Asymmetric Structure of the Yeast F₁ ATPase in the Absence of Bound Nucleotides*

Venkataraman Kabaleswaran†, Hong Shen††, Jindrich Symersky‡, John E. Walker§, Andrew G. W. Leslie*, and David M. Mueller†*‡

From the †Rosalind Franklin University of Medicine and Science, The Chicago Medical School, North Chicago, Illinois 60064, the ‡Medical Research Council (MRC) Dunn Human Nutrition, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 0XY, United Kingdom, and the *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, United Kingdom

The crystal structure of nucleotide-free yeast F₁ ATPase has been determined at a resolution of 3.6 Å. The overall structure is very similar to that of the ground state enzyme. In particular, the β₁DP and β₁TP subunits both adopt the closed conformation found in the ground state structure despite the absence of bound nucleotides. This implies that interactions between the γ and β subunits are as important as nucleotide occupancy in determining the conformational state of the β subunits. Furthermore, this result suggests that for the mitochondrial enzyme, there is no state of nucleotide occupancy that would result in more than one of the β subunits adopting the open conformation. The adenine-binding pocket of the β₁DP subunit is disrupted in the apo-enzyme, suggesting that the β₁DP subunit is responsible for unisite catalytic activity.

ATP synthase is a molecular motor driven by the sequential protonation and deprotonation of a conserved acidic residue (aspartate or glutamate) in the c subunit. Multiple copies of the c subunit form a ring within the membrane-bound portion of the enzyme, which rotates relative to the membrane anchored a subunit. The membrane-bound component of ATP synthase is known as F₀, and is linked to the catalytic component, denoted F₁, ATPase, by a central and a peripheral stalk. F₁ ATPase is composed of five different subunits with stoichiometry α₃β₃γδε and molecular mass of 350,000 Da (1). F₀ is minimalistically composed of three subunits with stoichiometry α₂β₃δε (2) and acts as a proton turbine, which drives the rotation of the central stalk of the ATP synthase, thereby effecting ATP synthesis in the catalytic F₁ component.

The asymmetrical elements of the enzyme are critical in the mechanism of ATP synthase. The F₁ portion is composed of an almost spherical headpiece formed by three pairs of alternating α and β subunits and a central stalk that runs through this assembly and is composed of the γ, δ, and ε subunits in the mitochondrial enzyme. The αβ subunit pairs form the three catalytic sites, but the structures of these sites are different, being influenced by the position of the central stalk. The asymmetric features of F₁ ATPase were seen in the ground state structure of bovine F₁ ATPase where the central stalk made unique contacts with each of the three catalytic sites (1, 3). The sites have different nucleotide occupancies, with one site containing AMP-PNP3 (denoted TP), one containing ADP (denoted DP), and one without any bound nucleotide (denoted E). The structures of the DP and TP sites are similar, and the corresponding β subunits are described as being in a closed conformation, but the structure of the E site differs, and this β subunit adopts an open conformation. Rotation of the central stalk in 120° increments is thought to convert the active sites in the sequence E, DP, TP when proceeding through ATP synthesis.

The hydrolytic activity of F₁ ATPase is thought to be the reverse of the synthesis reaction. Early studies indicated that the F₁ ATPase is highly cooperative with the binding of ATP showing a strong negative cooperativity and the hydrolytic activity displaying a highly positive cooperativity (4). Using enzyme depleted of nucleotide, three catalytic binding sites with different binding constants can be identified. The affinity for binding of the first nucleotide to F₁ ATPase is very high with a Kᵣ for the bovine enzyme of 10⁻¹² M, whereas the second and third sites have binding constants ranging from 30 to 150 μM (5). The rate of hydrolysis of ATP under conditions that ensure only a single nucleotide is bound, unisite conditions, is extremely slow with a kcat of 10⁻⁴ s⁻¹, whereas binding of the second or third ATP stimulates the rate by a factor of 10⁶ (6). Similar results have been obtained with the bacterial enzyme, although the values differ from those of the mitochondrial enzymes (7). The unisite catalytic activity is believed to involve the isoenergetic interconversion of ADP and Pᵢ to ATP, and this is central to conformational coupling hypothesis proposed by Boyer et al. (8) for the synthesis of ATP.

