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Conformational dynamics monitored by His-179 and His-200 of isolated thermophilic F_1-ATPase β subunit which reside at the entrance of the 'conical tunnel' in holoenzyme

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Abstract When monitored by ^{1}H NMR at various pH values, most of the C-2 proton signals from 12 His residues of the isolated β subunit of thermophilic F_1-ATPase (TF_1) could be separately observed. Two of them were assigned to His-179 and His-200 which reside at the entrance of a 'conical tunnel' to reach catalytic site in the crystal structure of F_1-ATPase. His-200 gave doublet, suggesting that this region is not a rigid α-helix in the isolated β subunit. The binding of Mg·AMP-PNP changed the chemical shifts of His-179 and His-200 significantly. Although His-119 located at the opposite side of the conical tunnel was not affected by the nucleotide-binding, it contributed to the stability of β subunit and the efficiency of the catalysis of the holoenzyme.

Key words: F_1-ATPase; Histidine residue; ^{1}H NMR; Ligand interaction; Site-directed mutagenesis; Thermophilic *Bacillus*

1. Introduction

F_OF_1-ATP synthase is responsible for ATP synthesis coupled with proton flow across a membrane and is easily and reversibly separated into two sectors, F_O and F_1. F_O is a proton channel integrated in the membrane and F_1 is a water-soluble part with ATP-hydrolyzing activity (F_1-ATPase). F_1-ATPase is composed of five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ [1–4]. The crystal structure of $\alpha_3\beta_3\gamma_1$ part of the bovine heart mitochondrial F_1-ATPase (MF_1) established that the α and β subunits have very similar folding topology and are aligned in an alternative way in a hexagonal arrangement [5]. The molecular weight of F_1 part is ~380 kDa and that of the isolated α and β subunits are ~55 kDa and ~52 kDa, respectively. The isolated α and β subunits can bind AT(D)P, but they have no ATPase activity [6]. The minimum set of subunits to show ATPase activity appears to be $\alpha_1\beta_1$ [7–10]. Abrahams et al. indicated the presence of a 'conical tunnel' leading to the nucleotide-binding site in the crystal structure of MF_1 [5]. His-177 and His-198, which are corresponding to His-179 and His-200 of thermophilic *Bacillus* PS-3 F_1-ATPase (TF_1), respectively, reside at the entrance of the conical tunnel. We have investigated the conformational dynamics of this region of TF_1-β subunit, monitoring His-179 and His-200 by nuclear magnetic resonance (NMR). His-119 located at the opposite side of the conical tunnel was used as a reference. The result indicates that the region, including His-200 of the β subunit, which forms an α-helical structure in the crystal structure of F_1-ATPase, takes two kinds of relatively stable conformations. The signals of His-179 and His-200 were affected by temperature, pH and substrate analogue in a similar manner. Although His-119 is remote from the catalytic site and at the long loop of the opposite side of the catalytic site, the substitution of the residue to Gln suppressed the ATPase activity and thermostability.

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Abbreviations: TF_1 and CF_1, F_1-ATPase from thermophilic *Bacillus* strain PS-3 and spinach chloroplasts, respectively; AMP-PNP, 5′-adenylylimidodiphosphate; HPLC, high-performance liquid chromatography.

2. Materials and methods

2.1. Materials, bacterial strains and subunit purification

Deuterium oxide (99.9 and 99.96 atom % D) were obtained from ISOTEC and Showadenko. 5′-Adenylylimidodiphosphate (AMP-PNP) and rabbit muscle pyruvate kinase were purchased from Sigma. ATP was obtained from Oriental Yeast; hog muscle lactate dehydrogenase and NADH were from Boehringer-Mannheim; and restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase and DNA polymerase were from Takara. All other chemicals were of analytical grade.

E. coli strain 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 TF_1-β subunit and mutant TF_1-β subunits, respectively. Other strains used for generation of oligonucleotide-directed mutants and production of α and γ subunits were described previously [11,12]. Cells were grown on nutrient-rich medium at 37°C and subjected to vigorous shaking. Subunits were purified from overproducing *E. coli* cells as described previously [11,13].

