Review

Dynamic mechanisms driving conformational conversions of the $\beta$ and $\epsilon$ subunits involved in rotational catalysis of F$_1$-ATPase

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Abstract: F-type ATPase is a ubiquitous molecular motor. Investigations on thermophilic F$_1$-ATPase and its subunits, $\beta$ and $\epsilon$, by NMR were reviewed. Using specific isotope labeling, $pK_a$ of the putative catalytic carboxylate in $O$ was estimated. Segmental isotope-labeling enabled us to monitor most residues of $O$, revealing that the conformational conversion from open to closed form of $O$ on nucleotide binding found in ATPase was an intrinsic property of $O$ and could work as a driving force of the rotational catalysis. A stepwise conformational change was driven by switching of the hydrogen bond networks involving Walker A and B motifs. Segmentally labeled ATPase provided a well resolved NMR spectra, revealing while the open form of $O$ was identical for $O$ monomer and ATPase, its closed form could be different. ATP-binding was also a critical factor in the conformational conversion of $\epsilon$, an ATP hydrolysis inhibitor. Its structural elucidation was described.

Keywords: F$_o$F$_1$-ATP synthase, nuclear magnetic resonance, segmental isotope-labeling, ATP binding, hydrogen bond network, X-ray crystallography

1. Introduction

F$_o$F$_1$ ATP synthase is a ubiquitous molecular motor involved in H$^+$-mediated energy conversion in organisms from bacteria to human and has been extensively investigated by a wide variety of methods.1)–9) A model image of a bacterial F$_o$F$_1$ ATP synthase is presented in Fig. 1. The H$^+$-driven ATP synthase converts the energy of the transmembrane electrochemical potential generated by proton gradient to that of ATP. It consists of a water-soluble F$_1$ sector and a membrane integrated F$_o$ sector. The former has catalytic sites for ATP synthesis/hydrolysis, and the latter mediates H$^+$ transport across membranes. The F$_1$ and F$_o$ sectors comprise multiple subunits. Basically, their compositions are $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ and $\alpha_1\beta_2\epsilon_n$ ($n=8–15$), respectively. Mitochondrial ATP synthases have more subunits. F$_1$ can be solubilized and the isolated F$_1$ functions only as ATPase.

The first crystal structure of F$_1$ from bovine heart mitochondria (MF$_1$) provided atomic information of the $\alpha_3\beta_3\gamma$ complex (PDB ID: 1BMF).10) The catalytic site of F$_1$-ATPase was found in the $O$ subunit facing the $\alpha/\beta$ interface. The subassembly $\alpha_3\beta_3$ formed a ring with long helices of the $\gamma$ subunit in the center, consistent with Boyer’s rotary-catalysis model.1) The tertiary structure of the $\beta$ subunit in the F$_1$-ATPase differed according to the state of its active site, namely, an empty, MgAMPPNP (5'-adenylyl-imidodiphosphate, an ATP analog, with Mg$^{2+}$) bound or MgADP (ADP with Mg$^{2+}$) bound state (designated as $\beta_E$, $\beta_{TP}$, $\beta_{DP}$, respectively). $\beta_{TP}$ and $\beta_{DP}$ took so-called closed forms because their hinged N- and C-terminal domains were closer to each other than those of $\beta_E$, the active site of which adopted a relatively open conformation (open form). Actually, the rotation of the $\gamma$ subunit in the fixed $\alpha_3\beta_3$ complex on hydrolysis of ATP was directly demonstrated by epifluorescence microscopy for the $\alpha_3\beta_3\gamma$ complex from thermophilic Bacillus PS3 (TF$_1$),11) which verified the Boyer’s model. This
method (single molecule analysis) has been very powerful to understand the molecular mechanism of the rotational catalysis. The first observation revealed that the rotation of $\gamma$ in F$_1$ was carried out in a 120° step-wise manner. Then, the 120° rotation turned out to comprise 80° and 40° rotational substeps, and the former and latter were attributed to ATP- and P$_i$-release and hydrolysis, respectively. The pauses before the substep rotations were designated as ATP-binding dwell and catalytic dwell, respectively. In the case of F$_0$F$_1$-ATP synthase, the $\gamma\epsilon\zeta$ complex rotates in the $\alpha_\beta\beta\delta\alpha_2$ stator coupled with H$^+$ translocation across a membrane (Fig. 1). Recently, single molecule analysis of human F$_1$-ATPase revealed a three-substep rotation, namely, 65°, 25°, and 30°, suggesting that the mechanism underlying the rotational catalysis of mitochondrial F$_1$-ATPase is different from the bacterial one.

In contrast to F$_1$-ATPase, structure and functional mechanism of F$_o$ is not yet well understood. It converts the electrochemical potential generated by the H$^+$-gradient across membranes into rotation of the c-subunit ring and then into that of the $\gamma\epsilon$-rotor in F$_1$, or vice versa. The c-ring comprises 8–15 c-subunits, depending on the biological species. The mechanism underlying the energy conversion at F$_o$ is one of the major unresolved issues for understanding F$_0$F$_1$-ATP synthase.

Biochemical, genetic, crystallographic, and single molecular investigations have been successful in conceptual elucidation of structure and function of F$_0$F$_1$-ATP synthase. We have been working on this enzyme using nuclear magnetic resonance (NMR) to clarify the dynamic mechanism at atomic resolution on the basis of the aforementioned knowledge. NMR is a unique methodology that can provide information bridging atomic structure and function of proteins under physiological conditions. However, a serious drawback in NMR was the size limit. Since F$_0$F$_1$-ATP synthase is a huge protein complex, we started with the subunits and proceeded to F$_1$. To overcome difficulty caused by the molecular size, we developed combined use of gene-engineering, isotope-labeling, and sophisticated solution NMR measurements. Although I do not touch on in this review, we also developed methodology in solid-state NMR to work on F$_o$c-ring, a membrane protein complex. We have been working on TF$_0$F$_1$-ATP synthase in close collaboration with Prof. Masasuke Yoshida and his colleagues.

**2. Targeting the heart of the $\beta$ subunit:**

**estimation of $pK_a$ of the putative catalytic carboxyl group**

The isolated F$_1$ hydrolyzes ATP to ADP and P$_i$. The smallest active complex of F$_1$ is $\alpha_3\beta_3\gamma$. The catalytic site is located in the $\beta$ subunit, although residues from the $\alpha$ subunit are also required for ATP hydrolysis. Modification of Glu190 of the $\beta$ subunit of TF$_1$ (from thermophilic Bacillus PS3) with dicyclohexylcarbodiimide (DCCD) and substitution of this residue by glutamine resulted in complete loss of the activity. The crystal structure of mitochondrial F$_1$-ATPase (MF$_1$) actually revealed that the carboxylate of Glu188, corresponding to Glu190 of TF$_1$, was involved in a hydrogen bond with a bound water molecule in the vicinity of the $\gamma$-phosphate of a substrate analogue, AMPmNP. Therefore, it was suggested that this carboxyl group would activate the water for nucleophilic attack of the $\gamma$-phosphate. Therefore, $pK_a$ of Glu190 is an important parameter for understanding the mechanism underlying the catalytic reaction.

Since NMR has an atomic resolution even in a solution, it is one of the most powerful methods to determine $pK_a$ of a particular residue in a protein. However, it was not possible more than two decades ago because of high molecular masses of the $\beta$ subunit (~52 kDa) and F$_1$-ATPase (~370 kDa). To overcome molecular-size problem, we developed a specific isotope-labeling method using a combination of amino acid replacement and chemical modification. Since there was no Cys residue in TF$_1$, Glu190 was replaced with Cys, followed by carboxymethylation with iodo[1,13C]acetic acid. As can be seen in Fig. 2B, the labeled carboxylate signal could be clearly identified. The pH titration of this signal provided $pK_a = 5.7 \pm 0.2$ in the absence of nucleotide.

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Fig. 1. A model image of a bacterial F$_0$F$_1$-ATP synthase composed of $\alpha_5\beta_7$ε$\delta$ab$\epsilon_\zeta$ complex. The subcomplex $\gamma\epsilon\zeta$-ring rotates in association with H$^+$ translocation, which leads to ATP synthesis at the catalytic site.
Further correction on the difference in pK_a between carboxymethylated Cys (CmCys) and glutamic acid
gave the estimation of pK_a of the carboxylate of
Glu190 in TF1β as 6.8 ± 0.5. In the presence of ADP,
pK_a shifted to lower pH by less than one. A pK_a value
of regular Glu residue in proteins falls in the range 3–5.20 Therefore, the estimated pK_a of Glu190 in the
isolated TF1β is unusually high, even taking into account its large error range.