The strong cooperativity of F₁ ATPase suggests that binding of the nucleotides has a pronounced effect on the conformation of the enzyme. This study investigates the role of nucleotides in modulating the structure of F₁ ATPase by determining the structure of the enzyme without any bound nucleotides. Analysis of the structure of the nucleotide-free enzyme also

---

* This work was supported, in whole or in part, by National Institutes of Health Grant R01-GM067091 (to D. M. M.). This work was also supported by a grant from the Medical Research Council (to J. E. W. and A. G. W. L.).
† The atomic coordinates and structure factors (code 3FKS) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
‡ Supported by American Heart Association Predoctoral Fellowship 0410020Z.
§ To whom correspondence should be addressed: Dept. Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, The Chicago Medical School, 3333 Green Bay Rd., North Chicago, IL 60064. Fax: 847-578-3240; E-mail: David.Mueller@RosalindFranklin.edu.

The abbreviation used was: AMP-PNP, 5’-adenylyl-β,γ-imidodiphosphate.
addresses the issue of the identity of the high affinity site observed in unisite conditions.

**MATERIALS AND METHODS**

Yeast F₁-ATPase was purified with a slightly amended procedure to that described earlier (9) in which the ATP in the buffer used for the Superdex 200 column was replaced with 2 mM sodium pyrophosphate. The fractions from the Superdex 200 column were pooled and precipitated with 70% saturated ammonium sulfate and stored at 4 °C. This procedure purified and partially removed the nucleotides from the enzyme.

Complete removal of nucleotides from the enzyme was achieved by separation on a Sephadex 25 superfine column (120 × 16 mm). The enzyme was collected by centrifugation and dissolved in 50 mM Tris–SO₄, 2 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride. The diffraction data were collected at 100 K on beamline ID-22, SER-CAT (Advanced Photon Source, Chicago, IL), at a wavelength of 1.000 Å using a Mar300 CCD detector. The diffraction data were indexed, integrated, and scaled using X-GEN (13).

Noncrystallographic symmetry restraints were applied in the three molecules in the crystallographic asymmetric unit. As with the ground state structure, the quality of the electron density map differs between the complexes with Complex I showing the best defined electron density and Complex III showing the poorest. The structures of Complex I, II, and III have all been analyzed, but the discussion will primarily be limited to that for Complex I except in the instances where features are unique or clearly differ between Complex I and either Complex II or Complex III.

Despite the limited resolution of the x-ray data, there is well defined electron density for the polypeptide backbone of almost all the residues included in the final model and for the great majority of the side chains, although some of these have very high temperature factors. The overall structure of the nucleotide-free yeast F₁-ATPase is very similar to that of the nucleotide-bound form, and therefore, the same naming convention (DP,

### RESULTS

The yeast mitochondrial F₁-ATPase devoid of nucleotides was crystallized under similar conditions to those reported for the ground state enzyme with bound nucleotides (9, 11). The major differences in the crystallization conditions were the absence of nucleotides and magnesium ions and the inclusion of 2 mM sodium pyrophosphate. Sodium pyrophosphate has been reported to bind to the noncatalytic but not to the catalytic sites (16–20) and was included to stabilize the enzyme. Sodium pyrophosphate was an essential component in the crystallization conditions even when it was present in the purification steps. Previous attempts to crystallize nucleotide-free bovine F₁-ATPase were unsuccessful as the nucleotide-free enzyme precipitates in the absence of high concentrations of glycerol.

The data collection and refinement statistics are shown in Table 1. The enzyme crystallized in space group P2₁, with unit cell dimensions similar to those of the ground state enzyme (11) and with three F₁ complexes in the crystallographic asymmetric unit. As with the ground state structure, the quality of the electron density map differs between the complexes with Complex I showing the best defined electron density and Complex III showing the poorest. The structures of Complex I, II, and III have all been analyzed, but the discussion will primarily be limited to that for Complex I except in the instances where features are unique or clearly differ between Complex I and either Complex II or Complex III.