2.2. Site-directed mutagenesis

Mutant TF_1-β subunits were obtained by oligonucleotide-directed mutagenesis [13,14]. The sequences of the antisense oligonucleotides used for generating βH119Q, βH179Q and βH200Q were 5′-AATTT-T*GGCGCG*GGACC**TT**GAATCGGGTC-3′, 5′-TCCCGCC**CT**GCTC-T*TGCGCA*ATGTTG**T**GAA-3′ and 5′-ATCTTTCATCTC**TT**G*ATA*-CAAGTC**G**TTTC-3′, respectively. The underlined letters indicate the mutated bases and the boldface letters indicate the Gln codons by which the His codons were substituted. The italic letters indicate the deleted *Bbe*I and *Rsa*I restriction sites by silent mutation in the Pro-121 and the Tyr-199 codons for βH119Q and βH200Q, respectively, and the inserted *Fsp*I restriction site by silent mutation in the Ile-175 and the Ala-176 codons for βH179Q.

2.3. ^{1}H NMR measurement

The protein concentration for NMR measurements was 26–52 mg/ml (0.5–1 mM). The protein was dissolved in a deuterated solution of 50 mM sodium phosphate buffer at the appropriate p^{2}H value. pH meter readings were not corrected for the deuterium isotope effect. Chemical shifts are shown relative to the internal sodium 2,2-dimethyl-2-silapen-

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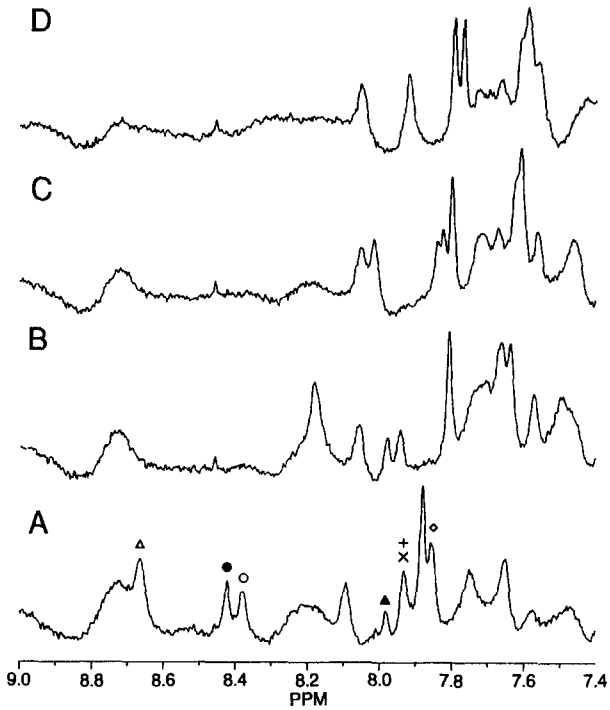

Fig. 1. ^{1}H NMR spectra of the wild-type TF_1-β subunit at various p^2H and 30 °C. p^2H values are 6.77 (A), 7.68 (B), 8.20 (C) and 9.49 (D). The symbols in A are associated with those of the titration curves in Fig. 2.

tane-5-sulfonate (DSS). For ligand or pH titration, a small amount of Mg·AMP-PNP[1] or NaO^2H solution was added directly to the protein solution in a sample tube. ^{1}H NMR spectra at 400 MHz were recorded on a Bruker AM400 spectrometer.

2.4. Other methods

Reconstitution of the β subunits with α and γ subunits was carried out according to the previous reports [7,12]. Reconstituted complexes were separated from isolated subunits by applying them to a gel permeation HPLC once and used for ATPase activity measurement without further purification. The ATPase activity was measured at 25°C in the presence of an ATP-regenerating system [12]. Protein concentration was determined by the method of Bradford [15] using bovine serum albumin as a standard.

3. Results

^{1}H NMR spectra of isolated wild-type β subunit were measured in a pH range of 6.50–10.00 (Fig. 1). The protein was aggregated at around pH 6.0. The signals that emerged in the range of 7.4 to 8.4 ppm at pH 7.68 (Fig. 1B) can be assigned to histidine C-2 protons, because the signals in that region disappeared by incorporation of deuterated histidine into the protein (Tozawa et al., unpubl. data). The signals were well resolved in spite of the high molecular weight of the molecule (~52 kDa) and about 12 peaks could be observed in this region. Since 12 histidines are scattered over the primary sequence, the histidine signals of TF_1-β subunit can be used as probes to monitor various local environments of the protein.