It was also possible to get a 13C-NMR
spectrum of an active TF1 subcomplex, α(C193S/W463F)β(E190[1-13C](CmCys))_3γ, at pD H 8.1 in spite
of its very high molecular mass (~370 kDa) as can be seen in Fig. 2C. It suggested that at least one
CmCys190 was under the chemical environment
equivalent to that in the isolated β(CmCys190).
Since ATPase activity of αβ(E190CmCys)βγ was 12% of
that of αβγ,30 the role of the carboxylate at the 190
position in the F_1-ATPase could be discussed. In the
ground state structure of mitochondrial F_1 at 1.9 Å
resolution, the carboxyl group corresponding to this
one formed a hydrogen bond with a water molecule in
the AMP-PNP bound β_{2P}.31 The oxygen of the water
molecule is located at 3.1 Å from the γ-phosphorus
of AMP-PNP in β_{2P}. The authors indicated that the
active site conformation of β_{2P} was close to the
transition state for γ-phosphate hydrolysis. The high
pK_a of Glu190 estimated in the TF1β monomer
suggests that the carboxylate may be involved in the
γ-phosphate hydrolysis of ATP. The lower activity of
the αβγ(E190CmCys)γ complex might be due to the
lower pK_a of CmCys190 as well as the structural
difference around the side chain. The exact mechanism
underlying the ATP hydrolysis/synthesis is still
under discussions.32,33

3. Characterization of a nucleotide-induced
conformational change of TF1β subunit

Crystallographic and single-molecule investigations
have promoted general understanding of the
rotational catalysis mechanism of F_0F_1 ATP
synthase. However, dynamic mechanism at atomic
resolution is not yet well established. Since the
catalytic site is localized in the β subunit, intrinsic
property of β monomer would be important to
understand the rotational catalysis of F_1. Although
the molecular size of TF1β (52 kDa) was a serious
problem, we started to work on TF1β monomer using
NMR more than two decades ago.

The crystal structures of TF1αβγ (PDB ID: 1SKY) complex and TF1αβγ (PDB ID: 4XD7)
have been reported.34,35 The structure of the β
subunit of the former took the open form and the
latter included two open and one closed form
structures. The open and closed forms were similar
to those of MF1-ATPase. The β subunit is composed
of roughly three domains, namely, the amino (N-)
terminal, catalytic and carboxyl (C-) terminal
domains (Fig. 5). Conformational difference between
the open and the closed form of β in the crystal
structures concentrates in the catalytic domain. At
first, aromatic ring protons of Tyr and His residues
(twelve each) were used for monitoring the conformational
change of the TF1β monomer on nucleotide
binding in solution, because of their relatively sharp
NMR signals on deuteration of the other ring
protons.36–38 The residues only in the catalytic
domain (Tyr-148, 199, 238, 307, and 341, and
His-173, 179, 200, and 324) revealed chemical shift
changes on AMP-PNP/ATP binding. In the crystal
structures, local conformations of a residue in the
open and the closed form were different only in the
catalytic domain. Therefore, the chemical shift
perturbation might reflect a conformational change
similar to that in MF1.

For elucidation of the origin of the chemical shift
perturbations of aromatic proton signals, detailed
information from all over the TF1β subunit was
required. However, broadening and overlapping of
the NMR signals due to its large molecular size

Fig. 2. 13C-NMR spectra of non-labeled TF1β(E190CmCys) (A),
TF1β(E190[1-13C](CmCys)) (B), and TF1 subcomplex α(C193S/
W463F)β(E190[1-13C](CmCys))_3γ (C) at 100 MHz.28) The signal
assigned to the labeled carboxyl group is indicated by an arrow
in B and C.
(52 kDa, 473 amino acid residues) hampered identification of individual signals. To make observable signals less and sharper, we used segmental isotope-labeling and protein deuteration along with two-dimensional NMR at high magnetic field.\(^{39}\) Segmental labeling by either intein splicing reaction or chemical ligation\(^{40,41}\) was reported. However, it was a challenge, since no application to a really large molecule such as the \(\beta\) monomer was carried out.

We used an intein, PI-PfuI, from hyperthermophilic *Pyrococcus furiosus* for segmental isotope-labeling.\(^{39}\) The PI-PfuI and TF\(_1\beta\) subunit genes were cut in the middle of the sequence, respectively. The N- and C-terminal fragments of the \(\beta\) subunit genes were ligated with those of the PI-PfuI genes in such a way that the \(\beta\) subunit fragments formed exteins by PCR (Fig. 3). The ligated genes of the intein-\(\beta\) subunit precursors (N and C) were cloned into different pET vectors and expressed in *E. coli* cell cultures separately. To label an N-terminal fragment of the \(\beta\) subunit, for example, the \(\beta\)-intein precursor (N) gene was expressed in a culture with \(^{15}\)NH\(_4\)Cl and \(^{13}\)C-sucrose as the sole nitrogen and carbon sources, respectively. The N- and C-terminal precursors were mixed together in a buffer including denaturant. After removal of denaturant, the intein with a nick was renatured (Fig. 3). Then, peptide splicing was carried out at 70 °C by the intein. The ligated \(\beta\) subunit was once unfolded by urea, then refolded to the intact structure by removing it. The segmentally isotope-labeled \(\beta\) subunit included additional residues, Gly-Gly-Gly-Thr-Gly, at the ligation site. After removal of denaturant, the intein with a nick was renatured (Fig. 3). Then, peptide splicing was carried out at 70 °C by the intein. The ligated \(\beta\) subunit was once unfolded by urea, then refolded to the intact structure by removing it. The segmentally isotope-labeled \(\beta\) subunit included additional residues, Gly-Gly-Gly-Thr-Gly, at the ligation site.

We prepared four kinds of TF\(_1\beta\) subunits labeled with \(^{13}\)N, \(^{13}\)C and \(^1\)H in the segments of residues 1–124, 1–271, 272–473, and 391–473, respectively. \(^1\)H/\(^{15}\)N TROSY-HSQC (Transverse relaxation-optimized spectroscopy—Heteronuclear single quantum coherence) spectra of the four kinds of segmentally isotope-labeled TF\(_1\beta\) monomers in the absence of nucleotide were presented in Fig. 4. TROSY-HSQC is a heteronuclear correlation spectroscopy specialized for a large molecule. The observed \(^1\)H/\(^{15}\)N signals completely coincided with the corresponding ones in the spectrum of the uniformly \(^{15}\)N labeled authentic \(\beta\) subunit except for those of the inserted amino acid residues (GGGTTG). Therefore, the ligated \(\beta\) subunits should take the intact structure. Sequential assignment was carried out using 3D TROSY-HNCO, 3D TROSY-HNCA, 3D TROSY-HN(CO)CA, 3D TROSY-HN(CO)CAB, 3D TROSY-HN(CO)CACB, and 3D \(^{15}\)N-edited NOESY (Nuclear Overhauser effect correlated spectroscopy) spectra. Here HNCO, for example, confers \(^1\)H, \(^{15}\)N, and \(^{13}\)C correlations of amide and carbonyl groups. NOESY provides the information on spatial proximity. To validate the sequential assignment, amino acid specific \(^{15}\)N labeling was performed for Tyr, Thr, Lys, Ser, and Gly. Thus, 89% of the NH (402/451), 89% of the C\(_\alpha\) (417/473), 83% of the C\(_\beta\) (357/431), and 90% of the CO (425/473) signals of TF\(_1\beta\) were assigned.

The residues 156–163, 188–201, 301–310, and 316–322 (colored white in Fig. 5) could not be assigned. They are located in the active site region and are involved in the conformational change and/or ATP hydrolysis activity. The stretch 156–163 is a part of so-called P-loop, the binding site of nucleotide phosphate.\(^{10}\) That of 188–201 includes a putative catalytic site Glu190 and other important residues. The regions of 301–310 and 316–322 are involved in a significant conformational change on nucleotide binding, namely, formation of a new \(\beta\) sheet area (\(\beta3\)-\(\beta7\), Fig. 5) and rearrangement of the surrounding area. Most unassigned signals (34 out of 49) were missing in the spectra. The major reason of loss of the signals should be the fast exchange of amide protons in the flexible regions. Those signals could not be recovered even on nucleotide binding. This fact strongly suggests that the four regions mentioned above are intrinsically flexible, which would be important for the global conformational change and the catalytic reaction.

A nucleotide-free TF\(_1\beta\) solution was titrated with MgADP. The signals of the 136–139, 164–186, 209–212, 311–314, 328–352, and 410–430 residues shifted on the titration (highlighted in red in Fig. 5, all data in Fig. 7A). The residues of 328–352 and 410–430 formed the binding pocket for the adenine ring of ADP in the crystal structure, suggesting that the chemical shift perturbations could be ascribed to direct interaction with adenine.\(^{10}\) The chemical shift change of the other residues could be ascribed to conformational changes induced by nucleotide binding. Actually, all of these residues fell in the catalytic domain. This result was consistent with our idea on a nucleotide-induced conformational change obtained from \(^1\)H-NMR analysis of Tyr and His residues.\(^{37,38}\)