The data collection and refinement statistics for the yeast F₁ ATPase are shown in Table 1. The crystallographic asymmetric unit. As with the ground state structure, the quality of the electron density map differs between the complexes with Complex I showing the best defined electron density and Complex III showing the poorest. The structures of Complex I, II, and III have all been analyzed, but the discussion will primarily be limited to that for Complex I except in the instances where features are unique or clearly differ between Complex I and either Complex II or Complex III.

### TABLE 1

| Data collection and refinement statistics for the yeast F₁ ATPase |
|---------------------------------------------------------------|
| **Data collection**                                           |
| Space group                                                   |
| Cell parameters (Å and °)                                      |
| Resolution                                                    |
| Number of reflections                                         |
| **Refinement statistics**                                     |
| R-factor (%)                                                  |
| Rmerge (%)                                                    |
| Rfree (%)                                                     |
| Completeness (%)                                              |
| Mean multiplicity (%)                                         |
| **Ramachandran plot**                                         |
| Most favored                                                  |
| Allowed                                                       |
| Generously allowed                                            |
| Disallowed                                                    |
| **Bond lengths (Å)**                                          |
| Bond angles (°)                                                |
| **Electron density map**                                      |
| Where n is the weighted mean intensity for all observations of reflections h after rejection of outliers. |
| Where n is the weighted mean intensity for all observations of reflections h after rejection of outliers. |

(1–47, 77–78, 91–104, 108–110, 112–124, 131–134, 136–156, 165–179, 209–276) δ (121–131), and ε (8–22, 34–43).
Structure of Nucleotide-free Yeast F$_1$-ATPase

The side chains of selected residues important for substrate binding and catalysis are shown.

**FIGURE 1.** Electron density at the catalytic sites of yeast F$_1$ ATPase in the absence of nucleotides. In each panel, the $2F_c - F$ map is shown (contoured at 1 σ) with the position of the nucleotide modeled from the ground state structure. The *inset plots* show the region where the nucleotide would bind and corresponding electron density. The main chain is represented as a ribbon in red and blue for the α and β subunits, respectively. The side chains of selected residues important for substrate binding and catalysis are shown. A, α$_{DP}$/β$_{DP}$ site; B, α$_{TP}$/β$_{TP}$ site; C, a noncatalytic (NC) site, all for Complex I. D, the phosphate-binding site in the α$_{E}/β_{E}$ site of Complex II.

TP, and E) for the α and β subunits and the catalytic sites will be retained although no nucleotides are bound.

The electron density map clearly indicates that the nucleotide was not present in any of the catalytic (α$_{DP}$/β$_{DP}$ and α$_{TP}$/β$_{TP}$) or noncatalytic (NC) sites (Fig. 1). Although there is no electron density for the purine base or the ribose moiety of the nucleotide, in Fig. 1, the *inset plots* show that there is some density in the region of the P-loop that overlaps with the position of the γ- or β-phosphates of AMP-PNP in the nucleotide-bound structure. This density may be due to the binding of phosphate, sulfate, or pyrophosphate. There is also a separate peak in the electron density in the E site for Complex II (E2) but not in Complex I or III. Corresponding density was observed in Complex II of the ground state structure and is thought to represent the phosphate-binding site. This suggests that the binding of phosphate to Complex II is independent of bound nucleotide but is related to conformation differences in Complex II relative to Complex I and III.

The overall structures of the nucleotide-free and nucleotide-bound enzymes are very similar (Fig. 2) with a root mean square deviation in α-carbon positions of 1.12 Å for all subunits. In particular, both the β$_{TP}$ and the β$_{DP}$ subunits adopt the “closed” conformation despite the absence of bound nucleotide.

