

Unusual pK_a of the carboxylate at the putative catalytic position of the thermophilic F_1 -ATPase β subunit determined by ^{13}C -NMR

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Abstract Glutamic acid-190 in the β subunit of F_1 -ATPase from thermophilic *Bacillus* PS-3 (TF₁) was reported to be essential for the ATPase activity. The mutant TF₁ β subunit in which Glu-190 had been substituted by cysteine was carboxymethylated with ^{13}C -labeled monoiodoacetic acid. The pK_a value of the carboxymethylene group at the 190 position was determined as 5.6 ± 0.4 by ^{13}C -NMR. On the basis of this value, the pK_a of the carboxylate of Glu-190 of the TF₁ β subunit was estimated to be 6.8 ± 0.5 . The unusually high pK_a could play a role in the catalytic mechanism of F_1 -ATPase.

Key words: F_1 -ATPase; pK_a ; Carboxymethylation; Glutamic acid; Specific ^{13}C labeling; ^{13}C -NMR; (Thermophilic *Bacillus*)

1. Introduction

F_0F_1 ATP synthase performs ATP synthesis coupled with H^+ movement driven by the proton gradient across the membrane of a bacterium, chloroplast or mitochondrion. It consists of two components, namely F_0 and F_1 . The F_0 portion acts as a proton channel and the F_1 portion carries catalytic sites. The isolated F_1 hydrolyzes ATP to ADP and P_i , and is called F_1 -ATPase. In many cases, F_1 comprises five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ [1,2]. The β subunit is thought to be the catalytic subunit although the α subunit is also required for expression of the ATP hydrolyzing activity [3]. Some amino acid residues of each subunit have been suggested to be involved in the enzyme function. In particular, the β subunit has been studied in detail using a variety of techniques, including chemical modification and site-directed mutagenesis. The modification of a glutamic acid residue (Glu-190) of the β subunit of F_1 from thermophilic *Bacillus* PS-3 (TF₁) with dicyclohexylcarbodiimide (DCCD) was found to inactivate the enzyme [4]. The substitution of this residue by glutamine also induced complete loss of the activity [5]. A crystallographic study on mitochondrial

F_1 -ATPase indicated that the carboxylate of a glutamic acid residue corresponding to Glu-190 of TF₁ β is arranged so as to activate a bound water molecule in the vicinity of the γ -phosphate of a substrate analogue, AMP-PNP [6]. Therefore, the pK_a of Glu-190 is an important parameter for understanding the mechanism of the catalytic reaction. Although pH titration of the NMR signal would make it possible to determine the pK_a value, it has not been performed because of the high molecular masses of the β subunit (~ 52 kDa) and F_1 ATPase (~ 370 kDa). In this study, we have developed a method for the ^{13}C labeling of a particular residue involving a combination of amino acid replacement and chemical modification. Thus, we could estimate the pK_a of the carboxylate at position 190, using the ^{13}C -carboxymethylated β subunit and ^{13}C -NMR. The pK_a of this carboxylate was shown to be much higher than the normal value.

2. Materials and methods

2.1. Materials, bacterial strains, and plasmids

Deuterium oxide deuterated at 99.96% and 99.996% was obtained from ISOTEC and CIL, respectively. Iodo[1- ^{13}C]acetic acid (99 atom%) and iodo[2- ^{13}C]acetic acid (99.5 atom%) were purchased from ICON. Dithiothreitol (DTT) was from Wako. N,N' -Dicyclohexylcarbodiimide (DCCD), 5,5'-dithiobis-2-nitrobenzoic (DTNB) acid, and 5'-adenylyl imidodiphosphate (AMP-PNP) were from Sigma. DEAE-Sephacel was from Pharmacia, and Toyopearl HW-55F was from Tosoh.

The *E. coli* strains and the plasmid carrying the gene of the β subunit of TF₁ used in this study were described previously [7]. The plasmid for expressing $\alpha_3\beta(\text{E190C})_3\gamma$ was constructed using expression plasmid $\alpha\gamma\beta/\text{pKK223-3}$, which harbors the $\alpha(\text{C193S}/\text{W463F})$, γ and β subunit genes of TF₁ [9]. $\beta(\text{E190C})$ and $\alpha(\text{C193S}/\text{W463F})$ correspond to mutant β in which Glu-190 was substituted by cysteine (Cys), and mutant α in which Cys-193 and Trp-463 were substituted by serine and phenylalanine, respectively. For expression of the mutant $\alpha_3\beta_3\gamma$ complex, *E. coli* strain JM103 $\Delta(\text{uncB-D})$ was transformed with the constructed plasmid [8].

