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# Functions and ATP-Binding Responses of the Twelve Histidine Residues in the $TF_1$-ATPase β Subunit[1]

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**The C2 proton signals of all (twelve) histidine residues of the $TF_1$ β subunit in the $^1$H-NMR spectrum have been identified and assigned by means of pH change experiments and site-directed substitution of histidines by glutamines. pH and ligand titration experiments were carried out for these signals. Furthermore, the ATPase activity of the reconstituted $\alpha_3\beta_3\gamma$ complex was examined for the twelve mutant β subunits. Two of three conserved histidines, namely, His-119 and 324, were found to be important for expression of the ATPase activity. The former fixes the N-terminal domain to the central domain. His-324 is involved in the formation of the interface essential for the $\alpha_3\beta_3\gamma$ complex assembly. The other conserved residue, His-363, showed a very low $pK_a$, suggesting that it is involved in the tertiary structure formation. On the binding of a nucleotide, only the signals of His-173, 179, 200, and 324 shifted. These histidines are located in the hinge region, and its proximity, of the β subunit. This observation provided further support for the conformational change of the β monomer from the open to the closed form on the binding of a nucleotide proposed by us [Yagi *et al.* (1999) *Biophys. J.* 77, 2175–2183]. This conformational change should be one of the essential driving forces in the rotation of the $\alpha_3\beta_3\gamma$ complex.**

**Key words: amino acid replacement, ATPase activity, nuclear magnetic resonance (NMR), nucleotide binding, reconstitution of the $\alpha_3\beta_3\gamma$ complex.**

The $H^+$-ATP synthase is a key protein in biological energy production. It is a huge membrane protein composed of a soluble catalytic part ($F_1$) and a membrane-embedded part ($F_o$). The $F_1$ portion ($F_1$-ATPase) has five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$, and the molecular size is ~380 kDa. The catalytic site of $F_1$-ATPase is located in the β subunit at the interface with the α subunit (*1–3*). The crystal structure of bovine mitochondrial $F_1$-ATPase ($MF_1$) shows that the subassembly $\alpha_3\beta_3$ forms a ring with long helices of the γ subunit in the center (*1, 4*). The tertiary structure of the β subunit in the $F_1$-ATPase differs according to the state of its active site, namely, an empty ($\beta_E$), Mg·AMP-PNP binding ($\beta_{TP}$), or Mg·ADP binding ($\beta_{DP}$) state (*1*). The empty and nucleotide binding forms are designated as the open and closed forms, respectively, because of their conformations, which are shown in Fig. 1. Furthermore, rotation of the γ subunit in the fixed $\alpha_3\beta_3$ complex on hydrolysis of ATP has been directly demonstrated by epifluorescence microscopy for the $\alpha_3\beta_3\gamma$ complex from thermophilic *Bacillus* PS-3 ($TF_1$) (*5, 6*). The rotation of the *c* subunit oligomer in $F_oF_1$-ATP synthase has also been shown in the same way for *Escherichia coli* and PS-3 (*7, 8*). Furthermore, the molecular architecture of the yeast $F_1c_{10}$ complex has been determined by X-ray crystallography (*9*). These reports strongly support the cyclical binding change mechanism of ATP synthesis (*10*). The mechanism of the rotational catalysis is an important problem to be solved. The mechanism of nucleotide binding to *E. coli* $F_1$-ATPase ($EF_1$) and $F_1F_o$-ATP synthase has been investigated in detail using tryptophan fluorescence (*11, 12*). Recently, it was suggested that the conformational change from the open to the closed form could be induced by nucleotide binding even in the monomer $TF_1$ β subunit (*13*). This conformational change can be one of the driving forces of the rotation.

Shirakihara and his colleagues reported the crystal structure of $TF_1$ nucleotide-free $\alpha_3\beta_3$ complex (*14*). The structure of the β subunit in the complex is similar to that of the equivalent subunit in the empty state (the open form) of $MF_1$. Therefore, it is possible to refer to the crystal structure of $MF_1$-ATPase when discussing the structure and function of $TF_1$-ATPase. We studied the conformational change on nucleotide binding by monitoring the ring proton NMR signals of the twelve tyrosine residues (*13*). This should be examined by means of independent experiments.

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Abbreviations: $MF_1$, $F_1$-ATPase from bovine heart mitochondria; $TF_1$, $F_1$-ATPase from thermophilic *Bacillus* strain PS3; $EF_1$, $F_1$-ATPase from *Escherichia coli*; AMP-PNP, 5′-adenylylimidodiphosphate.