Then, the structures of nucleotide-free and nucleotide-bound TF\(_1\beta\) monomers in solution should be characterized in the light of the crystal structures. The open and closed forms of \(\beta\) in the crystal structure of MF\(_1\) could be specified by the difference in relative orientation of the N- and C-terminal
domains. NMR is one of excellent methods to confer orientation information of chemical groups in a protein in solution. For example, a residual dipolar coupling (RDC) of $^{15}\text{N}$ and $^1\text{H}$ reveals information on the orientation of $^{15}\text{N}-^1\text{H}$ bond relative to the static magnetic field, provided that the protein is partially aligned along the static magnetic field. If residues of interest are located in an $\alpha$-helix, the orientation angle of the helix axis can be obtained. Judging from the secondary structures estimated from the assigned chemical shifts of $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, and carbonyl $^{13}\text{C}$ ($\text{C}’$) of TF$_1\beta$, the main folds of the N- and C-terminal domains are similar for the monomer and the crystal structure of TF$_1\alpha$3.$^{34}$ Since there was no chemical shift change in the N- and C-terminal domains on nucleotide binding, their conformations would not be affected by it. Therefore, changes in the orientation of NH bonds should originate from a change in the domain orientation. Thus, TF$_1\beta$ subunits labeled in the N-terminal (1–124) and C-terminal (390–473) domains were used to determine their relative orientations.
orientation. $^1$H-$^{15}$N RDCs were measured in the absence and presence of MgADP in liquid-crystal, which could induce partial alignment of TF$_1$$\beta$. In analysis of the RDC data of TF$_1$$\beta$ monomer, the $\beta$ subunit structure in the TF$_1$$\alpha_3$$\beta_3$ crystal was used as the monomer structure model. To avoid flexibility problem, the residues located in either $\alpha$-helices or $\beta$-sheets were used for the analysis. The number of RDCs used were 43 and 20 for the N- and C-terminal domains, respectively. The extent of alignment and the orientation angles of each domain relative to the reference frame were obtained on the fitting to the observed data. The results were similar for the N-terminal domains of the MgADP-bound and free $\beta$ subunits. In contrast, it was not the case for their C-terminal domains. The relative orientations between the N- and C-terminal domains were determined by complete superposition of the N-terminal domains of interest. In the case of MgADP-free $\beta$, the orientation of helix 399–409 in the C-terminal domain (Fig. 6 left, red) was different by 5° for the obtained solution structure and TF$_1$$\alpha_3$$\beta_3$ crystal structure. In view of the error ranges, these structures were practically the same. Since the crystal structure of TF$_1$$\beta$ in the $\alpha_3$$\beta_3$ complex is in good agreement with the open form of the $\beta$ subunit in the MF$_1$$\alpha_3$$\beta_3$$\gamma$ crystal structure (PDB ID: 1BMF), we can conclude that the $\beta$ subunit monomer in solution also takes on the open form in the absence of a nucleotide.

For comparison of the conformations of the nucleotide-bound and nucleotide-free $\beta$ monomers, the effect of MgADP on the relative orientations between the N- and C-terminal domains was examined. When an axis connecting the centers of mass of the N- and C-terminal domains (reference axis) was defined (vertical straight lines in Fig. 6), the angles between the reference axis and helix 399–409 axes were 110° and 144° for the MgADP-bound and free $\beta$ subunits, respectively (Fig. 6 left). On the other hand, the corresponding angles in the open and closed forms in the MF$_1$$\alpha_3$$\beta_3$$\gamma$ complex were 119° and 144°, respectively (Fig. 6 right). This result clearly revealed that the TF$_1$$\beta$ monomer took the open structure similar to that of MF$_1$$\beta$ in the MF$_1$$\alpha_3$$\beta_3$$\gamma$ crystal structure, and the binding of MgADP induced a conformational change from the open to the closed form (a bending motion) in TF$_1$$\beta$ monomer in solution as expected from the TF$_1$$\alpha_3$$\beta_3$$\gamma$ crystal structures.

The conformational change of TF$_1$$\beta$ monomer was further examined with MgATP instead of MgADP. The angle between the reference and helix 399–409 axes was also 110° in good agreement with that with MgADP. On the other hand, the orientation of the helix axis was different for the MgATP- and MgADP-bound $\beta$ monomers by 10° on the plane perpendicular to the reference axis. This reveals that there is a conformational variance even in the closed form depending on the bound nucleotide species. This was the first direct observation of the conformational change of the $\beta$ subunit on MgATP binding because F$_1$ could not be crystallized with MgATP. A similar conclusion was obtained from the chemical shift perturbation experiment with MgATP (Fig. 7B). Although small number of distinct differences were found, the general pattern of perturbed residues was similar to that with MgADP. An important conclusion of this section is that the conformational change from the open to the closed form of a $\beta$ subunit on nucleotide binding is its intrinsic property. Since the conformational change of $\beta$ on nucleotide binding is one of the most important elements in the rotational catalysis, this intrinsic property can contribute to the driving force of the rotation of the $\gamma$ subunit.

4. Stepwise mechanism driving the intrinsic bending motion of F$_1$$\beta$ subunit on nucleotide binding

Since the intrinsic conformational change is one of the essential driving forces of the rotational catalysis, elucidation of the atomic mechanism underlying the bending motion on nucleotide binding would be important for understanding F$_1$-ATPase. This was carried out on TF$_1$$\beta$ monomer using combination of NMR, information of crystal structures and gene engineering. In the crystal structure of MF$_1$, the phosphate groups of a nucleotide are tightly bound by the P-loop (GXXXGKT), which is the Walker motif A for the phosphate binding. In the presence of a bound nucleotide, the $\beta$ sheet composed of $\beta_3$ and $\beta_7$ strands (Fig. 5) became longer than that in its absence through the formation of additional hydrogen bonds of Leu156/Ile306, Gly158/Ile306, and Gly158/Val308 in the P-loop region. With the conformational change induced by nucleotide binding, four new hydrogen bonds involving the P-loop residues and phosphate were generated besides those between the nucleotide and the P-loop backbone that would be too local to induce a global change. The first is the side chain of Lys164 (162 for MF$_1$ numbering) forming hydrogen bonds with the backbone carbonyl group of Gly158 (156...
for MF1) along with the phosphate group of the nucleotide. The second was that between the hydroxyl group of Thr165 (163 for MF1) and the side chain carboxyl group of Asp252 (256 for MF1) in the Walker motif B. The third was that between the carbonyl group of Ala160 (158 for MF1) and \( \text{N}_2\)-H of Arg333 (337 for MF1), and the latter further formed a hydrogen bond with \( \text{O}_\gamma\) of Asp311 (315 for MF1). The fourth was that between Arg191 and the \(-\text{phosphate of AMPPNP.}\) Are they involved in the mechanism driving the global change from the open to the closed forms?

Fig. 6. Comparison of the relative orientations of the C-terminal domains in the nucleotide-bound and free forms for TF1\(1\) monomer in solution (left) and MF1\(1\) in MF1 crystal (right).\(39\) The N-terminal domains of the nucleotide-bound and free structures were superimposed for comparison of the C-terminal domain orientation. An axis connecting the centers of mass of the N- and C-terminal domains was defined as a reference axis (vertical straight lines). The relative orientations were compared using the angles between the reference and helix 399–409 axes. Red and green, nucleotide-free and bound forms, respectively. On the right, the structures in red and green are called the open and closed forms, respectively. Reprinted with permission from ref. 39. Copyright 2004 American Chemical Society.
Since the open and closed forms were similar for F1 and F1O monomer in terms of the relative orientation of the N- and C-terminal domains, we assumed that the hydrogen bonds essential for each form should be identical for the F1 and the O monomer. To identify the hydrogen bonds essential for the global conformational change of TF1O on nucleotide binding, we produced Ala-substituted proteins of O(K164A), O(T165A), O(R191A), O(D252A), O(D311A), and O(R333A).42) We used the aromatic proton signal of Tyr341 to monitor the nucleotide binding, because the aromatic ring of this residue stacked to the adenine ring of nucleotide in the crystal structure. For monitoring the conformational change of TF1O, those of Tyr148, Tyr199, and Tyr307 were used. The dissociation constant of AMPPNP (Kd) in the presence of Mg2+ was determined by the titration of Tyr341 signal, and was listed in Table 1 along with the ability of nucleotide-induced conformational change for the wild-type and mutant TF1O. While the binding affinity for β(D311A) and β(D252A) was similar to that of the wild-type β, it was reduced by two-, six- and 20-fold for β(T165A), β(R333A), and β(K164A), respectively. Therefore, the side chain of Lys164 should play a major role in the nucleotide binding as reported.44) The correlation between the nucleotide binding and the conformational change was examined by chemical shift changes of the conformation-relevant signals (Tyr148, Tyr199, and Tyr307) of the wild-type O as a function of the chemical shift change of Tyr341. In the case of Tyr148 and Tyr199, the chemical shift change was a linear function of the amount of the bound AMPPNP, ATP, or ADP, revealing that the conformational change was direct consequence of the nucleotide binding. Our previous RDC experiment on the ADP bound O subunit monomer revealed that this was the open/closed conformational change.39) In contrast, the change of the Tyr307 signal was negligible for MgADP, suggesting that the γ-phosphate of the nucleotide was involved in this conformational change.

In the case of β(R191A), β(D311A), and β(R333A), the plots for the Tyr199 and Tyr307 signals were similar to those of the wild-type β, respectively. Although the AMPPNP-binding affinity of β(R333A) was diminished, this mutation scarcely affected the open/closed conformational change induced by nucleotide binding. Therefore, we concluded that the hydrogen bonds of Arg191/phosphate, Ala160/Arg333 and Arg333/Asp311 were not essential for the open/closed conformational change.