Although the overall conformation is very similar to the ground state enzyme, there is a significant main chain shift in the region of the nucleotide-binding site of the β$_{TP}$ subunit, where the α-carbon of residue β-Phe-424 is shifted by 2 Å (Fig. 2D). The phenyl side chain of Phe-424 forms part of the adenine-binding pocket, and this displacement is likely to reduce the binding affinity for ATP. By contrast, this region of the β$_{DP}$ subunit is very similar in the two structures (Fig. 2). This suggests that the β$_{DP}$ subunit will have the highest affinity for nucleotide in the apo-enzyme and that this subunit is likely to be responsible for unisite catalytic activity.

We tested for possible model bias by making omit maps in this region, and the resulting $2F_c - F$ density omit maps were unchanged (Fig. 3). Omit maps were produced excluding residues βVal-371–Leu-391 (Fig. 3A) and βSer-340–Pro-350, and βGlu-422–Pro-428 (Fig. 3B), and the resulting $2F_c - F$ maps (1σ) were consistent with the final refined model. These results indicate that the models of these regions were not biased toward the structure of the ground state molecule, which was used in molecular replacement.

During catalysis, rotation of the central stalk, γδε, is responsible for the interconversion of the active site conformations in the β subunits.

The position and conformation of the γ subunit in the ground state and nucleotide-free structures were compared by superimposing the β-barrel domains of the α and β subunits, as these remain virtually unchanged in all of the known structures. The comparison (Fig. 4A) shows that the N-terminal and C-terminal helices of the γ subunits from the nucleotide-free and the ground state structures are very similar in both conformation and position relative to the α$_{E}$β$_{E}$ subassembly. The largest differences occur in the membrane-proximal regions of the coiled coil, which do not interact with the α or β subunits.

As in the ground state structure, phosphate was bound to the E site of Complex II, but not Complex I, in the nucleotide-free structure. As originally observed in the ground state structure, the two complexes also differ significantly in the conformation of the coiled-coil region of the γ subunit (Fig. 4B), but it remains unclear whether these differences result primarily from binding of phosphate or from crystal packing interactions.

**DISCUSSION**

The first crystal structure of bovine F$_1$ ATPase (1) revealed that the catalytic subunit with no bound nucleotide (β$_{E}$) adopted a conformation that was quite distinct from the other catalytic subunits, β$_{DP}$ and β$_{TP}$, that bound ADP and AMP-PNP, respectively. In the β$_{E}$ subunit, the C-terminal domain and the lower part of the nucleotide-binding domain had rotated away from the pseudo-three-fold axis of the complex by ~30° to give an “open” conformation for this subunit. The asymmetric position (relative to the pseudo-three-fold axis) and the curvature of the γ subunit provided a logical expla-
Structure of Nucleotide-free Yeast F₁-ATPase

The structure of nucleotide-free yeast F₁-ATPase has been determined to 3.2 Å resolution. The absence of nucleotide binding results in an open conformation of the β subunits, whereas the resulting distortion of the nucleotide-binding site was consistent with the low affinity for nucleotide.

Subsequently, the structure of the α₃β₉ subcomplex of PS3 F₁ ATPase was determined (21). In this case, the three β subunits were related by a crystallographic three-fold axis, and they all adopted an open conformation very similar to that of the β₉ subunit of the bovine structure. This result suggested that in the absence of nucleotide binding, an open conformation of the β subunits was energetically preferred, whereas nucleotide binding would favor a closed conformation. If this is the case, a nucleotide-free F₁ ATPase might be expected to adopt a conformation with all three β subunits in the open conformation.