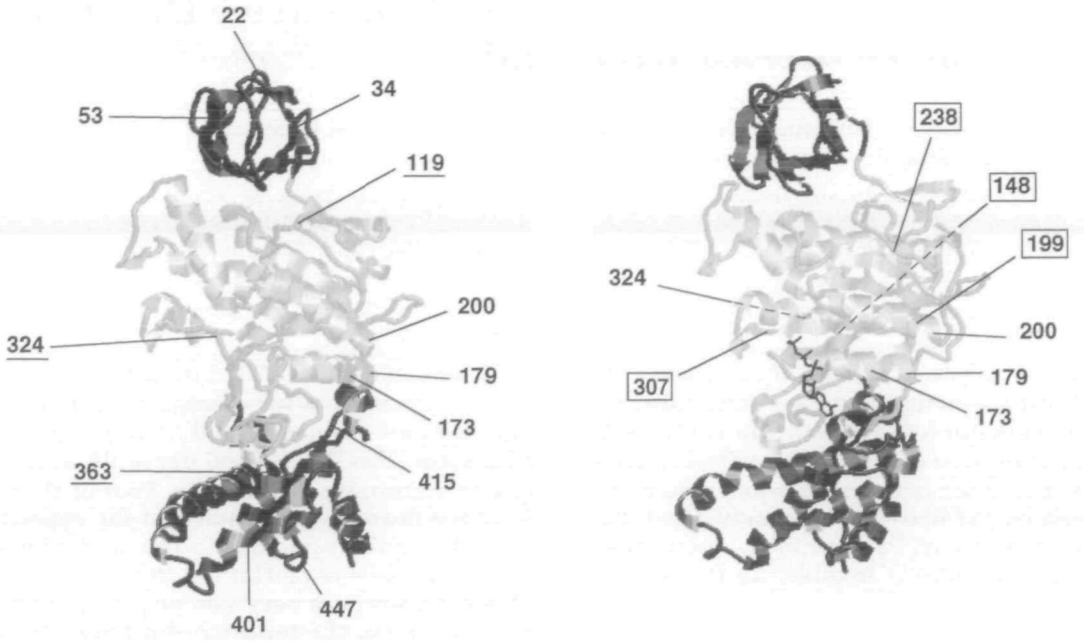


Fig. 1. **The crystal structures of the $MF_1$ β subunit in the empty (left, open form) and AMP-PNP binding (right, closed form) forms (*2*).** The locations of the twelve histidine residues are indicated on the left as the sequence numbers for the $TF_1$ β subunit. Conserved residues are underlined. The amino (N)-terminal, central (nucleotide-binding) and carboxyl (C)-terminal domains are shaded differently from top to bottom. The sequence numbers of histidine (this work) and tyrosine (*13*) residues, the chemical shift changes of which were observed on nucleotide titration, were indicated on the right. The boxed numbers represent the tyrosine residues.

There are twelve histidine (His) residues in the $TF_1$ β subunit. They are also scattered allover the $TF_1$ β subunit, namely, the amino (N)-terminal, central (nucleotide-binding) and carboxyl (C)-terminal domains, as shown in Fig. 1. Therefore, the histidine residues were used in this work to monitor the conformational change by $^1$H-NMR. In spite of the high molecular mass (~52 kDa), the imidazole C2 proton (C2H) signals from all twelve histidine residues were identified and assigned by means of site-directed mutagenesis. A conformational change from the open to the closed form of the β subunit on the binding of a nucleotide to the β monomer proposed by us (*13*) was confirmed. Furthermore, the functional roles of all histidine residues were investigated using the reconstituted $\alpha_3\beta(\text{mutant})_3\gamma$ complexes and pH titration of NMR spectra of the mutant β monomers. In particular, His-119, 324, and 363 (underlined in Fig. 1) are conserved in the primary sequences of the β subunits of all known $F_1$-ATPases. The conformational roles of these residues were discussed in connection with the catalytic mechanism of $F_1$-ATPase.