Table 1. The dissociation constants (Kd) and conformational change ability of the wild and mutated TF1β monomers with MgAMPPNP and the ATPase activity of the reconstituted TFαβγ(mutant) complex.42)

| β subunits | Kd/mM | ATPase activity1 | open/closed conformational change of β |
|------------|-------|------------------|-----------------------------------|
| Wild-type  | 0.96  | 6.0              | 100 yes                           |
| (D311A)    | 0.87  | 4.0              | 67 yes                            |
| (R333A)    | 6.1   | 7.7              | 128 yes                           |
| (K164A)    | 20.6  | <0.01            | - no                             |
| (T165A)    | 2.4   | 0.06             | 1 no                             |
| (D252A)    | 1.0   | 0.06             | 1 no                             |
| (R191A)    | 0.82  | 0.08             | 1 yes                            |
| (Y307P)    | 1.3   | <0.01            | - no                             |

1One unit is the activity that hydrolyzes 1 µmol of ATP/min at 298 K.
2A nucleotide-induced conformational change of the β monomer observed by 1H-NMR.

Fig. 7. Chemical shift perturbations induced by nucleotide for wild-type and mutated TF1β monomers.39),42) The used protein is specified in each panel. A, perturbations by MgADP; and B-E, perturbations by MgATP. The nucleotide/protein molar ratio was 5 for A and B, and 20 for C-E. The average chemical shift perturbations (Δδave) were obtained using the equation in the text. Residues with Δδave > 0.1 were classified as perturbed ones and those residues in A were mapped in Fig. 5. The regions of α-helices B and C are indicated in relevant panels.
although they might stabilize the closed form. Arg191 would be essential for the catalytic activity (Table 1). In contrast to these mutations, β(K164A), β(T165A), and β(D252A) revealed no chemical shift changes in the nucleotide titration of the Tyr148, Tyr199, and Tyr307 signals. This result indicated that the hydrogen bond networks involving the amino group of Lys164, the hydroxyl group of Thr165 and the carboxyl group of Asp252 were essential for the open/closed conformational change. Loss of any hydrogen bond in this network shut down this conformational change in spite of the presence of a bound nucleotide. The essential roles of these residues were also confirmed in F1 by the ATPase activity of TF1<sub>mutant</sub> (Table 1).

For detailed analysis of the effects of mutations on the nucleotide-induced conformational change, segmentally isotope-labeled β(K164A), β(T165A), and β(D252A) were examined by NMR. The <sup>1</sup>H-<sup>15</sup>N TROSY-HSQC spectrum of each mutant protein in the absence of nucleotide was in good agreement with that of the wild-type TF1<sub>mutant</sub> complex with β(K164A), β(T165A) or β(D252A) lost the activity (Table 1).

In the crystal structures of MF1 and TF1<sub>mutant</sub>, the Lys164 side chain formed a hydrogen bond with the side chain of Asp252 located in the β stride 6 (β6) in the open form (Fig. 8 left), but was attracted to the phosphate group of AMPPNP or ADP and formed hydrogen bonds with the phosphate and the carbonyl group of Gly158 in the P-loop, making the C=O angle of Gly158 suitable for a hydrogen bond with NH of Val308 (Fig. 8 middle) for extension of the β strads 3 and 7. Thus, the movement of the Lys164 side chain from Asp252 to the phosphate group should be the first essential step in the conformational change process on nucleotide binding. Without this, nothing happens except for the adenine binding.

In the cases of β(T165A) and β(D252A), an additional change was observed in the α helix B as mentioned above (Fig. 7D and E), which included Glu170. According to the crystal structure of MF1<sub>mutant</sub> and TF1<sub>mutant</sub>, the carboxyl group of Glu170 forms a hydrogen bond with the amide group of His415 in both open and closed forms. On the other hand, His415 is included in a stretch Phe414–Phe420,
forming a part of the adenine-binding pocket, in which a triple ring stacking of Tyr341:adenine: Phe420 stabilizes the adenine binding. A perturbed stretch of Leu410–Val430 in Figs. 5 and 7 connects the α helix B, the adenine binding pocket, and the C-terminal domain (at Leu410 and Val430). Lys164 and Thr165 on the α helix B are directly involved in the nucleotide binding. The fact that the stretch 168–174 was affected by nucleotide binding reveals that the structural perturbation was propagated to the α helix B through the stretch of Leu410–Val430, suggesting a strong structural correlation among the nucleotide binding site, stretch 410–430, and αB. They should work as a coupling device between the catalytic and C-terminal domains in the open/closed conformational change. Actually, a sliding of the α helix B was identified as a major factor in formation of the closed form by free energy simulations. The sliding of the helix would accompany a movement of the C-terminal domain through this coupling device. Without Thr165 or Asp252, the sliding of the whole helix could not take place.

The $K_d$ values of β(T165A) and β(D252A) were similar to that of the wild type (Table 1). Nevertheless, the nucleotide binding failed to induce an open/closed conformational change in the absence of either Thr165 or Asp252. The side chain of Thr165 formed a hydrogen bond with Glu201 in the open form and with Asp252 in the closed form in the crystal structures of MF1 and TF1. Namely, formation of the T165/D252 hydrogen bond should be the second essential step to the closed form. Since the side chain of Thr is much shorter than that of Lys, the Thr165/Asp252 hydrogen bonding brings up αB/P-loop together with the C-terminal domain, realizing the closed form (Fig. 8 right). For stabilization of the closed form, formation of the β-sheet structure between Leu156-Gly158 (Leu154-Gly156 for MF1) and Ile306-Val308 (Ile310-Val312) is indispensable, because a secondary-structure breaking mutation, Y307P, shut down the open/closed conformational change and ATPase activity of F1 (Table 1). Extension of the β-sheet should take place in concert with the Thr165/Asp252 hydrogen-bond formation. A model of stepwise mechanism of the conformational conversion from the open to the closed form base on our analysis is schematically presented in Fig. 8. The intermediate conformation in the figure has the features similar to those of the half-closed form of the β subunit in a crystal structure of MF1. In the half-closed form bound with ADP-AIF$_3$, O$_b$ of Thr165 is separated from O$_b$ of Asp252 by 3.43 Å (2.50 Å for β$_{TP}$) and from Glu201 by over 10 Å. This mechanism was supported by detailed free energy simulations. The open/closed conformational change must be driven by a collective fluctuation of the β monomer, and the interactions mentioned above should thermodynamically stabilize the closed form. Actually, the relative order parameter of the C-terminal domain was found as $S = 0.39$ in the RCD experiment, suggesting the presence of slow collective motions in the β monomer.

It should be noted that the Tyr307 aromatic signal shifted on MgATP or MgAMPPNP binding, but not on MgADP binding. However, the crystal structures reported so far gave no evidence for the involvement of Tyr307 in the interaction with γ-phosphate of MgAMPPNP. It was reported that although a chemical modification of Tyr307 of single β in F$_1$ suppressed ATPase activity for TF$_1$ and EF$_1$, a simple mutation such as Y307C did not impair the ATPase activity, suggesting that the ring itself was not an essential element for the activity. In the crystal structure of MF1 and TF$_1$, the aromatic ring of Tyr307 and the guanidyl group of Arg256 (Arg260 for MF1) are stacked to each other for β$_{TP}$ and β$_{DP}$. Arg256 is one of the conserved residues. Ahmad and Senior reported that Arg256 (Arg246 for EF$_1$) was critical for formation of transition state in catalysis and for recognition of the cleaved γ-phosphate in the EF$_1$ on the basis of their mutation experiments and reported crystal structures. They designated the area including Arg256 and Tyr307 as the P$_i$-binding pocket. This was also confirmed by a recent crystal structure. The chemical shift change of the Tyr307 ring proton described above suggested the interaction of the γ-phosphate of ATP with this pocket in the closed form of the β monomer. This might be explained by the difference in the closed form for the β subunit in F$_1$ and in monomer as presented in Fig. 6. The relative orientation angles were 119° and 110° for the former and latter, respectively. Since the structure of β monomer is more closed than that of β in F$_1$, the γ-phosphate of ATP may come close to the P$_i$-binding pocket without hydrolysis. Furthermore, the change of Tyr307 aromatic signal turned out to be an indirect effect of nucleotide binding from the Tyr307-vs-Tyr341 plots. Therefore, there should be a local conformational change following the global change, bringing the γ-phosphate of ATP close to the P$_i$-binding pocket. The final conformation would be similar to a conformation in the catalytic transition state suggested by Ahmad and Senior.
5. Structure and property of β subunit in TF1-ATPase in solution as studied by NMR

The information obtained with TF1β monomer should be examined with TF1. Although this was a challenge for NMR because of its huge molecular mass, this was successfully carried out. For better resolution, a segmentally isotope-labeled TF1β was used. For better efficiency, reconstitution of the TF1αβγε complex was carried out by a denaturation and refolding method in the presence of 10 mM MgAMPPNP and 3 mM MgADP. Furthermore, the C-terminal domain-truncated TF1ε subunit (residues 1–90, ε3Δε) and 200 mM arginine were added to suppress sample aggregation during NMR measurements. Thus, a segmentally isotope-labeled 360 kDa αβγε3Δε complex (TF1′) successfully provided well-resolved CRINEPT NMR spectra. The cross-relaxation enhanced polarization transfer (CRINEPT) pulse sequences were developed by Rick, Wüthrich et al. for NMR analysis of large molecular and supramolecular structures.

