The rotation of F₁-ATPase (F₁) is driven by the open/close bending motion of the β subunit. The mechanism underlying the bending motion was investigated for the F₁β monomer from thermophilic Bacillus PS3 (TF₁) in solution, using mutagenesis and NMR. The hydrogen bond networks involving the side chains of Lys-164 (numbering for TF₁β; 162 for mitochondrial F₁β in parentheses), Thr-165(163), Arg-191(189), Asp-252(256), Asp-311(315), and Arg-333(337) in the catalytic region are significantly different for the ligand-bound and free β subunits in the crystal structures of mitochondrial F₁. The role of each amino acid residue was examined by Ala substitution. β(K164A) reduced the affinity constant for 5′-adenyl-β,γ-imidodiphosphate by 20-fold and abolished the conformational change associated with nucleotide binding and the ATPase activity of αβ(164A)γβ(T165A) and β(D252A) exhibited no effect on the binding affinity but abolished the conformational change and the ATPase activity. The chemical shift perturbation of backbone amide signals of the segmentally labeled β(mutant)s indicated stepwise propagation of the open/close conversion on ligand binding. The key action in the conversion is the switching of the hydrogen-bonding partner of Asp-252 from Lys-164 to Thr-165. Residual dipolar coupling analysis revealed that the closed conformation of the β monomer was more closed than that in the crystal structure and was different for MgATP- and MgADP-bound β subunits. Actually, MgATP induced a conformational change around Tyr-307 (311 for MF₁β), whereas MgADP did not. The significance of these findings is discussed in connection with the catalytic rotation of F₁-ATPase.

F₁β-ATP synthase catalyzes ATP synthesis coupled with proton flow across membranes driven by the electrochemical potential of a proton gradient (1–4). F₁ is its aqueous portion, composed of α, β, γ, δ, and ε subunits. The α and β subunits form an αβ ring, into which the γ subunit penetrates (5). In the first reported crystal structure of mitochondrial F₁ (MF₁), three β subunits carrying catalytic sites exhibited different nucleotide occupancies and different conformations (5), namely MgAMPPNP-bound (βₚ₀), MgADP-bound (βₚ₀), and empty (β₀) ones. The conformation of the catalytic site with the nucleotide is more closed than that of the empty one. Thus, they are designated as the closed and open forms, respectively (5). In the latest reported structure of MF₁, in the ground state (6), MgADP is replaced by MgAMPPNP. Furthermore, a half-closed conformation of the β subunit was reported for MF₁ and was assumed to be a transient conformation during the release of ADP + P (7).

On the other hand, the 120° step rotation of the γ subunit in the fixed αβ₃ complex on hydrolysis of ATP has been demonstrated for the αβ₃γ complex from thermophilic Bacillus PS-3 (TF₃) (8–11). Yasuda et al. (12) found that the 120° step of the γ subunit rotation can be divided into roughly 90 and 30° substeps and suggested that these steps correspond to the ATP binding and the release of the hydrolyzed product, respectively. Later, these subsets were modified to 80 and 40° ones (13). Previously, we analyzed the chemical shift perturbation induced by ADP binding and determined the relative orientation between the N- and C-terminal domains of a monomeric β subunit using NMR residual dipolar couplings (RDCs) in combination with segmental isotope labeling (14). It was demonstrated that the conformational change of the β subunit from the open to the closed form (bending motion) upon nucleotide binding was similar to that found in MF₁ crystal structures and was an intrinsic property of the β subunit. This could be a driving force for the rotational catalysis. Recently, ATP binding to the β subunit was also taken as the driving force generating the torque for the rotation of the γ subunit in the single-molecule analysis (15). Since the properties of the β monomer were found to be closely correlated with those of F₁, the molecular mechanism of the bending motion of the isolated TF₁ β subunit induced by the ATP binding was investigated in this work. It should be noted that the conformation of the ATP-bound β subunit can be investigated only for the monomer because of the lack of ATPase activity.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.
Stepwise Propagation of F1-ATPase β Conformational Change