## MATERIALS AND METHODS

*Materials, Bacterial Strains, and Growth Media*—Deuterium oxide (99.9 and 99.96 atom% $^2$H) was purchased from ISOTEC and Showadenko. ATP, ADP, and 5′-adenylylimidodiphosphate (AMP-PNP) were obtained from Sigma. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase, and oligonucleotides used for introducing mutations were purchased from Takara Shuzo. Other chemicals were of analytical grade. *E. coli* strains HB101 [*supE*44, *hsdS*20(*r*B-*m*B-), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1, *leuB*6, *thi*-1] and DK8 [*bglR*, *thi*-1, *rel*-1, HfrPO1, Δ(*uncB-uncC*) *ilv*::Tn10] were used for the production of the wild-type and mutant $TF_1$ β subunits, respectively. Other strains used for the generation of oligonucleotide-directed mutants, and the production of α and γ subunits were described previously (*15, 16*). Cells were grown on a nutrient-rich medium at 37°C with vigorous shaking.

*NMR Measurements*—The wild-type and mutant β subunits of $TF_1$ expressed in *E. coli* were purified as described previously (*15, 17*). The exchangeable protons of the protein sample for NMR measurements were replaced with deuterons by repeated cycles of lyophilization. At the final step, each sample was dissolved in a 50 mM sodium phosphate $^2H_2O$ buffer, 0.25–1 mM protein concentration. However, the buffer was not used for pH titration experiments. The pH meter readings were not corrected for the deuterium isotope effect. Chemical shifts are referred to an internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). For ligand titration, a small amount of a concentrated ligand solution was directly added to the sample solution. The preparation of a Mg·nucleotide solution is described elsewhere (*13*). The addition of the ligand solution did not affect the p$^2$H of the NMR sample solution, which was confirmed after the titration experiment. $^1$H-NMR spectra were recorded at 400 MHz with a Bruker DRX400 NMR spectrometer. For most measurements, 256 transients were accumulated with a 1 s relaxation delay. The line-broadening factor was 1 Hz.

*Other Methods*—Mutated $TF_1$ β subunit genes were obtained by oligonucleotide-directed mutagenesis (*18*). Reconstitution of the $\alpha_3\beta_3\gamma$ complex from the α, β, and γ subunits, and assaying of its ATPase activity were carried out using the regeneration system described previously (*16, 19*). Protein concentrations were determined by the method of Bradford (*20*) with bovine serum albumin as a standard.

## RESULTS

*Resonance Assignment of Imidazole C2 Protons of Histidine Residues*—The $^1$H-NMR spectra of the $TF_1$ β subunit at various pHs are shown for a low field region in Fig. 2. Sharp signals can be observed in this region in spite of the high molecular mass (~52 kDa). They originate from the aromatic protons (mainly C2 protons) of histidine residues. The assignment of the C2H signals was confirmed by the spectra of the $TF_1$ β subunit specifically deuterated at the histidine residues (*21*). The assignment of the His-179 and His-200 signals has already been reported (*21*). The assignment of the other signals has been carried out through replacement of each histidine residue with a glutamine residue. The latter was used because the nature of its side chain is relatively similar to that of a histidine side chain. The histidine signals are well scattered at p$^2$H 6.78 and 30°C, as can be seen in Fig. 2D. Relevant regions in the spectra of the twelve mutant β subunits are presented in Fig. 3 in comparison with that of the wild-type one. Here, βH22Q, for example, stands for a mutant β subunit in which His-22 is replaced with a glutamine residue. The spectra of βH179Q and βH200Q are also included in this figure to confirm the earlier assignment. Since the chemical shifts and line-width (or apparent intensity) of a signal in the process of pH titration depends on its p$K_a$, and the rate of exchange between the protonated and deprotonated states, pH titration was also carried out for the assignment.

The assignment is straightforward for His-34, 53, 179, 200, 363, 401, and 415, the spectrum of a mutant protein being compared with that of the wild type one. This shows that the earlier assignment of His-179 and 200 is correct. In the case of His-22, two signals in the region of 7.9 to 8.0 ppm seemed to have disappeared. This problem was solved by comparison of this spectrum with those of βH34Q and βH53Q. The spectra of βH22Q, βH34Q, and βH53Q are the same as each other except for the mutated signal. It can be said, on the basis of these spectra, that the signal at 7.90 ppm in the spectrum of β wild shifts to a higher field, becoming a shoulder on the sharp signal at 7.88 ppm, and the signal at 7.92 ppm shifts to 7.98 ppm. The signal at 7.98 ppm has disappeared in the spectrum of βH22Q. Therefore, the signal at 7.98 ppm (7.92 ppm for β wild) can be ascribed to His-22. In the spectrum of βH119Q, the signal of His-22 overlaps that at 8.08 ppm, which was confirmed by the pH titration experiment. Thus, the signal at 7.90 ppm disappeared in this spectrum, leading to the assignment of this signal to His-119. The weak signals left in this

