 26/60 Superdex 200pg column, and 5 mM 2-mercaptoethanol was not included in the buffer. An ammonium sulfate precipitate of purified bovine mitochondrial F$_1$-ATPase was redissolved in minimal buffer. Crystals of F$_1$-ATPase were grown in microdialysis buttons (50 µl) with SpectraPor dialysis membranes (3500 molecular weight cut-off). An equal volume of inside buffer (100 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM MgCl$_2$, 2 mM AMP-PNP, 50 µM ADP, 0.004% (w/v) phenylmethylsulfonyl fluoride, and 14% (w/v) polyethylene glycol 6000 in D$_2$O) was added slowly to the protein, and the solution was mixed gently (final concentration 5 mg/ml). The samples were dialyzed against 3 ml of outside buffer (50 mM Tris-HCl, pH 8.2, 200 mM NaCl, 20 mM MgSO$_4$, 250 µM AMP-PNP, 5 µM ADP, 0.004% (w/v) phenylmethylsulfonyl fluoride, and 9% (w/v) polyethylene glycol 6000). After 48 h, this buffer was replaced with the same buffer containing polyethylene glycol 6000 from 10–12.5% (w/v) in 0.25% steps. Crystal growth was complete in 4 weeks.

Dehydration of Crystals—Crystals were harvested in a MicroMesh loop (MiteGen, Ithica, NY). Their diffraction properties were monitored during dehydration in a gradient of relative humidity of water from 99–94% in a free mounting system (Proteros Biostructures, GmbH, Martinsried, Germany) using established conditions for improved diffraction of crystals of bovine F$_1$-ATPase (8). The crystals were coated in a thin film of perfluoropolyether oil (Alfa Aesar, Heysham, UK), plunged into liquid nitrogen, and stored at 100 K.

Data Collection—Diffraction data for the N$_3$-free-F$_1$ crystals were collected to 1.9 Å resolution on a charge-coupled detector Q210 from Area Dector Systems Corp. (Poway, CA) at beamline ID14-2 (λ = 0.933 Å) at the European Synchrotron Radiation Facility (Grenoble, France). The diffraction data were processed with MOSFLM (9) and programs from the Collaborative Computational Project Number 4 (CCP4) suite (10) (see Table 1 for data collection and refinement statistics).

Solution and Refinement of the Structure—The structure was solved by molecular replacement with MOLREP (11) using the N$_3$-F$_1$ structure (6) (Protein Data Bank accession code 2CK3) as a search model (with ADP and AMP-PNP as bound ligands in β- and α-subunits, respectively). The refinement was carried out with REFMAC5 (12) alternating with manual rebuilding with COOT (13). Some water molecules were built with ARP/WARP (14). Stereochemistry was assessed with COOT (13), and Figs. 1–3 and 5 were produced with PyMol (15).

RESULTS AND DISCUSSION

The Structure of F$_1$-ATPase Determined with Crystals Grown in the Absence of Azide—As observed with the crystals of bovine F$_1$-ATPase grown in the presence of azide (6), the diffraction properties of the present crystals were improved substantially by controlled dehydration, allowing data to be collected to 1.9 Å, which is an exceptionally high resolution for such a large and mobile complex. The crystals belong to the space group P2$_1$2$_1$2$_1$. Their unit cell dimensions are a = 261.8 Å, b = 105.6 Å, and c = 123.1 Å, and there is one F$_1$ complex in the crystallographic asymmetric unit. The structure, known as N$_3$-free-F$_1$, was solved by molecular replacement