For analysis of TF1, the crystal structure of MF1 was used as a structural model, because the crystal structure of TF1β in the α3β3 hexamer is very similar to the open form of MF1β (16). To make the notation simple, the sequential numbering of TF1 is used throughout this paper, even for the crystal structure of MF1, unless otherwise mentioned. The MF1 numbering of the major amino acid residues is given in the legend to Fig. 1. In the crystal structure of MF1 (5,6), the phosphate groups of a nucleotide are tightly bound by the P-loop (GXXXGKKT), which is a conserved Walker motif for the phosphate binding (17,18). In the presence of a bound nucleotide, the β sheet composed of the β3 and β7 strands becomes longer than that in its absence through the formation of additional hydrogen bonds Leu-156/Ile-306, Gly-158/Ile-306, and Gly-158/Val-308 in the P-loop region (Fig. 1A). With the conformational change induced by the nucleotide binding, three new hydrogen bonds involving the P-loop residues are generated besides those between the nucleotide and the P-loop backbone. First, the side chain of Lys-164 forms hydrogen bonds with the backbone carbonyl group of Gly-158 along with the phosphate group of the nucleotide (Fig. 1A). The second is that between the hydroxyl group of Thr-165 and the side chain carbonyl group of Asp-252 in Walker’s motif B (17) (Fig. 1A). The third is that between the carbonyl group of Ala-160 and Nε-H of Arg-333, and the latter further forms a hydrogen bond with Oε1 of Asp-311 (Fig. 1B). These hydrogen bonds should play important roles in inducing a global change from the open to the closed form, because the interaction between the phosphates and the P-loop backbone is too local to induce a global change. In addition, Arg-191 was indicated to be important for catalysis along with the involvement of Arg-365 (Arg-373 for MF1) of the α subunit (5-7).

To elucidate the role of the hydrogen bond networks in the nucleotide-induced conformational change, a series of mutant TF1 β subunits were constructed. Using specific deuteration (19) and segmental isotope labeling (14,20), the conformational properties of the mutant proteins were investigated by means of NMR. On the basis of the results and ATPase activities of the reconstituted α3β(mutant)γ complexes, we propose stepwise propagation of the conformational change of the monomeric β subunit from the open to the closed form in solution. It was also found that the closed form in solution has a feature similar to the catalytically activated conformation.

EXPERIMENTAL PROCEDURES

Construction of Mutated β Subunit Genes and Preparation of Mutant Proteins—The expression plasmids of β(K164A), β(T165A), β(D311A), β(R333A), β(R191A), β(R191Q), β(R191E), and β(Y307P) were obtained from pUC118β by oligonucleotide-directed mutagenesis (21). For preparation of the specifically deuterated [3H-H, F, Y] proteins, cells were grown on a minimal medium with a mixture of amino acids. L-Phenylalanine, L-histidine, and L-tyrosine were replaced by [2,3,4,5,6-2H]phenylalanine, DL-[α-2,4-2H]histidine, and L-[2,6-2H]tyrosine, respectively. [3H-H, F, Y] represents the deuteriation of a protein by these amino acids. Glycophosphate was added to the culture medium to inhibit aromatic amino acid biosynthesis. The details are given elsewhere (19). All mutant proteins were purified according to previous reports (19,22).

Construction of Expression Plasmids for Segmentally Labeled Proteins—For the preparation of segmentally labeled β(K164A), β(T165A), and β(D252A), the genes of 1) the N-terminal fragment of the β subunit (1–271 residues) plus that of Pi-pifid intein (1–160) and 2) the C-terminal fragment of the Pi-pifid intein (161–454 residues) plus that of the β subunit (272–473) were constructed by PCR, respectively. Each gene was cloned into pET32a (Novagen) at the NdeI and BamHI restriction sites. The procedure for construction of expression plasmids was described in detail previously (14). The gene encoding the N-terminal fragment mutated at Lys-164, Thr-165, or Asp-252 was amplified by PCR, using a pUC118 (K164A, T165A, or D252A) β plasmid as a template.