The CRINEPT-HMQC-TROSY 15N-1H correlation spectrum of αβγε(391–473)3Δε is presented in Fig. 9. Here, βε(391–473) stands for the β subunit segmentally isotope-labeled in the residues 391–473. Signals were unexpectedly well resolved. Most chemical shifts of intense peaks were similar to those for the αβγε(391–473) monomer in the open form. Therefore, assignment could be performed on the basis of that of the αβγε(391–473) monomer. The assignment for αβγε(391–473)3Δε is shown in Fig. 9. Number of residues with Δδave (average chemical shift difference) between β monomer and TF1′ complex > 0.05 ppm was eleven, which included only two residues (V416 and F420) from the stretch sensitive to the open/closed conformational change (residues 410–428). This observation provided an important conclusion that at least one of three β subunits in...
TF₁’ took the open form (β₁) in solution and its structure was similar to that of the β monomer. The well-resolved spectrum indicated that the C-terminal domain of the β subunit in the open form was dynamically mobile even in TF₁’. In contrast, the CRINEPT-HMQC-TROSY ¹⁵N-¹H correlation spectrum of α₂β₃γ(1–124)γεΔC was poor, suggesting that the N-terminal domain was rigid in TF₁’.⁵³

On closer inspection of the spectrum some residues provided multiple signals, suggesting presence of the closed forms of β in TF₁’. Residues showing large chemical shift differences (Δν > 0.1 ppm) between the open and closed forms in the TF₁’ complex were F414, V416, E418, Q419, Q423, S426, and G457. Although most of them were located in the global-conformation sensitive stretch 410–428, they might be also sensitive to interactions with the neighboring α and/or γ subunits. However, since F414 and V416 are located inside the protein, they would be barely affected by the interaction with the α or γ subunit. The chemical shifts of their closed-form signals were different from those of TF₁β monomer. This strongly suggested that the closed form of the β subunit in the TF₁’ complex was different from that of the β monomer. This is in good agreement with the difference in the relative orientation of the N- and C-terminal domains of the nucleotide bound β between the TF₁β monomer in solution and MF₁ in crystal (Fig. 6).

The mechanism underlying the rotational catalysis has been extensively discussed on the basis of a variety of crystal structures and single molecule analysis.⁷,¹⁴,¹⁶,⁵¹,⁵⁶ Major conformational states of the α₂β₃γ subcomplex along the rotation of the γ subunit have been almost established, although there are still some conflicts. The largest rotation of the γ subunit (80° in TF₁) is induced by ATP-binding. The driving force of this rotation is the conformational change from the open to the closed form and the interaction with γ. The dynamic mechanism underlying the open/closed conformational change was elucidated by our work summarized in this review (Fig. 8) and a free energy and molecular dynamics simulations by Ikekuchi’s group.⁴⁵,⁵⁷ The conformational change proceeded stepwise. This was also consistent with a series of crystal structures. Representative structures were one in the ground state (GS),¹⁰,³¹ one of transition-state analogue (TS),⁴⁶ one in pre-nucleotide-release state (PNR),⁵⁸ and one in pre-phosphate-release state (PPR)⁵¹ of the bovine MF₁-ATPase (designation by Walker and his colleagues).⁷ Although the relationship with the rotational substeps is still not yet well established, they include interesting structures of β. GS comprises typical three states of β, namely, β₁ (empty, open form), β₁TP (MgATP analogue bound, closed form), and β₁DP (MgADP bound, closed form). In TS, β₁ takes a half-closed form with MgADP and phosphate analogue bound. While PNR includes an open form β₁ with ADP bound, PPR does an open form β₁ with phosphate analogue bound. This fact suggests that the structure of C-terminal domain of β₁ is flexible in comparison with the closed structures. It is evident from the NMR spectrum in Fig. 9 that the C-terminal domain of β₁ is mobile. The RDC experiment revealed that there is a collective fluctuation of the β monomer. Molecular dynamics and free energy simulations also revealed that the β monomer underwent low-frequency motions with propensity for global bending motions.⁴⁵,⁵⁷ Presence of a half-closed form is consistent with the stepwise conformational change of TF₁β monomer on nucleotide binding shown in Fig. 8. Stabilization of the intermediate structure would be warranted by the interaction with the γ subunit. Even though there are large number of crystal structures of F₁, there is no structure which indicates direct interactions between the γ phosphate of ATP analogue and the P₁-binding pocket including Arg256. In view of biochemical results on Arg256⁹⁰ and our observation on Tyr307, this kind of interaction would take place as an activated state in hydrolysis. It would not be easy to capture the activated state, because of its nature of unstable snapshot.

6. Structural basis of ATP-dependent suppression of ATP hydrolysis by the TF₁ε subunit

The ε subunit forms a ternary complex with the γ subunit and the ε subunit ring to convert the rotation of the c-ring in F₁α to that of γ in F₁ or vice versa. It is also known as an endogenous inhibitor of ATP hydrolysis activity of F₁ and F₁,Fₙ in bacteria and plants.⁵⁹–⁶¹ although its effect on ATP synthesis by F₁,Fₙ is not yet well understood.⁶² According to crystal⁶³ and solution⁶⁴ structures, the isolated ε from Escherichia coli (EF₁ε) comprised an N-terminal β-sandwich domain and a C-terminal domain (CTD). The two helices of CTD were folded into a hairpin form on top of the N-terminal domain (NTD). This structure was called the “down-state” because whole CTD was located at the bottom of the γ subunit (Fig. 1). However, cross-linking experiments found that CTD interacted with multiple subunits of F₁.⁶⁵,⁶⁶ This could not be explained by
just the down-state structure. Furthermore, cross-linking and fluorescence resonance energy transfer (FRET) studies on TF1 provided evidence for full extension of the CTD helices along the γ subunit under the conditions of ATPase-inhibition. Thus, the conformation responsible for the inhibition of ATP hydrolysis was called the “up-state”. The crystal structure of EF1γ′ε complex (γ′: truncated-γ) hinted such an ε conformation.

The efficiency of the conversion to the down-state was regulated by ATP concentration and membrane potential. Actually, specific ATP binding was observed for the isolated ε subunits of TF1 and Bacillus subtilis F1, although it was not clear for EF1. Since ATP-binding was assumed to be a critical factor regulating inhibitory function of TF1ε, the role of ATP in the conformational conversion of TF1ε was investigated by X-ray crystallography and NMR.

TF1ε was tried to be crystallized in the presence and absence of ATP. Crystals could be obtained only in the presence of ATP. The crystal structure of TF1ε presented in Fig. 10 (blue) carried an ATP, which was the most important distinction from the crystal structure of the folded EF1ε. No Mg2+ was found. Its overall backbone structure was similar to that of the folded EF1ε. TF1ε was composed of two domains, namely, an N-terminal β sandwich (residues 1–84) and a C-terminal α-helical domain (90–133). A 310 helix (85–87) was inserted in the short loop linking NTD and CTD. NTD was composed of ten β strands. Five strands (β1: 4–10, β2: 13–20, β5: 41–45, β8: 66–71, and β9: 74–79) formed one antiparallel β sheet except for a parallel pair, β1 and β9, and the other five strands (β3: 22–27, β4: 30–34, β6: 47–54, β7: 57–64, and β10: 82–84) formed another antiparallel β sheet. CTD comprised two α-helices (α1: 90–104 and α2: 113–130), and a short loop linking these α-helices. The hydrophobic contact surface between two helices was formed by an ‘alanine zipper’-like structure, as in the case of EF1ε. The relative orientation of NTD and CTD was slightly different for TF1ε and EF1ε. If TF1ε was superimposed on NTD of EF1ε, the CTD showed deviation by 8°. When NTD and CTD of two ε subunits were superimposed independently, they fitted well to each other except for the loop in CTD. A well-defined ATP-binding site was found in CTD. The ATP in TF1ε took on an unusual structure, in which the triphosphate chain bended at the β-phosphate. This ATP-specific binding site involved at least four residues. The carbonyl and amide groups of Asp89 specifically recognized the adenine ring through Watson-Crick type hydrogen bonds. The π electron system of the guanidyl group of Arg92 stacked on the adenine ring. Arg92 also formed hydrogen bonds with the ribose and γ-phosphate. These two residues should play key roles in specific recognition of ATP. Furthermore, Ile88 and Ala93 were conserved in the same bacterial ε subunits, and contributed to the adenine-binding pocket of TF1ε. Therefore, I(L)DXXRA (X, any amino acid residue) would be a new ATP-binding motif. Crystallization of ATP-free TF1ε was unsuccessful.

The interaction of TF1ε with ATP in solution was examined by NMR. 2D 1H-15N HSQC spectra of uniformly 15N labeled TF1ε were measured in the presence and absence of ATP. The two spectra were significantly different. In the presence of ATP, the 1H-15N HSQC spectrum was well resolved. All NH resonances except for those of Pro and the N-terminal two residues could be assigned. In the absence of ATP, however, the peak dispersion was worse than that in the presence of ATP. Nevertheless, the NH
resonances could be completely assigned except for Pro, the C-terminal six and the N-terminal two residues, which were not observed. Then, the solution structures of residues 88–133 and 88–127 of whole protein in the presence and absence of ATP were determined, respectively. Since NTD and CTD were structurally independent and the ATP-binding site was found in CTD in the crystal structure, we focused our attention on CTD. According to the crystal structure, CTD of TF₁ε can be defined as residues 90–133. The conformations of the CTD backbone in the presence of ATP were well converged and very similar to the crystal structure (Fig. 10, yellow lines superimposed with its crystal structure). The backbone RMSD of the 20 best structures (residues 90–131) was 0.50 Å. The Asp89 NH signal was observed at 11 ppm, which could be ascribed to hydrogen bonding with the adenine of ATP as in the crystal structure. In contrast, the best 20 out of 100 structures in the absence of ATP did not converge with the backbone RMSD > 4 Å (Fig. 10, white lines on top of the NTD β-sandwich starting from the linker). Nevertheless, the conformation was basically helical with two well defined helices (residues 90–102 and 113–117). When each helical region was superimposed, they were well defined with backbone RMSD of 0.39 Å and 0.28 Å, respectively (see Fig. 10 for the former). Inter-helix NOEs were not observed.