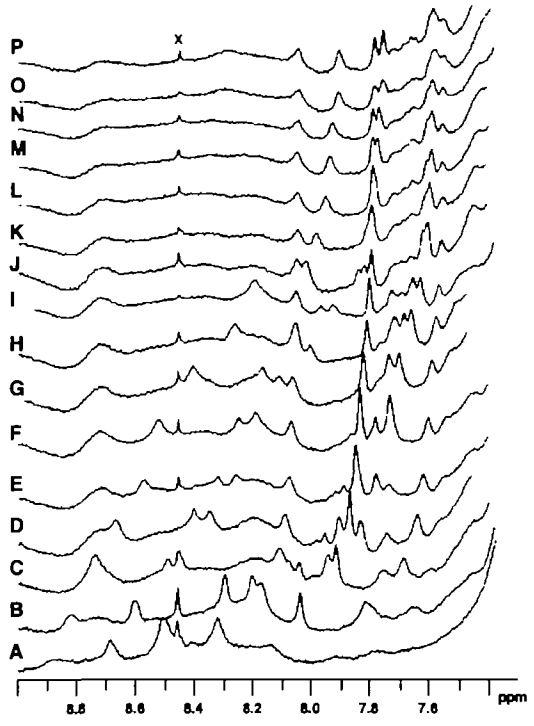


Fig. 2. **$^1$H-NMR spectra of the wild-type $TF_1$ β subunit at various p$^2$H, at 30°C.** The p$^2$H values were 5.61, 6.16, 6.53, 6.78, 6.93, 7.10, 7.21, 7.38, 7.67, 8.07, 8.21, 8.50, 8.70, 8.84, 9.62, and 9.98 for A to P, respectively. The peak indicated by X is a contaminating signal.

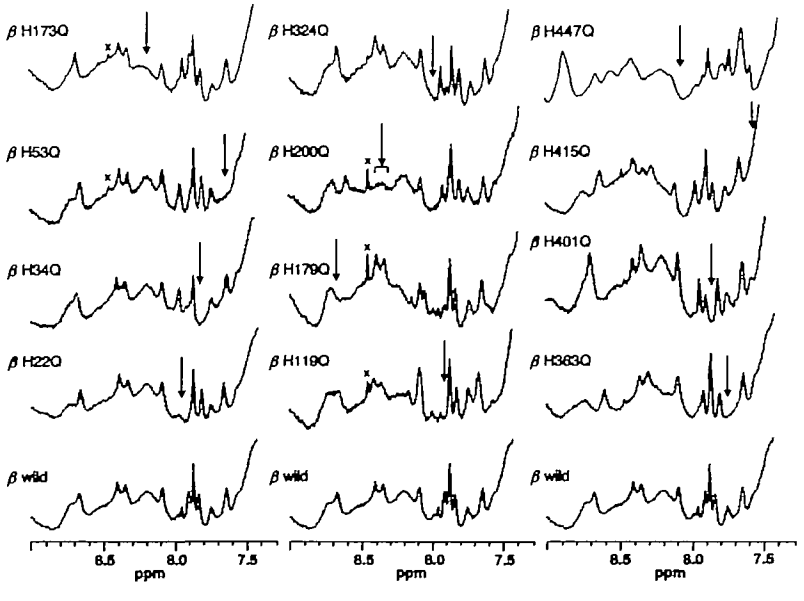


Fig. 3. **$^1$H-NMR spectra of the wild-type and twelve mutants in the relevant region at p$^2$H 6.78 and 30°C.** βH22Q, for instance, stands for the β subunit in which His-22 is substituted by glutamine. The arrows indicate the positions of the signals that disappeared. The spectrum of βH447Q was recorded at 50°C because it was too broad at 30°C.