Preparation of Segmentally Labeled β Subunits—Escherichia coli BL21 (ADE3) was transformed with each plasmid and then grown at 37 °C in 1 liter of LB medium (in the case of the unlabelled segment) or 80% H2O M9 minimal medium (in the case of the labeled segment) containing 0.5 g of [15]NH4Cl and 2 g of 13C-glucose as the sole nitrogen and carbon sources, respectively. The intein splicing reaction and purification of segmentally labeled proteins were carried out according to the reported procedures (14).
Stepwise Propagation of F$_1$-ATPase β Conformational Change

NMR Measurements—Specifically deuterated mutant β subunits were dissolved at 0.5 mM in 50 mM sodium phosphate $^2$H$_2$O buffer, pH 7.8. The exchangeable protons of the protein were replaced with deuterons by repeated cycles of lyophilization and dissolution in $^2$H$_2$O. For MgAMPPNP titration, an aliquot of a 200 mM MgAMPPNP solution was directly added to 0.5 mM mutant β subunits. The MgAMPPNP solution was prepared by dissolving equimolar amounts of AMPPNP and MgCl$_2$ in the same buffer. 1H NMR spectra were recorded with 15N $^2$H$_2$O. Sets comprised 3072 (15N) points with a data size of 256 (1H) real points with digital resolutions of 0.84 (15N) and 3.9 (1H) Hz/point. All NMR data were processed using NMRPipe and NMRDraw (25) and analyzed with Sparky, a program developed by T. D. Goddard and D. G. Kneller (University of California, San Francisco).

Determination of the Alignment Tensor—The alignment tensors of weakly oriented samples were determined with the program Module (26). Residual dipolar couplings between spins $i$ and $j$, $D_{ij}$, can be described in terms of the orientation ($\theta, \phi$) of the internuclear vector relative to a common alignment tensor for the molecule,

$$ D_{ij} = -\frac{\gamma_i \gamma_j \mu_{ij}}{16 \pi^2 r_{ij}^3} \left[ A_x (3 \cos^2 \theta - 1) + \frac{3}{2} A_y \sin^2 \theta \cos 2\phi \right] $$

(Eq. 2)

where $A_x$ and $A_y$ represent the axial and rhombic components of the alignment tensor, $r_{ij}$ is the internuclear distance, and $S$ is the order parameter. $A_{\alpha}$, $A_\beta$, and the Euler angles ($\alpha$, $\beta$, and $\gamma$) describing the orientation of the tensor relative to the Protein Data Bank frame were obtained by least squares minimization of the target function, $\chi^2$, over all couplings associated with a given domain,

$$ \chi^2 = \sum_n (D^{exp}_{ij} - D^{calc}_{ij})^2/\sigma^2_{ij} $$

(Eq. 3)

where $\sigma_{ij}$ is the uncertainty of the experimentally measured coupling. The standard coordinate file of the β subunit obtained from the crystal structure of the α$_\beta_2$γ complex (Protein Data Bank entry 1SKY) (16) was converted to a new file that includes proton coordinates generated with Insight II. The experimental uncertainty, $\sigma_{ij}$, was estimated to be 0.5 Hz for all signals.

ATPase Assay—Reconstitution of the α$_\beta_2$γ complex from α, β, and γ subunits and assaying of its ATPase activity were carried out as described previously (27, 28). Protein concentrations were determined by the Bradford method (29), using bovine serum albumin as a standard. The ATPase activity of the TF$_1$β monomer at 5 μM in the presence of 2 mM ATP was negligible during observation for 20 min.

RESULTS

Roles of Lys-164, Thr-165, Asp-252, Asp-311, and Arg-333 in the Conformational Change—To identify the hydrogen bonds essential for the formation of the closed conformation of TF$_1$β, we generated Ala-substituted proteins, β(K164A), β(T165A), β(D252A), β(D311A), and β(R333A). Every mutant protein was specifically deuterated for the aromatic protons of His, Phe, and Tyr (except for 3,5H). This allowed us to analyze the shift change of the Tyr-341 signal was in the fast exchange regime for the molecule,
Stepwise Propagation of F$_1$-ATPase β Conformational Change

FIGURE 2. MgAMPPNP titration of the tyrosine signals of the TF$_1$ [H, F, H, Y] β subunits. A, the $^1$H NMR spectra of the wild-type β subunit in the aromatic region. The aromatic rings of Phe, His, and Tyr (only at the 2- and 6-positions) of the β subunit were deuterated (19). The molar concentration ratios of nucleotide to protein are 0, 0.1, 0.2, 0.5, 0.7, 1.0, 2.0, and 3.0, from the bottom to the top. ×, Tyr-148; ⊖, Tyr-199; ○, Tyr-307; ■, Tyr-341. B, titration curves of the Tyr-341 signal for the wild-type and mutant β subunits as a function of the nucleotide/protein ratio. The symbols are the observed points, and the solid lines are the best fit curves obtained by nonlinear least squares fitting. ■, β (wild type); □, β(R164A); ▲, β(T165A); ◯, β(D252A); △, β(D311A); ▼, β(R333A).

and MgADP, the dissociation constant ($K_d$) was determined only for MgAMPPNP.