TABLE 1
Data processing and refinement statistics

| Space group       | P2$_1$2$_1$2$_1$ |
|-------------------|------------------|
| Unit cell dimensions (Å) a, b, c | 261.8, 105.6, 123.1 |
| Resolution range (Å)          | 20.0-1.9 |
| No. of unique reflections     | 194,999 |
| Multiplicity$^a$ (%)          | 3.1 (1.5) |
| Completeness$^a,b$ (%)         | 72.8 (18.8) |
| R$_{merge}$$^{a,c}$ (%)        | 0.060 (25.7) |
| (I/σ(I))$^{a}$              | 15.9 (1.7) |
| B-factor (from Wilson) (Å$^2$) | 23.5 |
| Water molecules               | 2340 |
| R-factor$^a$ (%)              | 17.5 |
| Free R-factor$^a$ (%)          | 22.0 |

$^a$ Statistics for the highest resolution bin (1.96-1.9 Å) are shown in parentheses.

$^b$ The completeness is 88.2% at 2.27 Å; the completeness to 1.9 Å is low as these data were only recorded in the corners of the detector.

$^c$ $R_{merge} = \sum h |F_{o} - F_{c}| / \sum h |F_{o}|$, where $F_{c}$ and $F_{o}$ are the observed and calculated structure factor amplitudes.

$^d$ $R_{free} = \sum h |F_{o} - F_{c}| / \sum h |F_{o}|$, where $F_{c}$ and $F_{o}$ are the observed and calculated structure factor amplitudes and $T$ is the test set of data omitted from refinement (5% in this case).

r.m.s.$^e$ deviations

| Bonds (Å) | 0.008 |
| Angles (°) | 1.1 |

$^e$ r.m.s., root mean square.

FIGURE 1. The β$_{162}$ binding site in the structure of the azide-free F$_1$-ATPase from bovine heart mitochondria. The view is stereo. The difference electron density ($F_o - F_c$) for the γ-phosphate, contoured at 3σ before its inclusion in the model, is shown as a green mesh. The 2$F_o - F_c$ densities for ADP and αArg 373 are shown as a blue mesh contoured at 1.5σ. The red and green spheres represent the catalytic water molecule and a magnesium ion, respectively.
Azide-free $F_{1}$-ATPase

![Comparison of the catalytic sites in the $\beta_{\text{TP}}$-subunits of the azide-free $F_{1}$-ATPase and $N_{3}$-$F_{1}$ structures.](image)

The view (in stereo) was made by superimposing the $\gamma$-loops of the two structures. The catalytic site in the azide-free structure is in color, and that in the $N_{3}$-$F_{1}$ structure is gray.

![Superimposition of the catalytic sites in the $\beta_{\text{TP}}$- and $\beta_{\text{DP}}$-subunits in the structure of the azide-free $F_{1}$-ATPase.](image)

The view is in stereo, and the $\beta_{\text{TP}}$ and $\beta_{\text{DP}}$ catalytic sites are colored and gray, respectively. In $A$, the view is taken from an aspect similar to that shown in Fig. 1. In $B$, the view illustrates the movement of $\alpha$Arg-373 by $\sim 1$ Å. As a result, the water molecule in the $\beta_{\text{DP}}$ catalytic site is positioned optimally for in-line nucleophilic attack of $\gamma$-phosphate in the $\beta_{\text{DP}}$-subunit.

using data to 1.9 Å. (The structure of $F_{1}$-ATPase inhibited with azide is known as $N_{3}$-$F_{1}$.) Data processing and refinement statistics are summarized in Table 1. The final model contains the following residues: $\alpha_{\text{TP}}$ 23–401 and 410–510; $\alpha_{\text{DP}}$ 21–510; $\alpha_{\text{E}}$ 24–510; $\beta_{\text{TP}}$ 9–474; $\beta_{\text{DP}}$ 9–475; $\beta_{\text{P}}$ 9–387 and 396–474; $\gamma_{1}$–47, 67–86, 105–116, 127–148, 159–173, and 206–273; $\delta$ 17–29, 35–40, 58–65, 76–81, and 91–145; and $\epsilon$ 1–25. The shrinkage of the unit cell parameter of the crystal in the $a$ dimension, brought about by controlled dehydration, stabilizes the central stalk subunits ($\gamma$, $\delta$, and $\epsilon$), as has been observed in other structures (16, 17). However, the concomitant reduction of the $c$ dimension by $\sim 20$ Å to a value of 123.1 Å leads to the $\beta$-sandwich domain of the $\delta$-subunit becoming disordered, because stabilizing crystal contacts in structures with a $c$ cell edge of $\sim 140$ Å are lost.