region of the spectrum of βH119Q can be ascribed to His-324. Because of its weak intensity and overlapping with other signals, the spectrum of βH324Q failed to provide clear evidence for the assignment. However, ligand titration experiments enabled us to assign the signal of His-324, as will be described later. In the spectrum of βH173Q, the intensity of a broad peak at about 8.2 ppm decreased in comparison with that of β wild. For the mutant protein of βH415Q, a less broad signal was seen in this region (Fig. 6). Thus, this signal can be ascribed to His-173. A sharp signal at 8.08 ppm was not affected by the eleven mutations described so far. Therefore, this signal is a candidate for His-447. However, the spectrum of βH447Q was too broad to identify the relevant signals at 30°C, suggesting that this substitution affects a large part of the β subunit. A reasonable spectrum was obtained at 50°C, as shown at top right of Fig. 3. The spectrum is different from that of the wild-type in the region of 7.5 to 7.9 ppm because of the temperature difference. Nevertheless, the lack of the signal at 8.08 ppm observed for β wild is clear. On the basis of these observations, we assigned the signal at 8.08 ppm to C2H of His-447. All the assignments are presented in Fig. 4 for the spectra at p$^2$H 6.78 and 7.67 at 30°C.

The pH titration curves of these signals are presented in Fig. 5. His-173 is not included because of its very broad linewidth. These curves can be placed in four categories. His-363, 415, and 447 show very low p$K_a$. His-53 and 401 show relatively low p$K_a$. The p$K_a$ values of His-22, 34, 119, and 324 are normal. Those of His-179 and 200 are relatively high. While the signal of His-200 is a singlet below pH 6.0 and above 8.70, it is a doublet in the region between these values. The high p$K_a$ of His-179 and 200 should be due to the presence of negative charges around them, namely, Glu-178 and 201, respectively. The p$K_a$ values obtained by least squares fitting were 7.22 ± 0.03, 7.09 ± 0.01, and 6.97 ± 0.01 for His-179, 200, and 200′, respectively. Since the C2H signals of His-173, 324, and 415 are very weak, the rates of exchange between the protonated and deprotonated states should be much slower than those of the other nine histidine residues. It is also interesting that all histidine residues in the C-terminal domain show low p$K_a$.

*ATPase Activity of the $\alpha_3\beta_3\gamma$ Complex*—The $\alpha_3\beta_3\gamma$ complex was reconstituted using each mutant β subunit. The yield of the complex formation was less than 4% for βH324Q, judging from the results of HPLC. Otherwise, the yield of the complex was in the range of 55–68%. The obtained ATPase activities are summarized in Table I. To examine the stability of the complex, the activity at 25°C after incubation at 60°C for 10 min was also measured. These data are also included in Table I. The ATPase activity was seriously affected in the cases of βH119Q and βH324Q. His-119 and 324 are conserved in all β subunit sequences so far reported. In contrast, the activity was enhanced in the case of βH173Q. The stability at 60°C was relatively low for βH53Q, βH119Q, and βH324Q, and relatively high for βH363Q and βH447Q.

*The Effect of Nucleotide Binding to the β Monomer*—Mg·ATP titration was carried out for the wild-type and

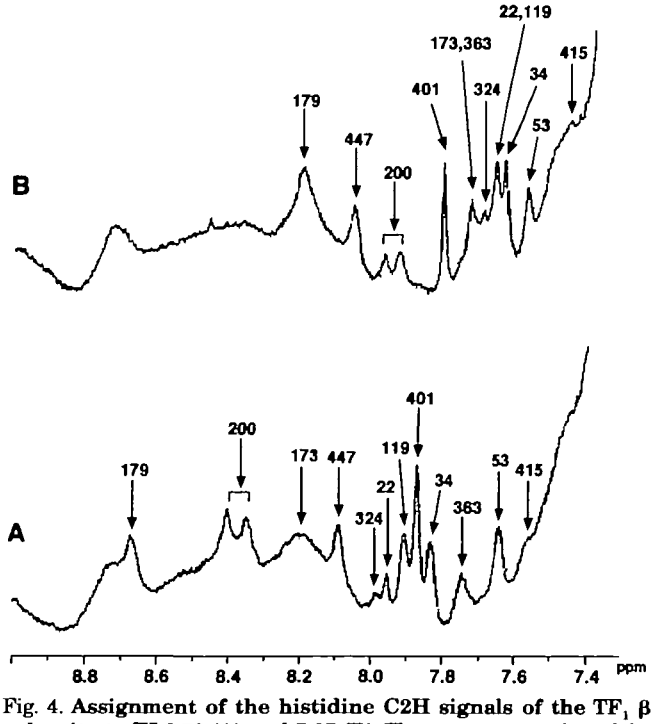


Fig. 4. **Assignment of the histidine C2H signals of the $TF_1$ β subunit at p$^2$H 6.78 (A) and 7.67 (B).** The sequence number of the histidine residue is shown at the top of each signal. The temperature was 30°C.