The MgAMPPNP titration curve of the Tyr-341 signal is presented in Fig. 2B for the wild-type and mutant β subunits. $K_d$ was obtained from each curve, using Equation 1, as summarized in Table 1. Although the binding affinity is similar to that of the wild-type β for β(D311A) and β(D252A), it is reduced by 2-, 6-, and 20-fold for β(T165A), β(R333A), and β(K164A), respectively. This indicates that the side chain of Lys-164 is important for the nucleotide binding, in accordance with the previous report (30). To examine the relationship between the nucleotide binding and the conformational change, the chemical shifts ($\Delta\delta_h$) of the shifting signals (Tyr-118, Tyr-199, and Tyr-307) of the wild-type β were plotted as a function of the chemical shift change ($\Delta\delta_h$) of Tyr-341 for MgAMPPNP, MgATP, and MgADP. Since Tyr-118 and Tyr-199 behave in the same manner, the plots for Tyr-199 and Tyr-307 are presented in Fig. 3, A and B, respectively. In the case of the former, the chemical shift change is a linear function of the amount of the bound nucleotide, showing that the conformational change represented by

### Table 1

| β subunits | $K_d$ (mM) | ATPase activity$^a$ |
|------------|-----------|----------------------|
| Wild type  | 0.96      | 6.0 100              |
| β(D311A)  | 0.87      | 4.0 67               |
| β(D252A)  | 6.1       | 7.7 128              |
| β(T165A)  | 2.4       | -0.01 1              |
| β(R191A)  | 1.0       | 0.06 1               |
| β(R191Q)  | 0.82      | 0.08 1               |
| β(R191E)  | 0.78      | 0.08 1               |
| β(Y307P)  | 2.4       | -0.01 1              |

$^a$ 1 unit is the activity that hydrolyzes 1 μmol of ATP/min at 298 K.
Stepwise Propagation of F₁-ATPase β Conformational Change

Tyr-199 \( \Delta \delta_{14} \) is induced through the nucleotide binding. Our previous RDC experiment on the MgADP-bound β subunit monomer revealed that this is the open/close conformational change (14). In contrast, the Tyr-307 signal started to change at \( \Delta \delta_{14}(\text{Tyr-341}) = \) about 0.25 ppm for MgAMPPNP and MgATP, suggesting that this is a second event following the major conformational change. In the case of MgADP, however, the change of the Tyr-307 signal is negligible, suggesting that the \( \gamma \)-phosphate of the nucleotide is involved in this conformational change.

The plots for Tyr-199 and Tyr-307 of each mutant are also summarized in Fig. 3, C and D, respectively. In the cases of \( \beta(D311A) \) and \( \beta(R333A) \), the plots for the Tyr-199 and Tyr-307 signals are similar to those of the wild-type \( \beta \), respectively. Although the MgAMPPNP-binding affinity of \( \beta(R333A) \) decreased, this mutation scarcely affected the conformational change induced by the nucleotide binding. This result indicates that the hydrogen bonds Ala-160/Arg-333 and Arg-333/Asp-252 play essential roles in the conformational change.

The nucleotide-binding affinity is similar to that of the wild type for \( \beta(R191A) \) and \( \beta(R191Q) \), and \( \beta(R191E) \) were produced. Their \( K_v \) values are shown in Table 1. The nucleotide-binding affinity is similar to that of the wild type for \( \beta(R191A) \) and \( \beta(R191Q) \) and one-half that for \( \beta(R191E) \). The chemical shifts of the Tyr-199 and Tyr-307 signals are plotted as a function of Tyr-341 \( \Delta \delta_{14} \), respectively, in supplemental Fig. 1. They are similar to those of the wild type, showing that Arg-191 is not essential not only for the nucleotide binding but also for the global conformational change and a further change in the region around Tyr-307. However, the ATPase activity of the \( \alpha_\beta(\text{R191X})_3 \gamma \) complex (where \( X \) represents Ala, Gln, or Glu) was decreased to 1% of the original level (Table 1) in accordance with previous results (32, 33). Therefore, Arg-191 should only contribute to the catalytic reaction.