On the basis of the structural analysis, it can be concluded that although the ATP-bound hairpin structure of TF₁ε CTD held in solution, the helices were not folded anymore in the absence of ATP. Analysis of relaxation parameters (NOE, T₁ and T₂) of TF₁ε also supported this conclusion. In the case of the ATP-bound TF₁ε, NTD and CTD formed a rigid body through strong interdomain interactions mediated by ATP. In contrast, the two domains revealed completely different dynamic properties in the absence of ATP. While NTD was a rigid body, CTD got more and more flexible with approaching to the C terminus. The conformational change induced by ATP binding can be inferred from Fig. 10.

We also examined ATP binding to the isolated EF₁ε by NMR. ATP titration experiments revealed that Asp90, Arg93, and Ala94 (corresponding to Asp89, Arg92, and Ala93 for TF₁ε) were included in the major affected residues. Especially, the NH signal of Asp90 disappeared at the molar ratio of ATP/ε = 1. This result strongly suggested that the ATP binding site of EF₁ε was the same as that of TF₁ε, although the affinity is significantly lower (Kd = 22 ± 1 mM at 25 °C). Kd of the TF₁ε/ATP complex was 1.4 μM and 0.67 mM at 36 and 65 °C, respectively. The latter is the physiological temperature for TF₁ε.

The structure of ε in the up-state was determined later for EF₁αβεγζ and TF₁αβεγζ by X-ray crystallography. As can be seen in Fig. 11, εCTD was basically extended along the γ subunit in both structures. While two helices of TF₁ε CTD lined up almost in a straight manner, the first helix of EF₁ε CTD shifted to the β barrel of γ, forming a hook structure. In both cases, the second helix of ε CTD was inserted into the central cavity of F₁, interacting with αDP, αE, βDP, βTP, and γ. An important consequence of these interactions was non-closed conformations of βTP (Fig. 11). It took on the half-closed and open conformations in EF₁ and TF₁, respectively. This would cause the inhibition of ATP hydrolysis in F₁. Why the structures of EF₁ε CTD and TF₁ε CTD are different? It is not clear so far. However, this may be ascribed to the difference in the role of ε inhibition in two bacteria. There is a clear difference in ATP-binding affinity. In the case of TF₁ε, ATP binding converts the extended CTD (the up-state) to the folded one (the down-state). We also found the ATP binding site in EF₁ε CTD as mentioned above. Actually, FRET experiments suggested that 1 mM MgATP or 1 mM MgAMPPNP induced generation of the folded conformation to
some extent, although the extended conformation was the dominant population in the absence of nucleotides and in the presence of ADP and P$i$. The dissociation constants of the $\varepsilon$/ATP complex under physiological conditions were 22 and 0.67 mM for EF$_1^{\varepsilon}$ and TF$_1^{\varepsilon}$, respectively. Namely, efficiency of ATP in conformational conversion of $\varepsilon$CTD depends on the bacterial species presumably because of different survival strategies. In the case of E. coli, the extended form of $\varepsilon$CTD turned out to inhibit not only ATP-hydrolysis but also ATP-synthesis. Nevertheless, the major population of $\varepsilon$CTD is supposed to take the extended conformation. Thus, it is possible for EF$_1$ to synthesize ATP in the presence of the extended $\varepsilon$CTD under the physiological conditions. The hook structure of EF$_1^{\varepsilon}$ would be more flexible to cope with effects of the rotation driven by the proton motive force (pmf) during the ATP synthesis than the structure of TF$_1^{\varepsilon}$. The intermediate structure proposed on the basis of EF$_1^{\varepsilon}$-ATP crystal structure may be included in this flexibility as well. In the case of TF$_1$, $\varepsilon$CTD would mainly take the folded conformation in the presence of physiological concentration of ATP, although the extended conformation was reported to inhibit the ATP synthesis to some extent as well. There is no ATP-binding motif in the sequences of $\varepsilon$ subunits of chloroplasts and cyanobacteria. Their $\varepsilon$ subunits revealed very strong inhibition of ATP hydrolysis in vitro, suggesting that most $\varepsilon$CTDs took on the extended structure under the physiological conditions. Since the structure of an isolated $\varepsilon$ subunit from a cyanobacterium, Thermosynechococcus elongatus, determined by NMR was found in a folded structure, some driving forces including pmf should work for the conformational conversion.

### 7. Concluding remark

In this review, I have tried to convince the readers that solution NMR provided unique information for understanding the mechanism of the rotational catalysis in F$_1$-ATPase with the help of crystal structures. Using a variety of isotope labeling methods, we could get reasonable spectra even for 360 kDa TF$_1^{\prime}$. Segmental isotope-labeling of TF$_1^{\beta}$ enabled us to get the information from most residues of TF$_1^{\beta}$. It turned out that the conformational change from the open to the closed form of the $\beta$ subunit on nucleotide binding was its intrinsic property and could work as a driving force of the rotational catalysis. The conformational change does not need energy. The conformational change was initiated by nucleotide binding and driven in a stepwise manner by switching of the hydrogen bond networks involving Walker A and B motifs. Segmentally labeled TF$_1$ provided a well resolved NMR spectra, revealing while the open form of $\beta$ was identical for the TF$_1^{\beta}$ monomer and TF$_1$, its closed form could be different. The activated state in the hydrolysis reaction was suggested in connection with the closed form of the $\beta$ monomer. ATP-binding was also found to be a critical factor in the regulation of TF$_1$ activity by the $\varepsilon$ subunit. The structures of the ATP-bound and ATP-free TF$_1^{\varepsilon}$ were determined by X-ray crystallography and NMR, which revealed a structural conversion of TF$_1^{\varepsilon}$ depending on ATP concentration. Thus, the works reviewed here contributed to elucidation of the basic mechanisms underlying important elements of rotational ATP hydrolysis by F$_1$.

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### References

1. Boyer, P.D. (1997) The ATP synthase—a splendid molecular machine. Annu. Rev. Biochem. 66, 717–749.
2. Yoshida, M., Muneyuki, E. and Hisabori, T. (2001) ATP synthase—a marvellous rotary engine of the cell. Nat. Rev. Mol. Cell Biol. 2, 669–677.
3. Senior, A.E., Nadanaciva, S. and Weber, J. (2002) The molecular mechanism of ATP synthesis by F$_1$F$_{\varepsilon}$-ATP synthase. Biochim. Biophys. Acta 1553, 188–211.
4. Futai, M. (2006) Our research on proton pumping ATPases over three decades: their biochemistry, molecular biology and cell biology. Proc. Jpn. Acad., Ser. B 82, 416–438.
5. Dimroth, P., Von Ballmoos, C. and Meier, T. (2006) Catalytic and mechanical cycles in F-ATP synthase. EMBO Rep. 7, 276–282.
6. Kagawa, Y. (2010) ATP synthase: from single molecule to human bioenergetics. Proc. Jpn. Acad., Ser. B 86, 667–693.
7. Walker, J.E. (2013) The ATP synthase: the understood, the uncertain and the unknown. Biochem. Soc. Trans. 41, 1–16.
8. Hisabori, T., Sunamura, E., Kim, Y. and Konno, H. (2013) The chloroplast ATP synthase features the characteristic redox regulation machinery. Antioxid. Redox Signal. 19, 1846–1854.
9. Nakanoishi-Matsui, M., Sekiya, M. and Futai, M. (2016) ATPase from Escherichia coli: Mechanism of rotational catalysis, and inhibition with the $\varepsilon$ subunit and phytopolyphenols. Biochim. Biophys.
Acta 1857, 129–140.

10) Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria. Nature 370, 621–628.

11) Noji, H., Yasuda, R., Yoshida, M. and Kinosita, K. Jr. (1997) Direct observation of the rotation of F1-ATPase. Nature 386, 299–302.

12) Yasuda, R., Noji, H., Yoshida, M., Kinosita, K. Jr. and Itoh, H. (2001) Resolution of distinct rotational substeps by submillisecond kinetic analysis of F1-ATPase. Nature 410, 989–904.

13) Shimabukuro, K., Yasuda, R., Muneyuki, E., Harra, K.Y., Kinosita, K. Jr. and Yoshida, M. (2003) Catalysis and rotation of F1 motor: cleavage of ATP at the catalytic site occurs in 1 ms before 40° substep rotation. Proc. Natl. Acad. Sci. U.S.A. 100, 14731–14736.

14) Adachi, K., Ohwada, K., Nishizawa, T., Furuike, S., Noji, H., Itoh, H., Yoshida, M. and Kinosita, K. Jr. (2007) Coupling of rotation and catalysis in F1-ATPase revealed by single-molecule imaging and manipulation. Cell 130, 309–321.