The pH-titration curves of C-2 proton chemical shifts can be roughly classified into two groups. The signals belonging to one group show rather normal or higher pKa values. The titration curves of these signals are presented in Fig. 2. The signals of the other group do not show significant change in the chemical shifts in the pH range examined, suggesting that these histidine residues are located inside of the protein.

Signals caused by His-179 and His-200 were used to investigate the conformational dynamics of the entrance of the conical tunnel region of the isolated TF_1-β. His-119 was selected as a reference, because it is positioned in a long loop on the opposite side of the subunit and is remote from the catalytic site. For the assignment of the C-2 proton signals of these residues, three mutant β subunits (βH119Q, βH179Q and βH200Q) were used in which His-119, His-179 and His-200 were substituted by Gln, respectively. Their spectra are compared with those of wild-type β (Fig. 3). The resonance at 8.14 ppm and the two resonances at 7.92 and 7.95 ppm in the spectrum of wild-type β are unambiguously absent in those of βH179Q and βH200Q, respectively, indicating that the former resonance is assigned to His-179 and the latter two resonances arise from His-200. The appearance of two signals from a single residue is the evidence for the presence of two different environments around His-200, exchanging at a slow rate. Although the spectrum of βH119Q is different from that of the wild-type β to some extent, there is a good correlation between the signals of the two spectra. The correlation could be confirmed by the pH dependence on the signals. In the spectrum of βH119Q, the signal intensity at about 7.66 ppm diminished significantly in comparison to that in the spectrum of the wild-type β (data not shown). Although the exact assignment is difficult because of severe overlapping

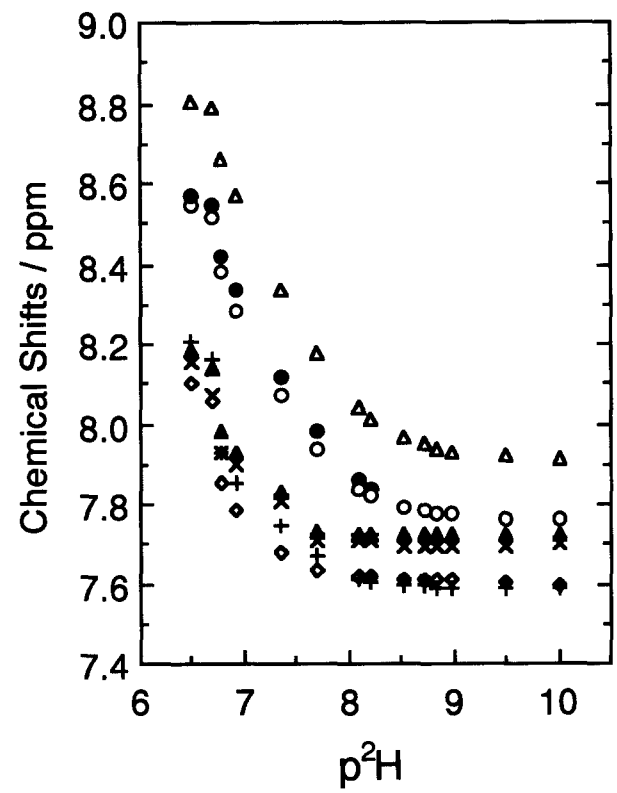

Fig. 2. Titration curves of the histidine proton signals of the wild-type TF_1-β subunit at 30°C. Only the signals which shifted in this p^2H range are plotted. Each symbol represents the signal indicated by the same symbol in Fig. 1A. ● and ○ are assigned to His-200. △ is assigned to His-179.

[1]Mg·AMP-PNP, an equimolar mixture of $MgCl_2$ and AMP-PNP.