MgATP-induced Conformational Changes of \( \beta \) Subunits Monitored through the Backbone Amide Signals—So far, information was obtained only from the side-chain signals of Tyr residues of the \( \beta \) subunit because of its large molecular mass (52 kDa). We can also get information from the main-chain signals of most residues of the \( \beta \) subunit by use of segmental isotope labeling (14). To compare the results on the TF, \( \beta/MgADP \) interaction (14), we have carried out MgATP titration of the TROSY-HSQC spectra for two kinds of segmentally \( ^{15} \)N-labeled \( \beta \) subunits that are labeled in the regions of residues 1–271 (\( \beta(1–271) \)) and 272–473 (\( \beta(272–473) \)), respectively. The assignment of their signals has already been established (14). The average chemical shift perturbation (\( \Delta \delta_{\text{ave}} \)) of each NH signal was calculated as described under "Experimental Procedures." The value of \( \Delta \delta_{\text{ave}} \) observed at MgATP/\( \beta = 5 \) is presented as a function of the sequence in Fig. 4A (top). The perturbed residues with \( \Delta \delta_{\text{ave}} > 0.1 \) ppm are 136–138, 164–176, 181–184, 207–212, 247–250, 311–314, 329–345, and 408–428, which are mapped on the crystal structure of MF₁β₁Thr in Fig. 4B. There are distinct differences (e.g. residues 247–250 in Walker’s motif B) in comparison with the reported results of MgADP titration (14) (Fig. 4A, bottom). However, the general pattern of the perturbation is similar in the two cases. This suggests that the binding of MgATP induces a conformational change in the \( \beta \) subunit monomer similar to that induced by the MgADP binding. The residues with different perturbations including 247–250 will be associated with the conformational change induced by \( \gamma \)-phosphate recognition.

We have further determined the relative orientation between the N- and C-terminal domains in the presence of MgATP by RDC analysis. \(^{1}H^{–}{^{15}} \)N RDCs were measured for the \( \beta \) subunits.
Stepwise Propagation of $F_1$-ATPase $\beta$ Conformational Change

A segmentally labeled in the N- and C-terminal domains, namely $\beta(1\text{–}124)$ and $\beta(390\text{–}473)$. The alignment tensor was obtained from the fitting of the values calculated from the crystal structure (1SKY) (16) to the experimental data. Since the alignment tensor of the C-terminal domain was smaller than that of the N-terminal domain, an order parameter was included in the analysis of the C-terminal domain. The alignment tensors, the Euler angles describing the orientation of the tensor relative to the Protein Data Bank frame, the order parameter, and the target function of over all couplings ($\chi^2$) are summarized in supplemental Table 1 along with the reported results for the free and MgADP-bound $\beta$ subunits (14). When $S = 1$ was assumed for the N-terminal domain, the order parameter was 0.39 for the C-terminal domain, suggesting the presence of slow collective motions in the $\beta$ subunit (34). The order parameter did not affect the relative orientation. Comparison of the observed RDC values with the calculated ones is shown in supplemental Fig. 2.

The relative orientation between the N- and C-terminal domains in the presence of MgATP is presented in Fig. 4C. Here, the N-terminal domain is completely superimposed on that of the nucleotide-free $\beta$ subunit determined previously (14). The axis connecting the centers of masses of the N- and C-terminal domains is shown as a solid straight line in Fig. 4C. The angle between this axis and the helix axis of residues 399–409 in the C-terminal domain (dashed line) was found to be 110° in the presence of MgATP. This is in good agreement with that in the presence of MgADP (110°) (14). Since the angle is 144° for the nucleotide-free state, a conformational change from the open to the closed form also takes place on MgATP binding in the $\beta$ subunit monomer as on MgADP binding. 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 mass center axis in Fig. 4C. This reveals that there is a conformational variance in the closed form depending on the bound nucleotide species. This is the first direct observation of a conformational change of the $\beta$ subunit on MgATP binding, because $F_1$ cannot be crystallized in the presence of MgADP (110°) (14). Since the angle is 144° for the nucleotide-free state, a conformational change from the open to the closed form also takes place on MgATP binding in the $\beta$ subunit monomer as on MgADP binding. 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 mass center axis in Fig. 4C. This reveals that there is a conformational variance in the closed form depending on the bound nucleotide species. This is the first direct observation of a conformational change of the $\beta$ subunit on MgATP binding, because $F_1$ cannot be crystallized with MgATP. It should be noted that the orientation angle for $\beta_{399}$ in the MF1 crystal was 119° (14). This means that the closed form of the TF $\beta$ monomer in solution is more closed than that in the MF1 crystal.