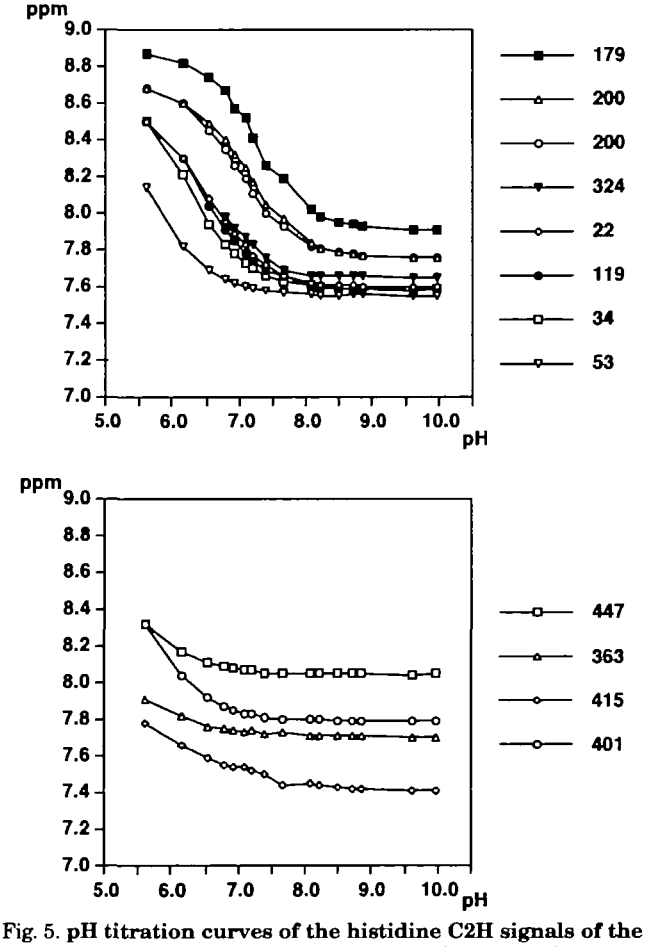


Fig. 5. **pH titration curves of the histidine C2H signals of the $TF_1$ β subunit at 30°C.** Residue numbers and corresponding symbols are shown on the right.

TABLE I. **ATPase-specific activity of the reconstituted $\alpha_3\beta_3\gamma$ complex.** One unit is taken as the activity hydrolyzing 1 μmol of ATP per min at 25°C. The activity was measured under two different conditions, namely, after preincubation at 60°C for 10 min, and without preincubation.

| Mutant β in $\alpha_3\beta_3\gamma$ | Activity of the complex after preincubation (unit/mg protein) | |
|---|---|---|
| | None | 60°C |
| Wild | 4.22 (100)[b] | 2.80 (100)[b] |
| H22Q | 3.38 (81) | 2.26 (81) |
| H34Q | 3.84 (91) | 1.60 (57) |
| H53Q | 8.63 (205) | 2.55 (91) |
| H119Q | 0.62 (15) | 0.15 (5) |
| H173Q | 10.5 (249) | 6.08 (217) |
| H179Q | 5.19 (123) | 2.65 (95) |
| H200Q | 4.44 (104) | 2.86 (102) |
| H324Q[a] | 0.99 (23) | 0.38 (13) |
| H363Q | 3.32 (76) | 2.78 (99) |
| H401Q | 5.58 (132) | 3.98 (142) |
| H415Q | 3.09 (73) | 2.26 (81) |
| H447Q | 3.25 (77) | 2.98 (106) |

[a]The yield of $\alpha_3\beta_3\gamma$ complex formation was very poor. [b]The values in parentheses are percentages as compared with the activity of the wild-type under the same conditions.

mutant proteins. The spectra for the wild-type are presented in Fig. 6. Only four signals were found to shift as a function of the nucleotide/protein molar ratio. Three of them are the signals of His-179 and 200, which was reported earlier (*21*). The other one at 7.98 ppm could not be assigned in the first stage because it is weak and sometimes overlaps the signal of His-22. However, it was found that no signal shifts in this region of the βH324Q spectrum on the addition of Mg·ATP. Therefore, the signal at 7.98 ppm is a candidate for His-324. This assignment was confirmed by Mg·ATP titration of βH119Q (data not shown). In the spectrum of βH119Q, this signal can be seen explicitly without any overlapping (see Fig. 3). The two weak signals left in this region actually shifted on the addition of Mg·ATP. This also suggests that the C2H signal of His-324 is a doublet like that of His-200. Furthermore, the His-173 signal shifted on the binding of a nucleotide to βH415Q, as can be seen in G and H of Fig. 6. The imidazole of His-173 forms a hydrogen bond with the imidazole of His-415 in the crystal structure (*14*). The replacement of His-415 with glutamine makes the His-173 signal clearer. This enabled us to detect the chemical shift change of the His-173 signal on the binding of a nucleotide.