After one round of refinement, positive density peaks (height 12 and 14) representing the $\gamma$-phosphate of AMP-PNP were observed in the difference map for the $\beta_{\text{DP}}$- and $\beta_{\text{TP}}$-subunits, respectively (Fig. 1). The noncatalytic nucleotide binding sites contained AMP-PNP also. Thus, the $\beta_{\text{DP}}$-subunits in the $N_{3}$-$F_{1}$ and azide-free structures differ in so far as the former binds ADP and azide and the latter AMP-PNP. Nonetheless, their overall conformations differ very little; the root-mean-square difference between $C_{\alpha}$ chains of the $\beta_{\text{DP}}$-subunits is 0.22 Å, and the positions of the adenosine, $\alpha$- and $\beta$-phosphates, and catalytic side chains are very similar (Fig. 2). The root-mean-square deviation between the two $F_{1}$-ATPase complexes is 0.44 Å.

Superimposition of the P-loop residues of the $\beta_{\text{TP}}$-subunit onto those of the $\beta_{\text{DP}}$-subunit in the azide-free structure reveals small but significant differences between the two sites (Fig. 3). In the $\beta_{\text{DP}}$-subunit, a movement of the guanidinium group of $\alpha$Arg-373 toward the $\gamma$-phosphate of 1.0–1.5 Å relative to the $\beta_{\text{TP}}$-subunit rotates the
γ-phosphate of AMP-PNP and positions it for in-line nucleophilic attack by the water molecule that is polarized by βGlu-188. In addition, the nucleophilic water molecule in the β_{DP}-subunit has moved 0.7 Å closer to the γ-phosphate than in the β_{TP}-subunit of the azide-free structure (see Figs. 3 and 4). Similar changes have been noted previously in the β_{DP}-subunit in the structure of bovine F{\textsubscript{1}}-ATPase where ADP-BeF{\textsubscript{3}} is bound to both the β_{DP} and β_{TP} sites (18).

Recently, the structure of the F{\textsubscript{1}}-ATPase from Saccharomyces cerevisiae has been described at 2.8 Å resolution based on crystals grown in the presence of AMP-PNP and ADP and in the absence of azide (19). In this structure, AMP-PNP is also bound to both the β_{DP}- and β_{TP}-subunits. Superposition of the structures of the P-loops of the β_{DP}-subunits of the bovine azide-free F{\textsubscript{1}}-ATPase and the yeast F{\textsubscript{1}}-ATPase shows that the positions of the nucleotides and of the key side chains in the nucleotide binding sites are very similar (supplemental Fig. 1).

Position of αArg-373 in the β{\textsubscript{E}} Catalytic Site—The highly accurate electron density map of the azide-free F{\textsubscript{1}}-ATPase has provided evidence that αE-Arg-373, the arginine-finger residue in the β{\textsubscript{E}} catalytic site, has two conformations, each at about 50% occupancy (Fig. 5 and supplemental Fig. S2). One conformation of the side chain of this residue is oriented away from the catalytic site in the β_{E}-subunit, and the other is oriented toward it. The inward pointing conformation is very similar to the conformation of the equivalent side chain of αE-Arg-375 in yeast F{\textsubscript{1}}-ATPase (19), which is involved in the coordination of a phosphate (or sulfate) ion. It seems likely that the side chain in the bovine enzyme alternates between the two conformations and assumes the inward position when phosphate binds. Why phosphate (sulfate) is not bound in a similar way in the bovine N\textsubscript{3}-free-F{\textsubscript{1}} structure is unclear, especially because its concentration during crystallization of the bovine enzyme was 40 times greater than that employed in the crystallization of the yeast enzyme.