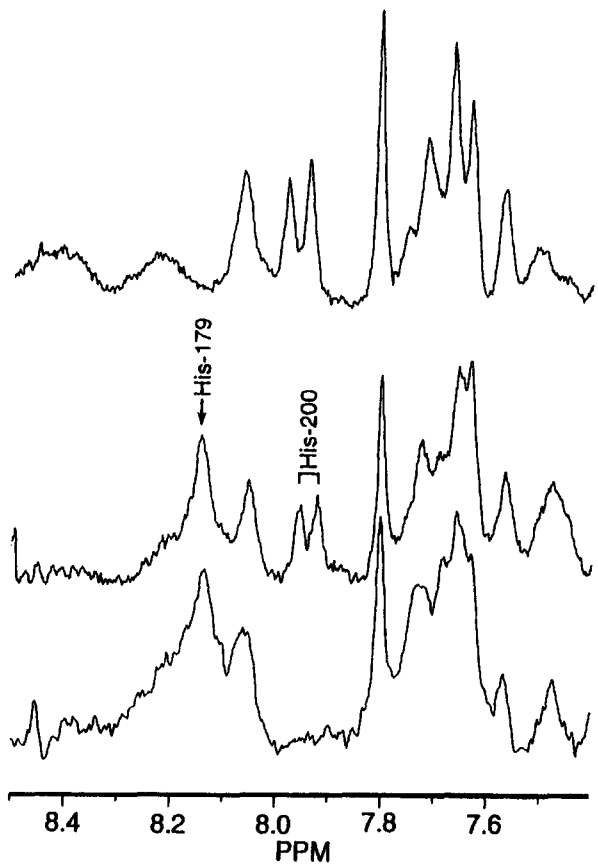

Fig. 3. Comparison of the ^{1}H NMR spectra of the wild-type with those of mutant TF_1-β subunits. βH179Q (top), wild-type β (middle) and βH200Q (bottom) at 30°C and p^{2}H 7.68. The assigned resonances are indicated in the spectrum of the wild-type β subunit.

of the signal, His-119 signal should be located in the region from 7.60 to 7.68 ppm.

The intensity ratio of the split resonance of His-200 was constant in the pH range of 6.50 to about 8. The doublet coalesced to one peak at pH higher than 8.5. Two explanations are possible, i.e. the rate of the exchange of the two conformations becomes faster in comparison with the frequency difference of the two signals, or that the chemical shift values of two conformations become very close to each other.

An addition of a substrate analogue, Mg·AMP-PNP caused the change of the signals of His-179 and His-200 of the wild-type TF_1-β. With the increase of Mg·AMP-PNP, the signals of His-200 shifted downfield, retaining the doublet and finally coalesced to the resonance at 8.07 ppm (Fig. 4). The signal of His-179 also shifted downfield and slightly broadened with the addition of Mg·AMP-PNP. Other histidine signals, including His-119 region, did not show a significant change other than broadening. An addition of $MgCl_2$ did not induce such an effect that Mg·AMP-PNP did.

To know further structural and functional properties of TF_1-β and their mutants, the spectra of the three mutant proteins were measured at various temperatures (data not shown). The spectra of βH179Q and βH200Q mutants showed that the temperature dependence was similar to that of the wild-type β except for the absence of His-179 and His-200 signals, respectively, suggesting that substitution of His-179 and His-200 to Gln has little effect on the β subunit structure. In contrast, some of the histidine resonances of βH119Q show more significant temperature dependence than those of the wild-type β. Phenylalanine resonances changed to some extent, suggesting that the amino acid replacement at His-119 affects the structure in a certain region. Moreover, βH119Q was aggregated at above 45°C, although aggregation took place at 60°C for the wild-type β. This indicates that the replacement of His-119 with glutamine destabilizes the protein structure.