Segmental Labeling Analysis of $\beta$(K164A), $\beta$(T165A), and $\beta$(D252A)—Since Lys-164, Thr-165, and Asp-252 were found to be essential for the global conformational change, segmental labeling analysis was carried out for the $\beta$ (K164A), $\beta$ (T165A), and $\beta$ (D252A) mutants to examine the behavior of each amino acid residue upon nucleotide binding. Two kinds of segmentally labeled $\beta$ subunits, $\beta$(1–271) and $\beta$(272–473), were used for each mutant protein. The $^1$H–$^1$5N TRSY-HSQC spectra of each mutant protein in the absence of MgATP were in good agreement with those of the wild-type $\beta$, indicating that these mutations did not affect the tertiary structure of the nucleotide-free $\beta$ subunit (see supplemental Fig. 3 for $\beta$(K164A) as an example). We have assigned the signals by transferring those of the wild-type spectra. The spectra in the absence and presence of 20-fold MgATP were compared. The average chemical shift perturbation ($\Delta\delta_{ave}$) of each NH signal is shown in Fig. 5, A–C. The $\Delta\delta_{ave}$ values of the Tyr-341 signals of $\beta$(K164A), $\beta$(T165A), and $\beta$(D252A) were 0.40, 0.42, and 0.44 ppm, respectively. These $\Delta\delta_{ave}$ values are similar to that of the wild-type $\beta$, 0.43 ppm, induced by 5-fold MgATP. The perturbed residues ($\Delta\delta_{ave} > 0.1$ ppm) are 328–344 and 412–426 for $\beta$(K164A);
Stepwise Propagation of F1-ATPase β Conformational Change

The conformational change of the β subunit from the open to the closed form induced by nucleotide binding is considered to be the essential driving force of F1 rotation (5, 14, 15). The relative orientation between the N- and C-terminal domains of the β subunit monomer determined in this work demonstrated that ATP induces this bending motion. Extensive mutational studies have revealed the important residues for nucleotide binding and catalysis (30–33, 35–38). Combining mutagenesis and NMR, we can elucidate the mechanism underlying the bending motion in terms of the conformational roles of the key residues.

The chemical shift changes of the Tyr aromatic signals of the wild-type and mutant β subunits indicated that the hydrogen bond network involving the side chains of Lys-164 (Lys-162 for MF1), Thr-165 (Thr-163), and Asp-252 (Asp-256) is important for the nucleotide-induced conformational change. Each Ala-substituted mutation suppressed the conformational change, although a nucleotide still binds to the mutant β. Two-dimensional NMR analysis of the segmentally labeled β subunits revealed that the extent of the suppression could be classified into two categories.

Loss of the Lys-164 side chain suppresses the whole conformational change except that in the adenine pocket. Namely, the phosphate groups of the bound nucleotide cannot induce a conformational change even in the P-loop without the involvement of Lys-164. In the crystal structure of MF1 (5, 6), the Lys-164 side chain forms a hydrogen bond with the side chain of Asp-252 in the open form (Fig. 1A) but is attracted to the phosphate group of AMPPNP or ADP and forms hydrogen bonds with the phosphate and the carbonyl group of Gly-158 in the closed form, making the C=O angle of Gly-158 suitable for a hydrogen bond with NH of Val-308 (Fig. 1A) for extension of the β3 and β7 strands. Thus, the movement of the Lys-164 side chain from Asp-252 to the phosphate group should be an essential step for the conformational change on nucleotide binding. Without this, nothing happens except for the adenine binding. This should be the case also for TFγ, because the ATPase activity of αβ(K164A)γ was negligible (Table 1).