Roles of the β_{DP}- and β_{TP}-Subunits in Catalysis—The structure of azide-free bovine F{\textsubscript{1}}-ATPase described here, where AMP-PNP is bound in preference to ADP to the nucleotide binding sites in both the β_{DP}- and β_{TP}-subunits, illustrates the similarities between the two sites. These similarities, and the selectivity for AMP-PNP over ADP at the concentrations of nucleotides employed in the crystallization experiments, are consistent with the K_{d} values measured in the Escherichia coli enzyme, which show that AMP-PNP and ATP have a much higher affinity for both sites than ADP (20, 21). However, despite these similarities, in all of the structures that have been described of the bovine F{\textsubscript{1}}-ATPase (1, 6, 16, 18, 22–25) and in that of the yeast enzyme (19), the β_{DP}- and β_{TP}-nucleotide binding sites are not equivalent to each other. This structural inequivalence arises because the structure of F{\textsubscript{1}}-ATPase is inherently asymmetrical. Therefore, the interactions of β_{DP}- and β_{TP}-subunits with adjacent α-subunits differ, they occupy different positions in the structure relative to the β{\textsubscript{E}}-subunit, and they are adjacent to different aspects of the asymmetrical centrally located γ-subunit. These structural differences between β_{DP}- and β_{TP}-subunits make them functionally inequivalent, as various pieces of structural evidence show. First, the current structure and that determined in

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**FIGURE 4. Schematic representations of the nucleotide binding sites in the β_{DP}- and β_{TP}-subunits in the structure of the azide-free F{\textsubscript{1}}-ATPase. A, β_{DP}-subunit; B, β_{TP}-subunit. The distances between atoms are given in angstroms, and possible hydrogen bonds and interactions involving the magnesium ion are shown as dotted lines.**

**FIGURE 5. Comparison of the positions of the arginine-finger residue relative to the β{\textsubscript{E}}-subunits in the structures of azide-free bovine F{\textsubscript{1}}-ATPase and in yeast F{\textsubscript{1}}-ATPase. In the bovine enzyme, the side chain of αE-Arg-373 has two conformations (shown in green), each at 50% occupancy. The position of the equivalent side chain of αE-Arg-375 in the yeast enzyme is gray.**
the presence of azide (6) show that the βDP site, and not the βTP site, binds ADP and azide very strongly, but it can bind AMP-PNP also. In contrast, the βTP site does not bind ADP and azide, but it has a high affinity for AMP-PNP. Second, in both the current structure (see Fig. 4) and in the BeF3-F1 structure (18), the nucleophilic water molecule in the βDP subunit is significantly closer to the γ-phosphate of ATP (or rather its analogs) than in the βTP subunit. Third, in a transition state analog structure formed in the presence of ADP and aluminum fluoride (the (AlF3;ADP)-F1 structure (25)), the transition state analog of ATP hydrolysis has formed in the βDP site, whereas ATP (AMP-PNP) is bound to the βTP subunit. This last observation and the previous one show that the ATP bound to the βDP site, and not the ATP bound to the βTP site, is the nucleotide that is poised to form the transition state and then be hydrolyzed. Subsequently, hydrolysis at this site leads to the release of products and the regeneration of the βE state.

Thus, the structure of the azide-free F1-ATPase described here represents a highly detailed snapshot of three different structures of β-subunits representing intermediates that form together in the ground state of the catalytic cycle of ATP hydrolysis by F1-ATPase. From this structure and that of N3-F1, it is clear that the reference structure (and the more detailed structure of N3-F1) represents a state closely related to the ADP-inhibited state of the enzyme and that this conformation is also an accurate representation of an intermediate in the active catalytic cycle.

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