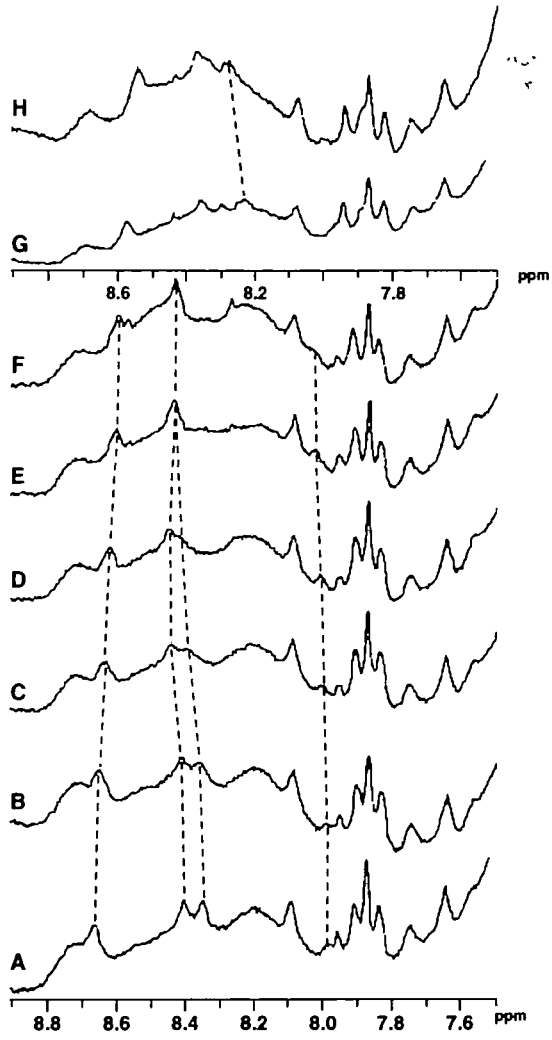


Fig. 6. **Titration of the histidine C2H signals of the $TF_1$ β subunit and a mutant protein, βH415Q, as a function of the Mg·ATP/protein ratio.** The concentration ratios of Mg·ATP to βwild were 0, 0.1, 0.33, 0.5, 1.0, and 2.0 for A to F, respectively. The protein concentration was 1 mM. The spectra of βH415Q in the absence and presence of Mg·ATP are presented at the top (G and H). The concentration ratio of Mg·ATP to the mutant protein was 0.5.

## DISCUSSION

It has been shown that conserved histidine residues His-119 and 324 are important for the ATPase activity. The imidazole of His-119 forms a hydrogen bond with the carboxyl group of Glu-49 (*14*), which is also conserved. Since His-119 and Glu-49 are located in the central (nucleotide-binding) and N-terminal domains, respectively, this hydrogen bond should be involved in the fixing of the N-terminal domain to the central domain. This important role of His-119 was also indicated for *E. coli* $F_1$-ATPase (His-110 for $EF_1$). The replacement of His with Ala or Asp at this position caused defective assembly of $F_1$, while on that with Arg the assembly was maintained with a decrease in ATPase activity like in the case of the replacement with Gln in this work (*22*). Glu-49 (Glu-41 for $EF_1$) cannot form a hydrogen bond with the side chain of either Ala or Asp (because of electrostatic repulsion). The elimination of this hydrogen bond might damage the $F_1$ assembly. Gln-119 or Arg-119 may form a hydrogen bond with Glu-49. However, their side chains have much more freedom as to internal rotation than that of histidine. This kind of freedom would cause ambiguity in the interdomain interaction. Therefore, it can be concluded that the hydrogen bond between His-119 and Glu-49 is essential for $F_1$ to fully implement the rotational catalysis. The structural destabilization of the $\alpha_3\beta_3\gamma$ complex observed for βH119Q at 60°C supports the idea that His-119 is important for maintenance of the active conformation.