However, the structure of the chloroplast F₁ ATPase determined at 3.2 Å resolution (22) suggested that all three β subunits adopted a closed conformation in the absence of bound nucleotide (1FX0). This enzyme was crystallized in the presence of 0.02 mM ADP and 1 mM AMP-PNP, but magnesium was not present, and there was no evidence of nucleotide binding to either the α or the β subunits. As in the PS3 crystal structure (1SK4), the three α subunits and the three β subunits are related by a crystallographic three-fold axis, which means that the γ subunit is statistically disordered, and because of the limited resolution of the x-ray data, it could not be modeled. Also, it was not possible to rule out the possibility of some statistical disorder involving the α or β subunits. Indeed, it is not physically possible for all three β subunits to adopt the conformation of the model as deposited in the Protein Data Bank (ID 1FX0) because this does not leave sufficient space for the coiled coil of the γ subunit to exit the cavity in the center of the α₃β₉ assembly. In all likelihood, one of the β subunits will adopt a different conformation to accommodate the coiled-coil region of the γ subunit, but this could not be detected in the electron density because of difficulties arising from the statistical disorder and limited resolution. Despite these issues, it was clear that all three β subunits did not adopt the open conformation and that, on average, the conformation was very close to the closed form seen in the bovine structure.

Although the current structure of nucleotide-free yeast enzyme is only at 3.6 Å resolution, there is no evidence for any type of statistical disorder, and the β subunits are not related by a crystallographic symmetry axis. It is clear for the first time from this structure that both the β₉ and the β₉ subunits adopt a closed conformation even when no nucleotide is bound, whereas the β₉ subunit adopts the conventional open conformation. The difference between this result and that for the PS3 α₉β₉ subcomplex suggests that the γ subunit, in addition to the presence or absence of nucleotide, has an influence on the β subunit conformation. There are significant interactions between the γ subunit and both the β₉ and the β₉ subunits involving the lower parts of the nucleotide-binding domain and the C-terminal domains. For the yeast F₁ ATPase structure, the buried surface area for these interactions is 380 and 500 Å² for the β₉ and β₉ subunits, respectively. Maintaining these interactions (which would be lost if these subunits adopted the open conformation) is apparently energetically favorable in the absence of bound nucleotide. Given the degree of similarity in overall conformation between the nucleotide-free structure and that of the ground state (with nucleotide bound to β₉ and β₉ subunits), it seems unlikely that any intermediate nucleotide occupancy will result in a dramatically different tertiary or quaternary structure.

These results are rather different from those obtained for the thermoalkaliphilic TA2 F₁ ATPase crystallized in the absence of nucleotides and magnesium (23). In this structure, all three β subunits adopt the open conformation, whereas the γ subunit adopts a highly asymmetric position that has not been observed in any other F₁ ATPase structures. This may be the result of salt bridge interactions between the γ subunit and the β₉ subunit that involve residues that are not conserved in the mitochondrial enzymes, which are also believed to prevent this species of ATP synthase from operating in the hydrolysis direction.

Although there is no evidence for nucleotide binding at any of the catalytic or noncatalytic sites in the structure described here, there is a peak of electron density close to the P-loop residues, approximately in the position of the β-phosphate.

FIGURE 2. Comparison of the structures of ground state and nucleotide-free yeast F₁ ATPase. Pairs of α/β subunits are shown after superposition of the N-terminal β-barrel domains. The ground state structure is shown in yellow, and the structures of the α and β subunits of the nucleotide-free enzyme are shown in red and blue, respectively. Panels A, C, and E show the α-carbon traces for the α,β₀,α₀β₀, and α₀β₀ subunit pairs, respectively. In panels A and C, shaded regions show the location of the nucleotide-binding sites. Panels B and D show the region around the active site with the side chains shown only for the ground state structure. The arrow indicates the shift of the Cα backbone at residue βPhe-424.
when nucleotide is bound, in all six nucleotide-binding sites. In both position and size, these peaks resemble those observed in the \( \beta_E \) subunit in all bovine F\(_1\) ATPase crystal structures, except when ADP is bound to this subunit, and in both \( \alpha \) and \( \beta \) subunits of the nucleotide-free PS3 \( \alpha_3\beta_3 \) subcomplex. In previous structures, this peak has been identified as a poorly ordered phosphate or sulfate ion. Because the primary role of the P-loop is to bind the nucleotide phosphate groups, it is not surprising that, in the absence of bound nucleotide, this site is occupied by other anions. The crystallization medium for nucleotide-free yeast F\(_1\) ATPase included 2 mM sodium pyrophosphate, which is known to bind to the \( \alpha \) subunits of Escherichia coli F\(_1\) ATPase (but not to the \( \beta \) subunits). It is therefore possible that these peaks, at least in the \( \alpha \) subunits, represent pyrophosphate rather than phosphate or sulfate, but the limited resolution of the x-ray data did not allow an unambiguous distinction between these alternatives. The possibility that anion binding to the \( \beta_{1P} \) and \( \beta_{1P} \) subunits in nucleotide-free yeast F\(_1\) ATPase promotes or stabilizes the observed closed conformation of these subunits cannot be ruled out. However, anion binding at the P-loop site was also found in the \( \alpha \) subunits of the PS3 \( \alpha_3\beta_3 \) subcomplex, which adopt an open conformation, suggesting that this is probably not the case.