To see the effect of the amino acid substitution of β subunit on the function of $\alpha_3\beta_3\gamma$ complex, the steady-state ATPase activity of the complexes containing βH119Q, βH179Q and

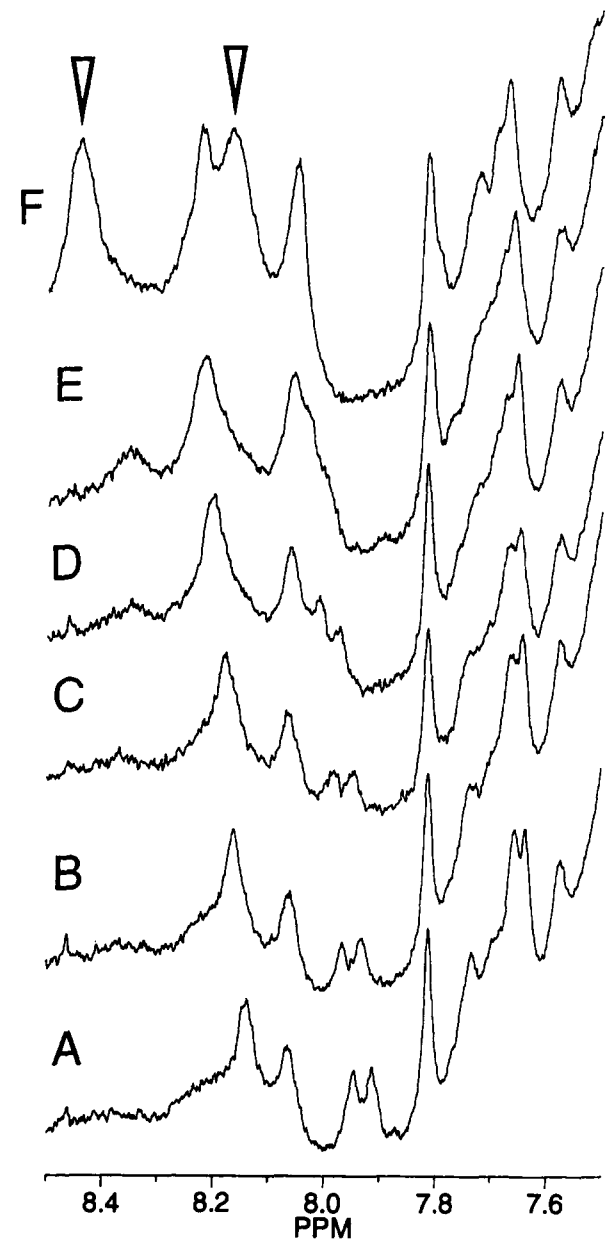

Fig. 4. Mg·AMP-PNP titration spectra of the wild-type TF_1-β subunit at 30°C and p^{2}H 7.68. Molar ratios of the substrate analogue to protein are 0 (A), 0.12 (B), 0.23 (C), 0.54 (D), 1.00 (E) and 2.35 (F). The resonances from adenine ring protons of AMP-PNP are indicated by arrow heads in F.

Table 1
ATPase activity of the reconstituted $\alpha_3\beta_3\gamma$ complexes containing βH119Q, βH179Q or βH200Q

Preincubation	Activity (unit/mg protein)			
	$\alpha_3\beta$(wild)$_3\gamma$	$\alpha_3\beta$(H119Q)$_3\gamma$	$\alpha_3\beta$(H179Q)$_3\gamma$	$\alpha_3\beta$(H200Q)$_3\gamma$
25°C, 30 min	6.59	0.975	8.10	6.94
0°C, 30 min	5.99	0.716	7.14	6.37
65°C, 10 min	4.49	0.233	4.26	4.58

Each complex was purified by gel-permeation HPLC as described in text. Solutions containing 70–140 μg/ml protein in 50 mM Tris sulfate, pH 7.0, were preincubated as indicated and the remaining activity was measured at 25°C. The activities were calculated using the data from 3 to 5 min after starting the reaction.

βH200Q mutants were measured. Specific activities of the $\alpha_3\beta$H119Q$_3\gamma$, $\alpha_3\beta$H179Q$_3\gamma$ and $\alpha_3\beta$H200Q$_3\gamma$ complexes at 25°C were 14.8%, 123% and 105% of that of the wild-type complex, respectively (Table 1). This indicates that the replacement of His-119 with Gln suppresses the ATPase activity, while that of His-179 rather increases the activity. Thermostability of the complexes were examined by the preincubation at 0 and 65°C. Preincubation of $\alpha_3\beta$H119Q$_3\gamma$ at 65°C for 10 min induced a significant decrease in ATPase activity (23.9% of that of the same complex at 25°C). Therefore, the thermolability of βH119Q is retained in the $\alpha_3\beta_3\gamma$ complex. Stability of $\alpha_3\beta$H200Q$_3\gamma$ was similar to that of the wild-type $\alpha_3\beta_3\gamma$. ATPase activities of these complexes are sensitive to azide inhibition since ATP hydrolysis was stopped within 1 min when sodium azide at final concentration of 1 mM was added to the reaction mixtures (data not shown).