His-324 was found to be important for the $\alpha_3\beta_3\gamma$ complex formation. His-324 is close to the surface of the β subunit that faces the noncatalytic nucleotide–binding site of the α subunit in the $\alpha_3\beta_3\gamma$ complex. This region of the β subunit surface was indicated to be important for the $\alpha_3\beta_3\gamma$ complex formation (*13*). Elimination of the hydrogen bond between

either Tyr-277 and Asp-315, or Gln-289 and Gln-304 led to loss of the complex formation. His-324 is very close to the Gln-289/Gln-304 pair. The imidazole is not involved in hydrogen bonding, but sandwiched by the side chains of Gln-289 and Ala-323 in the crystal structures of the $\alpha_3\beta_3\gamma$ complex (*1*) and $\alpha_3\beta_3$ complex (*14*). This fact suggests that the interaction of aliphatic groups ($CH_3$ and $CH_2$) with the $\pi$ electron ring plays an important role in the formation of the interface for the $\alpha_3\beta_3$ assembly. In the crystal structure of $MF_1$, the imidazole ring of His-324 changes its position relative to the side chains of Gln-289 and Ala-323 in the $\beta_{TP}$, $\beta_{DP}$, and $\beta_E$ states, respectively. This means that the aromatic ring of histidine slips, depending on the conformational change of the β subunit. This is consistent with the observed change in the chemical shift of the C2H signal of this histidine on nucleotide binding. This kind of soft interaction should be important in a molecular machine such as ATP synthase.

The third conserved residue, His-363, showed a very low $pK_a$. This is reasonable in view of the crystal structures, because it is almost completely embedded in the β subunit (*1, 14*). This would be mainly involved in the tertiary structure formation. The imidazole group of His-415 is half-embedded in the β subunit in the crystal structures (*1, 14*). The exposed nitrogen is involved in the hydrogen bonding with His-173 and the other nitrogen is located inside the protein. The higher ATPase activity of $\alpha_3(\beta H173Q)_3\gamma$ suggests that the hydrogen bond between His-173 and 415 suppresses the catalytic activity to some extent. This hydrogen bond bridges the central and C-terminal domains. Flexibility in this region would be preferred for ATPase activity. His-447 also shows a very low $pK_a$. The chemical shift of the deprotonated C2H signal is larger than the normal one. The substitution by Gln affected a large part of the structure. These observations suggest that the side chain of His-447 is not free. In the crystal structure (*1, 14*), however, it is almost freely exposed on the surface. The NMR result is inconsistent with the crystal structure in this case, suggesting that the conformation around His-447 is different for the complex in crystal and the β monomer in solution.

On Mg·ATP titration, only the signals of His-173, 179, 200, and 324 out of the twelve histidine residues shifted. All of them fall in the hinge region, and its proximity, of the β subunit, as shown on the right of Fig. 1. It has been reported that only the signals of Tyr-148, Tyr-199, Tyr-238, and Tyr-307 out of the twelve tyrosine residues shift downfield on binding of Mg•ATP or Mg•AMP-PNP (*13*). All of them have also been mapped in the hinge region and its proximity (Fig. 1, boxed numbers on the right). Thus, the conformational responses of two kinds of amino acid residues to nucleotide binding lead to the same conclusion. In the crystal structure of $MF_1$, the relative orientation of the N- and C-terminal domains of the β subunit differs for the empty state (open form) and the nucleotide binding state (closed form). The conformational variations between the open and closed forms are only located in the hinge region and its proximity (*1*). Since the area including the conformational variations is almost the same for the β monomer and the $\alpha_3\beta_3\gamma$ complex, the conformational change observed on NMR can be ascribed to the conformational change from the open to the closed form. This conclusion indicates that the nucleotide binding is directly associated with the conformational change between the open and closed form even in the β monomer. Yasuda *et al.* (*6*) recently demonstrated that the 120° rotation of the γ subunit in the $\alpha_3\beta_3\gamma$ complex could be divided into 90° and 30° substeps. The 90° and 30° rotations were interpreted on the basis of the conformational changes induced by binding of ATP and dissociation of ADP, respectively. The intrinsic conformational change in the β subunit shown by us is consistent with their interpretation. Therefore, the conformational change induced by the nucleotide binding should be one of the essential driving forces for the $F_1$ rotation, as suggested in our previous paper (*13*).

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