In the cases of β(T165A) and β(D252A), an additional change was observed in the αβ helix (Fig. 5). According to the crystal structure of MF1 (5, 6), the carboxyl group of Glu-170 on the αβ helix forms hydrogen bonds with the amide groups of Gln-415 and Val-416, and the alkyl groups of Glu-170 interact with the aromatic ring of Phe-414 in both the open and closed forms. Phe-414—Val-416 are located in the adenine-binding pocket. Therefore, the αβ helix is closely involved in the adenine-binding pocket. On the other hand, the P-loop is the direct neighbor of the αβ helix. This helix should be the key element for the cooperative conformational change in the adenine- and phosphate-binding sites. We do not have information on the P-loop because of loss of the signals. However, the K_d values of β(T165A) and β(D252A) are similar to that of the wild type (Table 1). This strongly indicates that the interactions of Lys-164 with the phosphate and the P-loop facilitate formation of the phosphate-bound P-loop conformation, which affects the αβ helix. Nevertheless, the nucleotide binding fails to induce a

![FIGURE 5. The chemical shift perturbations induced by MgATP for β(K164A), β(T165A), and β(D252A). A–C, average chemical shift perturbations of the backbone amide signals (Δδ_ave) on MgATP binding to β(K164A) (A), β(T165A) (B), and β(D252A) (C). The ratio of MgATP to protein is 20. Residues without lines were not observed. D–F, the residues of β(K164A) (D), β(T165A) (E), and β(D252A) (F) with Δδ_ave > 0.1 ppm are mapped on the βTP form in the MF1 β subunit (6) (Protein Data Bank code 2JDI). The colors are the same as in Fig. 4B.](image)
global conformational change in the absence of either Thr-165 or Asp-252. The side chain of Thr-165 forms a hydrogen bond with Glu-201 in the open form and with Asp-252 in the closed form (5, 6, 39). This means that the lack of the Thr-165/Asp-252 hydrogen bond blocks the global change to the closed form despite the formation of the phosphate-bound P-loop conformation. Therefore, the formation of the Thr-165/Asp-252 hydrogen bond is the other essential step for the change to the closed form. This should also be the case for TF1, because the ATPase activities of the $\alpha_3\beta(T165A)_3\gamma$ and $\alpha_3\beta(D252A)_3\gamma$ complexes were negligible (Table 1). Since Lys-164 and Thr-165 share the same hydrogen-bonding partner, Asp-252, in the open and closed forms, respectively, the two essential steps should take place sequentially. Here, Asp-252 in the Walker’s motif B plays a role as a switch for the open/close conversion.

The significance of the hydrogen bond switch in the global change is associated with the change in the length of the side chain. Since the side chain of Thr is much shorter than that of Lys, the Thr-165/Asp-252 hydrogen bond brings the C-terminal domain closer to the catalytic one, yielding the closed form. However, this is not enough for stabilization of the closed form. The effect of the Y307P mutation revealed that the formation of the $\beta$-sheet structure between Leu-156 and Gly-158 (Leu-154 and Gly-156 for MF$_1$) and Ile-306 and Val-308 (Ile-310 and Val-312) is essential for the open/close conversion, even if Lys-164, Thr-165, and Asp-252 are intact. The $\beta$-sheet formation should take place in concert with the Thr-165/Asp-252 hydrogen bond formation.

The mechanism underlying the conformational conversion from the open to the closed form based on our results is schematically presented in Fig. 6B. The intermediate conformation in the figure has features similar to those of the half-closed form of the subunit in a crystal structure of MF$_1$ (7). In the half-closed form bound with ADP-AlF$_3$O$_2$, Thr-165 is separated from O$_\beta$ of Asp-252 by 3.43 Å (2.50 Å for $\beta_{TP}$) and from Glu-201 by over 10 Å. Here, the global conformation is similar to the open form in the absence of the Thr-165/Asp-252 hydrogen bond, although the phosphate-bound P-loop conformation is formed. We have also carried out two-dimensional NMR analysis of the segmentally labeled mutant $\beta$ subunits bound with MgADP. It revealed similar chemical shift perturbations to those in Fig. 5 (data not shown). Therefore, the mechanism presented in Fig. 6B should be applicable to the ADP-induced conformational change as well.