In summary, the structure reported here suggests that, at least for the mitochondrial enzyme, there is no nucleotide state in which more than one of the three catalytic \( \beta \) subunits adopts an open conformation. Interactions between the central \( \gamma \) subunit and the C-terminal domains of the \( \beta \) subunits result in a closed conformation for two of the three \( \beta \) subunits even in the absence of nucleotide binding.
4. Kayalar, C., Rosing, J., and Boyer, P. D. (1977) *J. Biol. Chem.* **252**, 2486–2491
5. Grubmeyer, C., Cross, R. L., and Penefsky, H. S. (1982) *J. Biol. Chem.* **257**, 12092–12100
6. Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) *J. Biol. Chem.* **258**, 12101–12105
7. Weber, J., Wilke-Mounts, S., Lee, R. S., Grell, E., and Senior, A. E. (1993) *J. Biol. Chem.* **268**, 20126–20133
8. Boyer, P. D., Cross, R. L., and Momsen, W. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 2837–2839
9. Mueller, D. M., Puri, N., Kabaleeswaran, V., Terry, C., Leslie, A. G. W., and Walker, J. E. (2004) *Protein Expression Purif.* **37**, 479–485
10. Garrett, N. E., and Penefsky, H. S. (1975) *J. Biol. Chem.* **250**, 6640–6647
11. Kabaleeswaran, V., Puri, N., Walker, J. E., Leslie, A. G., and Mueller, D. M. (2006) *EMBO J.* **25**, 5433–5442
12. Mueller, D. M., Puri, N., Kabaleeswaran, V., Terry, C., Leslie, A. G. W., and Walker, J. E. (2004) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **60**, 1441–1444
13. Howard, A. (2000) in *Crystallographic Computing 7: Proceedings from the Macromolecular Crystallographic Computing School* (Bourne, P., and Wartenpaugh, K. eds) pp. 88–99, Oxford University Press, Oxford, UK
14. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54**, 905–921
15. Emsley, P., and Cowtan, K. (2004) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **60**, 2126–2132
16. Milgrom, Y. M., and Cross, R. L. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 13831–13836
17. Weber, J., and Senior, A. E. (1995) *J. Biol. Chem.* **270**, 12653–12658
18. Murataliev, M. B. (1992) *Biochemistry* **31**, 12885–12892
19. Milgrom, Y. M., and Cross, R. L. (1993) *J. Biol. Chem.* **268**, 23179–23185
20. Kalashnikova, T., Milgrom, Y. M., and Murataliev, M. B. (1988) *Eur. J. Biochem.* **177**, 213–218
21. Shirakihara, Y., Yohda, M., Kagawa, Y., Yokoyama, K., and Yoshida, M. (1991) *J. Biochem.* **109**, 466–471
22. Groth, G., and Pohl, E. (2001) *J. Biol. Chem.* **276**, 1345–1352
23. Stocker, A., Keis, S., Vonck, J., Cook, G. M., and Dimroth, P. (2007) *Structure (Camb).* **15**, 904–914

**Structure of Nucleotide-free Yeast F1-ATPase**

APRIL 17, 2009 • VOLUME 284 • NUMBER 16 • JOURNAL OF BIOLOGICAL CHEMISTRY