15) Watanabe, R., Iino, R. and Noji, H. (2010) Phosphatase release in F1-ATPase catalytic cycle follows ADP release. Nat. Chem. Biol. 6, 814–820.

16) Suzuki, T., Tanaka, K., Nakayashiki, C., Saita, E. and Yoshida, M. (2014) Chemomechanical coupling of human mitochondrial F1-ATPase motor. Nat. Chem. Biol. 10, 930–936.

17) Fillingame, R.H., Angevine, C.M. and Dmitrovic, O.Y. (2003) Mechanics of coupling proton movements to c-ring rotation in ATP synthase. FEBS Lett. 555, 29–34.

18) Stock, D., Leslie, A.G. and Walker, J.E. (1999) Molecular architecture of the rotary motor in ATP synthase. Science 286, 1700–1705.

19) Seelert, H., Poetsch, A., Deucher, N.A., Engel, A., Stahilberg, H. and Muller, D.J. (2000) Proton-powered turbine of a plant motor. Nature 405, 418–419.

20) Mitome, N., Suzuki, T., Hayashi, S. and Yoshida, M. (2004) The membrane ATP synthase has a decamer c-ring: indication of noninteger 103 H+/ATP ratio and permissive elastic coupling. Proc. Natl. Acad. Sci. U.S.A. 101, 12159–12164.

21) Meier, T., Polzer, P., Diederichs, K., Welte, W. and Dimroth, P. (2005) Structure of the rotor ring of F-type Na+/ATPase from Ilyobacter tartaricus. Science 308, 659–662.

22) Matthis, D., Preiss, L., Klyszewko, A.L., Muller, D.J., Cook, G.M., Vonck, J. and Meier, T. (2009) The c13 ring from a thermoaalkaliphilic ATP synthase reveals an extended diameter due to a special structural region. J. Mol. Biol. 388, 611–618.

23) Watt, J.N., Montgomery, M.G., Runswick, M.J., Leslie, A.G. and Walker, J.E. (2010) Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. Proc. Natl. Acad. Sci. U.S.A. 107, 16823–16827.

24) Nesci, S., Trombetti, F., Ventrella, V. and Pagnialani, A. (2016) The c-ring of the F1F0-ATP synthase: facts and perspective. J. Membr. Biol. 249, 11–21.

25) Miwa, K. and Yoshida, M. (1989) The αβ complex, the catalytic core of F1-ATPase. Proc. Natl. Acad. Sci. U.S.A. 86, 6484–6487.

26) Yoshida, M., Poser, J.W., Allison, W.S. and Esch, F.S. (1981) Identification of an essential glutamic acid residue in the β subunit of the adenosine triphosphatase from the thermophilic bacterium PS3. J. Biol. Chem. 256, 148–153.

27) Ohtsubo, M., Yoshida, M., Ohta, S., Kagawa, Y., Yohda, M. and Date, T. (1987) In vitro mutated β subunits from the F1-ATPase of the thermophilic bacterium, PS3, containing glutamine in place of glutamic acid in positions 190 or 201 assembles with the α and γ subunits to produce inactive complexes. Biochem. Biophys. Res. Commun. 146, 705–710.

28) Tozawa, K., Ohbuchi, H., Yagi, H., Amano, T., Matsui, T., Yoshida, M. and Akutsu, H. (1996) Unusual pKa of the carboxylate at the putative catalytic position of the thermophilic F1-ATPase β subunit determined by 13C-NMR. FEBS Lett. 397, 122–126.

29) Kohda, D., Sawada, T. and Inagaki, F. (1991) Characterization of pH titration shifts for all the nonlabile proton resonances in a protein by two-dimensional NMR: The case of mouse epidermal growth factor. Biochemistry 30, 4896–4900.

30) Amano, T., Tozawa, K., Yoshida, M. and Murakami, H. (1994) Spatial precision of a catalytic carboxylate of F1-ATPase β subunit probed by introducing different carboxylate-containing side chains. FEBS Lett. 348, 93–98.

31) Bowler, M.W., Montgomery, M.G., Leslie, A.G.W. and Walker, J.E. (2007) Ground state structure of F1-ATPase from bovine heart mitochondria at 1.9 Å resolution. J. Biol. Chem. 282, 14238–14242.

32) Beke-Somfai, T., Lincol, P. and Norden, B. (2011) Double-lock ratchet mechanism revealing the role of ααα-344 in F2F1 ATP synthase. Proc. Natl. Acad. Sci. U.S.A. 108, 4832–4837.

33) Hayashi, S., Ueno, H., Shaikh, A.R., Umemura, M., Kaniya, M., Itou, Y., Ibeguchi, M., Komoriya, Y., Iino, R. and Noji, H. (2012) Molecular mechanism of ATP hydrolysis in F1-ATPase revealed by molecular simulations and single-molecule observations. J. Am. Chem. Soc. 134, 8447–8454.

34) Shirakihara, Y., Leslie, A.G.W., Abrahams, J.P., Walker, J.E., Ueda, T., Sekimoto, Y., Kambara, M., Saika, K., Kagawa, Y. and Yoshida, M. (1997) The crystal structure of the nucleotide-free αβ34 complex, of F1-ATPase from the thermophilic Bacillus PS3 is a symmetric trimer. Structure 5, 825–836.

35) Shirakihara, Y., Shiratori, A., Tanikawa, H., Nakasako, M., Yoshida, M. and Suzuki, T. (2015) Structure of a thermophilic F1-ATPase inhibited by an ε-subunit: deeper insight into the ε-inhibition mechanism. FEBS J. 282, 2895–2913.

36) Tozawa, K., Sekino, N., Soga, M., Yagi, H., Yoshida, M. and Akutsu, H. (1995) Conformational dynam-
ics monitored by His-179 and His-200 of isolated thermophilic F$_1$-ATPase $\beta$ subunit which reside at the entrance of the “conical tunnel” in Holoenzyme. FEBS Lett. 376, 190–194.

37) Yagi, H., Tozawa, K., Sekino, N., Iwabuchi, T., Yoshida, M. and Akutsu, H. (1999) Functional conformational changes in the TF$_1$-ATPase $\beta$ subunit probed by twelve tyrosine residues. Biophys. J. 77, 2175–2183.

38) Tozawa, K., Yagi, H., Hisamatsu, K., Ozawa, K., Yoshida, M. and Akutsu, H. (2001) Functions and ATP-binding responses of the twelve histidine residues in the TF$_1$-ATPase $\beta$ subunit. J. Biochem. 130, 527–533.

39) Yagi, H., Tsujimoto, T., Yamazaki, T., Yoshida, M. and Akutsu, H. (2004) A conformational change of H$^+$-ATPase $\beta$ monomer revealed on segmental isotope labeling NMR spectroscopy. J. Am. Chem. Soc. 126, 16632–16638.

40) Yamazaki, T., Otomo, T., Oda, N., Kiyokura, Y., Uegaki, K., Ito, N., Ishino, Y. and Nakamura, H. (1998) Segmental isotope labeling for protein NMR using peptide splicing. J. Am. Chem. Soc. 120, 5591–5592.

41) Xu, R., Ayers, B., Cowburn, D. and Muir, T.W. (1999) Chemical ligation of folded recombinant proteins: Segmental isotopic labeling of domains for NMR studies. Proc. Natl. Acad. Sci. U.S.A. 96, 388–393.

42) Yagi, H., Kajiwara, N., Iwabuchi, T., Izumi, K., Yoshida, M. and Akutsu, H. (2000) Stepwise propagation of the ATP-induced conformational change of the F$_1$-ATPase $\beta$ subunit revealed by NMR. J. Biol. Chem. 274, 2374–2382.

43) Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 945–951.

44) Löbau, S., Weber, J., Wilke-Mounts, S. and Senior, A.E. (1997) F$_1$-ATPase, roles of three catalytic site residues. J. Biol. Chem. 272, 3648–3656.

45) Ito, Y., Oroguchi, T. and Ikekuchi, M. (2011) Mechanism of the conformational change of the F$_1$-ATPase $\beta$ subunit revealed by free energy simulations. J. Am. Chem. Soc. 133, 3372–3380.

46) Menz, R.I., Walker, J.E. and Leslie, A.G.W. (2001) Structure of Bovine Mitochondrial F$_1$-ATPase with nucleotide bound to all three catalytic sites: Implications for the mechanism of rotary catalysis. Cell 106, 331–341.

47) Tolman, J.R., Flanagan, J.M., Kennedy, M.A. and Prestegard, J.H. (1997) NMR evidence for slow collective motions in cyanometmyoglobin. Nat. Struct. Biol. 4, 292–297.

48) Yoshida, M. and Allison, W.S. (1990) The ATPase activity of the $\alpha_3\beta_3$ complex of the F$_1$-ATPase of the thermophilic bacterium PS3 is inactivated on modification of tyrosine 307 in a single $\beta$ subunit by 7-chloro-4-nitrobenzofuran. J. Biol. Chem. 265, 2483–2487.

49) Ahmad, Z. and Senior, A.E. (2004) Mutagenesis of residue $\beta$Arg-246 in the phosphate-binding subdomain of catalytic sites of Escherichia coli F$_1$-ATPase. J. Biol. Chem. 279, 31505–31513.