4. Discussion

When isolated β subunit of TF_1 was exposed to protease, the region covering His-200 was easily cleaved [12]. In the crystal structure of the MF_1, the corresponding residue is located in an α-helix, which forms the conical tunnel to the nucleotide-binding site [5]. If this is the case with TF_1-β subunit as well, this region should not be protease susceptible. The doublet signal in the NMR spectrum showed that there is a slow exchange between the two environments around His-200 in the isolated β subunit. Since the exchange is slow, the barrier between the environments should be relatively high. If the environment involved in the exchange is restricted to the side-chain of His-200, hydrogen-bonding interaction or salt-bridge formation of the imidazole group would be responsible for the barrier. Since these interactions should be affected by the charge of the imidazole group, the intensity ratio of the doublet signal should be altered by pH. However, the observed intensity ratio was constant in the pH range, covering pKa of His-200. Therefore, the difference in the environment is not so minor as that in the side-chain conformation of His-200. The doublet should be explained by the presence of two more globally different conformations around His-200, suggesting that this region is not a rigid α-helix. This observation explains the protease-susceptible nature of this region in the isolated β subunit [12]. Probably, intersubunit interactions in the complex fix this flexible conformation to α-helix.

Binding of Mg·AMP-PNP significantly affected the chemical shift of the His-179 and the His-200 of TF_1-β. This can be explained in two ways. One, that His-179 and His-200 are directly involved in a conformational change induced by the ligand-binding. The other, that the presence of a negative charge of Mg·AMP-PNP changes pKa of His-179 and His-200, leading to an increase of the protonated fractions of the imidazole groups. In the MF_1 crystal structure, the bound nucleotide is not as close to the amino acid residues corresponding to TF_1-β His-179 and His-200. Therefore, the former explanation is more likely. This explanation is also consistent with the fact that the region around the entrance of the conical tunnel in the crystal structure takes different conformations depending on the substrate-binding in spite of its relatively distant location from the substrate-binding site [5]. The conformational flexibility in the region around His-200 may offer the possibility for the conformational change responding the substrate-binding. It was also reported that a mutation at the catalytic site can be suppressed by mutations at the entrance of the conical tunnel [16]. The binding of Mg·AMP-PNP did not change the intensity ratio of the His-200 doublet, suggesting that the binding affinity of Mg·AMP-PNP is similar for β subunits in two conformations. The substitution of His-179 and His-200 to Gln did not change the NMR spectrum significantly. Since $\alpha_3\beta$H179Q$_3\gamma$ and $\alpha_3\beta$H200Q$_3\gamma$ complex showed uninhibited and stable ATPase activity, the interactions at the subunit interface which stabilize $\alpha_3\beta_3\gamma$ structure should not be affected by the amino acid substitution at all. Thus, the side-chain of His-179 and His-200 may not be directly involved in the structure formation.

The amino acid residue corresponding to His-119 of TF_1-β is located in the longest loop in β subunit, which is far from the catalytic site [5]. Nevertheless, the ATPase activity of $\alpha_3\beta$H119Q$_3\gamma$ diminished significantly even at room temperature. The replacement of His-119 with glutamine changed the NMR spectrum and the denaturation temperature to a certain extent, suggesting that the side-chain of this residue is involved in the stabilization of the tertiary structure. It is obvious that the substitution of His-119 induces a conformational change around this loop region. It would impair the formation of active complex by affecting the intersubunit interactions, although the yield of the $\alpha_3\beta_3\gamma$ complex formation itself is similar to that of the wild-type $\alpha_3\beta_3\gamma$. This region has never been carefully examined so far. However, the conformation of this loop turned out to be important in the formation of the active complex and His-119 was shown to be involved in maintaining of this conformation. Actually, this histidine residue is conserved in all of the known sequences of β subunits from a variety of sources.

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