It should be noted that the Tyr-307 aromatic signal shifts upon ATP binding but not upon ADP binding (Fig. 3). Tyr-307 is located in the switch II region. Masaik et al. (40) suggested that the switch II region might be involved in the $\gamma$-phosphate binding in TF$_1$. Although the crystal structures reported so far provide no evidence of the involvement of Tyr-307 in the binding of ATP $\gamma$-phosphate, this is the case for the TF$_1$ $\beta$ monomer in solution. It has been well known that a chemical modification of Tyr-307 of a single $\beta$ blocked the ATPase activity for TF$_1$ and EF$_1$ (41, 42). However, a simple mutation, such as Y307C, did not impair the ATPase activity (31). In the crystal structure of MF$_1$ (6), the aromatic ring of Tyr-307 and the guanidyl group of Arg-256 (Arg-260 for MF$_1$) are stacked on each other for $\beta_{TP}$ and $\beta_{2TP}$ (Fig. 6C). Arg-256 is one of the conserved residues. Ahmad and Senior (42) reported that Arg-256 (Arg-246 for EF$_1$) was an essential residue for recognition of P$_i$, the cleaved $\gamma$-phosphate, in the EF$_1$ $\beta$ subunit on the basis of mutations at this residue. According to close inspection of the crystal structures of MF$_1$, they designated the area including Arg-256 and Tyr-307 as the P$_i$-binding pocket. The chemical shift change of Tyr-307 observed in this work suggests the interaction of the $\gamma$-phosphate of ATP with this pocket in the closed form of the $\beta$ monomer. This would occur in the $\beta$ monomer because of the more closed conformation than that of $\beta_{TP}$. The Arg-256/P$_i$ interaction is supposed to play an important role in the catalytically activated conformation (42). Therefore, the ATP-bound conformation of the $\beta$ monomer in solution seems to have a feature similar to that of the catalytically activated conformation. The formation of this conformation takes place after the major global change, as can be seen in Fig. 3. A conformational difference between the MgATP- and MgADP-bound $\beta$ monomers in the closed forms was actually confirmed by RDC analysis in this work.

Our previous works revealed that the properties of the $\beta$ monomer were closely correlated to those of F$_1$ (14, 19, 22). This was confirmed more precisely in this work. For example, the nucleotide-binding site is almost identical for F$_1$ and the $\beta$ monomer. The binding affinity is not necessarily the same, because of involvement of the $\alpha$ and $\gamma$ subunits in F$_1$. Nevertheless, the dissociation constant ($K_d$) of the MgATP-bound TF$_1$ $\beta$ monomer is 15 $\mu$M (43), and the largest $K_{dL}$ ($K_{dL}$ of TF$_1$) is 35 $\mu$M (44), suggesting that the binding mechanism is similar for the monomer and one binding site of F$_1$, despite the presence of other subunits. As Weber et al. reported (45), however, there are much smaller $K_{dL}$ and $K_{d2L}$ values for F$_1$. The properties of these binding sites should be determined by the interaction with the $\alpha$ subunit and the involvement of the $\gamma$ rotation. The significance of the properties of the $\beta$ monomer was also verified by the fact that the key residues identified in this work were always essential for the ATPase activity of the TF$_1$ $\alpha_3\beta_3\gamma$ con-
plex. Therefore, we can discuss the impact of our results on TF₁ as far as the elementary structure of the β subunit is concerned.

Single molecule analysis of TF₁ has revealed three (80° + 40°)-step rotation of the γ subunit in the αβ3 ring (12, 13, 15). Adachi et al. (15) elucidated the following kinetic states for the TF₁ rotation. If we set the ATP-waiting dwell as the starting point (0°), the first 80° rotation of the γ subunit is induced by the ATP binding. ATP hydrolysis takes place after the second 80° rotation (at 200°). The third 80° rotation (starting at 240°) is associated with ADP release. The phosphate release is coupled with either the second or third 40° rotation. Our results can provide a conformational insight into these kinetic states. In F₁, with either the second or third 40° rotation. Our results can provide a conformational insight into these kinetic states. In F₁, associated with ADP release. The phosphate release is coupled with the interaction between the Arg-conformation in Fig. 6 and the γ-phosphate of ATP, leading to the catalytically activated state. After the hydrolysis, ADP starts to be released at 240° rotation (15). The DP conformation in the crystal structure is not suitable for the release of ADP instantly. Actually, the conformation at 240° would not be DP if the αβ unit takes on the DP-like conformation at 320° rotation as proposed (46). Most likely, the conformation of the αβ unit at 240° is similar to the half-closed form in the MF₁ crystal structure (7) or the intermediate conformation in Fig. 6B, which is ready to release ADP.

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