50) Odaka, M., Kiribuchi, K., Allison, W.S. and Yoshida, M. (1993) In vivo affinity label of a protein expressed in Escherichia coli coenzyme A occupied the AT(D)P binding site of the mutant F$_1$-ATPase $\beta$ subunit (Y307C) through a disulfide bond. FEBS Lett. 336, 231–235.

51) Bason, J.V., Montgomery, M.G., Leslie, A.G.W. and Walker, J.E. (2015) How release of phosphate from mammalian F$_1$-ATPase generates a rotary substep. Proc. Natl. Acad. Sci. U.S.A. 112, 6009–6014.

52) Kobayashi, M., Yagi, H., Yamazaki, T., Yoshida, M. and Akutsu, H. (2008) Dynamic inter-subunit interactions in thermophilic F$_1$-ATPase subcomplexes studied by cross-correlated relaxation-enhanced polarization transfer NMR. J. Biomol. NMR 40, 165–174.

53) Kobayashi, M., Akutsu, H., Suzuki, T., Yoshida, M. and Yagi, H. (2010) Analysis of the open and closed conformations of the $\beta$ subunits in thermophilic F$_1$-ATPase by solution NMR. J. Mol. Biol. 398, 189–199.

54) Riek, R., Wider, G., Pervushin, K. and Wüthrich, K. (1999) Polarization transfer by cross-correlated relaxation in solution NMR with very large molecules. Proc. Natl. Acad. Sci. U.S.A. 96, 4918–4923.

55) Riek, R., Fiaux, J., Bertelsen, E.B., Horwich, A.L. and Wüthrich, K. (2002) Solution NMR techniques for large molecular and supramolecular structures. J. Am. Chem. Soc. 124, 12144–12153.

56) Watanabe, R. and Noji, H. (2014) Timing of inorganic phosphate release modulates the catalytic activity of ATP-driven rotary motor protein. Nat. Commun. 5, 3486.

57) Ito, Y. and Ikekuchi, M. (2010) Molecular dynamics simulations of the isolated $\beta$ subunit of F$_1$-ATPase. Chem. Phys. Lett. 490, 80–83.

58) Rees, D.M., Montgomery, M.G., Leslie, A.G.W. and Walker, J.E. (2012) Structural evidence of a new catalytic intermediate in the pathway of ATP hydrolysis by F$_1$-ATPase from bovine heart mitochondria. Proc. Natl. Acad. Sci. U.S.A. 109, 11139–11143.

59) Laget, P.P. and Smith, J.B. (1979) Inhibitory properties of endogenous subunit $\varepsilon$ in the Escherichia coli F$_1$ ATPase. Arch. Biochem. Biophys. 197, 83–89.

60) Sternweis, P.C. and Smith, J.B. (1980) Characterization of the inhibitory (\varepsilon) subunit of the proton-translocating adenosine triphosphatase from Escherichia coli. Biochemistry 19, 526–531.

61) Kato-Yamada, Y., Baid, D., Koike, M., Motohashi, K., Hisabori, T. and Yoshida, M. (1999) $\varepsilon$ Subunit, an endogenous inhibitor of bacterial F$_1$-ATPase, also inhibits F$_0$F$_1$-ATPase. J. Biol. Chem. 274, 33991–33994.

62) Iino, R., Hasegawa, R., Tabata, K.V. and Noji, H. (2009) Mechanism of inhibition by C-terminal $\alpha$-
helices of the $\varepsilon$ subunit of \textit{Escherichia coli} F$_1$F$_0$-ATP synthase. J. Biol. Chem. \textbf{284}, 17457–17464.

63) Uhlin, U., Cox, G.B. and Guss, J.M. (1997) Crystal structure of the $\varepsilon$ subunit of the proton-translocating ATP synthase from \textit{Escherichia coli}. Structure \textbf{5}, 1219–1230.

64) Wilkens, S., Dahlquist, F.W., McIntosh, L.P., Donaldson, L.W. and Capaldi, R.A. (1995) Structural features of the $\varepsilon$ subunit of the \textit{Escherichia coli} ATP synthase determined by NMR spectroscopy. Nat. Struct. Biol. \textbf{2}, 961–967.

65) Dahlmann, H.G., Flynn, T.G. and Dunn, S.D. (1992) Determination of the 1-ethyl-3-[(3-dimethylaminomethyl)propyl]-carbodiimide-induced cross-link between the $\beta$ and $\varepsilon$ subunits of \textit{Escherichia coli} F$_1$-ATPase. J. Biol. Chem. \textbf{267}, 18553–18560.

66) Tsunoda, S.P., Rodgers, A.J.W., Agyeler, R., Wilce, M.C.J., Yoshida, M. and Capaldi, R.A. (2001) Large conformational changes of the $\varepsilon$ subunit in the bacterial F$_1$F$_0$-ATP synthase provide a ratchet action to regulate this rotary motor enzyme. Proc. Natl. Acad. Sci. U.S.A. \textbf{98}, 6560–6564.

67) Suzuki, T., Murakami, T., Iino, R., Suzuki, J., Ono, S., Shirakihara, Y. and Yoshida, M. (2003) F$_1$F$_0$-ATPase/synthase is geared to the synthesis mode by conformational rearrangement of $\varepsilon$ subunit in response to proton motive force and ADP/ATP balance. J. Biol. Chem. \textbf{278}, 46840–46846.

68) Iino, R., Murakami, T., Izuka, S., Kato-Yamada, Y., Suzuki, T. and Yoshida, M. (2005) Real-time monitoring of conformational dynamics of the $\varepsilon$ subunit in F$_1$-ATPase. J. Biol. Chem. \textbf{280}, 40130–40134.

69) Rodgers, A.J.W. and Wilce, M.C.J. (2000) Structure of the $\gamma$-$\varepsilon$ complex of ATP synthase. Nat. Struct. Biol. \textbf{7}, 1051–1054.

70) Kato-Yamada, Y. and Yoshida, M. (2003) Isolated $\varepsilon$ subunit of thermophilic F$_1$-ATPase binds ATP. J. Biol. Chem. \textbf{278}, 36013–36016.

71) Kato-Yamada, Y. (2005) Isolated $\varepsilon$ subunit of \textit{Bacillus subtilis} binds ATP. FEBS Lett. \textbf{579}, 6875–6878.

72) Yagi, H., Kajiwara, N., Tanaka, H., Tsukihara, T., Kato-Yamada, Y., Yoshida, M. and Akutsu, H. (2007) Structures of the thermophilic F$_1$-ATPase $\varepsilon$ subunit suggesting ATP-regulated arm motion of its C-terminal domain in F$_1$. Proc. Natl. Acad. Sci. U.S.A. \textbf{104}, 11233–11238.

73) Cingolani, G. and Duncan, T.M. (2011) Structure of the ATP synthase catalytic complex (F$_i$) from \textit{Escherichia coli} in an autoinhibited conformation. Nat. Struct. Mol. Biol. \textbf{18}, 701–708.

74) Borsch, M. and Duncan, T.M. (2013) Spotlighting motors and controls of single F$_i$F$_1$-ATP synthase. Biochem. Soc. Trans. \textbf{41}, 1210–1226.

75) Masaike, T., Suzuki, T., Tsunoda, S.P., Konno, H. and Yoshida, M. (2006) Probing conformations of the $\beta$ subunit of F$_i$F$_1$-ATP synthase in catalysis. Biochem. Biophys. Res. Commun. \textbf{342}, 800–807.

76) Yagi, H., Konno, H., Murakami-Fuse, T., Isu, A., Oroguchi, T., Akutsu, H., Ikeguchi, M. and Hisabori, T. (2010) Structural and functional analysis of the intrinsic inhibitor subunit $\varepsilon$ of F$_i$-ATPase from photosynthetic organisms. Biochem. J. \textbf{425}, 85–94.

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Profile

Hideo Akutsu was born in 1944 in Tokyo. He graduated from the University of Tokyo in 1967 and received Doctor of Science degree in 1973. He was appointed as an assistant professor at Institute for Protein Research, Osaka University in 1972 to work with Prof. Y. Kyogoku, then moved to Faculty of Engineering, Yokohama National University as an associate professor in 1985. Meanwhile, he joined the group of Prof. J. Seelig at University of Basel as a research fellow of Japan Society for the Promotion of Science (JSPS) and EMBO from 1978 to 1980 to work on membrane structure by solid-state NMR. He became a professor in 1991 at the same university and moved back to Institute for Protein Research in 2000. He served as director of the institute from 2004 to 2006. He was appointed to be a WCU professor at Seoul National University from 2009 to 2013, and to be director of the JSPS Stockholm Office from 2014 to 2016. His research has been focusing on NMR structural biology of lipids and proteins involved in energy transduction. He elucidated electron transport mechanisms in a variety of biological processes involving tetraheme cytochromes, and the mechanism underlying the nucleotide-induced conformational change of the $\beta$ subunit, driving the rotational catalysis of F$_1$-ATPase. In order to analyze huge biomolecular complexes such as F$_i$F$_1$-ATP synthase, he has developed biological solid-state NMR, which was used for the investigation of F$_i$F$_0$-subunit ring in membranes to elucidate the mechanism underlying proton translocation across the membrane.