2.2. Purification and carboxymethylation of $\beta(\text{E190C})$ and $\alpha_3\beta(\text{E190C})_3\gamma$

$\beta(\text{E190C})$ was purified as described previously [7]. The sulfhydryl group of Cys-190 was carboxymethylated as reported by Amano et al. [9]. Completion of carboxymethylation was confirmed by quantitative analysis of sulfhydryl groups using DTNB [10]. The overproduction and purification of $\alpha_3\beta(\text{E190C})_3\gamma$ were carried out as reported by Matsui et al. [8]. Urea was added to a solution containing 250 mg of the complex at more than 8 M, followed by the addition of 5 mole equivalents of an iodo[1- ^{13}C]acetic acid solution. The solution was diluted to 1 mg/ml, and then dialyzed against 20 mM Tris- SO_4 , pH 8.0, containing 200 mM NaCl to remove urea and excess IAA. The modified $\alpha_3\beta(\text{E190C})_3\gamma$ was subjected to gel filtration (Sephacel CL-4B; Pharmacia). Ultrafiltration was carried out to concentrate the solution and to replace the solvent with 25 mM deuterated phosphate buffer, pH 8.0. An aliquot of the concentrated solution (~ 1 $\mu\text{g}/\mu\text{l}$)

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Abbreviations: AMP-PNP, 5'-adenylyl imidodiphosphate; $\alpha(\text{C193S}/\text{W463F})$, mutant α subunit in which Cys-193 and Trp-463 are substituted by serine and phenylalanine, respectively; $\beta(\text{E190C})$, mutant β subunit in which Glu-190 is substituted by cysteine; CmCys, S-carboxymethylcysteine; Cm β , S-carboxymethylated at Cys-190 of $\beta(\text{E190C})$; DCCD, N,N' -dicyclohexylcarbodiimide; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; IAA, monoiodoacetic acid; Mg-AMP-PNP, an equimolar mixture of MgCl_2 and AMP-PNP; TF₁, F_1 -ATPase from thermophilic *Bacillus* strain PS-3; TF₁ β , β subunit of TF₁-ATPase

was used for the ATPase activity assay. The activity assay method was reported elsewhere [7]. Protein concentrations were determined using the method of Bradford [11].

2.3. NMR measurements

^1H - and ^{13}C -NMR spectra were obtained at 400 and 100 MHz, respectively, with a Bruker AM-400 at 30°C. The concentrations of ^1H -NMR samples were 0.25–1 mM in $^2\text{H}_2\text{O}$. A pH titration experiment was carried out by adding aliquots of a ^2HCl or NaO^2H solution. The sample solutions for ^{13}C -NMR measurements were concentrated to 1.8–1.9 ml. The concentrations of the isolated $\beta(\text{E190C})$ and $\alpha_3\beta(\text{E190C})_3\gamma$ were about 1.28 and 0.06 mM, respectively. Chemical shifts are presented relative to the internal DSS signal. The ^{13}C -NMR measurements were performed under proton decoupling using a composite pulse. The repetition time was 10 s. The given pH values are the direct readings with a pH meter.

3. Results

The $\text{TF}_1 \alpha_3\beta_3\gamma$ complex containing the mutant β subunit in which Glu-190 was substituted by *S*-carboxymethylcysteine exhibited 12% of the ATPase activity of the wild-type $\alpha_3\beta_3\gamma$ complex [9]. Therefore, this β subunit was used to characterize the chemical nature of the carboxylate at position 190. The $\text{TF}_1 \beta(\text{E190C})$ mutant protein was modified using unlabeled and ^{13}C -labeled monoiodoacetic acids (IAA) to obtain carboxymethylated $\beta(\text{E190C})$ ($\text{Cm}\beta$) and $[2\text{-}^{13}\text{C}]$ - or $[1\text{-}^{13}\text{C}]\text{carboxymethylated } \beta(\text{E190C})$ ($\text{Cm}[2\text{-}^{13}\text{C}]\beta$ or $\text{Cm}[1\text{-}^{13}\text{C}]\beta$), respectively. Since the TF_1 - β subunit has no cysteine residue, the cysteine at position 190 of the $\beta(\text{E190C})$ subunit could be specifically carboxymethylated. The ^{13}C -NMR spectra of $\text{Cm}\beta$ and $\text{Cm}[1\text{-}^{13}\text{C}]\beta$ are presented in Fig. 1. By comparing the two spectra, the resonance at 179.9 ppm could be assigned to the labeled carboxylate carbon of the modified $\beta(\text{E190C})$. Although IAA can also react with other amino acid species, the reactivity should be much lower than that with the cysteine residue under the experimental conditions we have employed [12]. In view of the complete loss of the sulfhydryl group of the single cysteine residue in the β subunit, the major signal should originate from the *S*-carboxymethyl group. Actually, there was only one major signal as can be seen in Fig. 1. This justifies the assignment given above. A

similar comparison of the spectra of $\text{Cm}\beta$ and $\text{Cm}[2\text{-}^{13}\text{C}]\beta$ allowed us to assign the signal at 40.4 ppm to the labeled methylene carbon of the modified $\beta(\text{E190C})$ (data not shown).

To determine the pK_a of the carboxylate, the ^{13}C -NMR spectra of both $\text{Cm}[2\text{-}^{13}\text{C}]\beta$ and $\text{Cm}[1\text{-}^{13}\text{C}]\beta$ were measured at various pH values. Since the protein was precipitated at low pH because of its isoelectric point, the lower limit of the titration was p^2H 5.5. The $1\text{-}^{13}\text{C}$ signal was sharper than the $2\text{-}^{13}\text{C}$ signal, and the chemical shift value of the former changed more significantly than that of the latter during pH titration. Therefore, the data set for $\text{Cm}[1\text{-}^{13}\text{C}]\beta$ should be more reliable for pK_a determination. The chemical shift value of the $\text{Cm}[1\text{-}^{13}\text{C}]\beta$ signal is plotted as a function of p^2H in Fig. 2A. On non-linear least-squares fitting to two sets of independent observations, the best-fit pK_a value of the carboxylate was found to be 5.7 ± 0.2 . The lack of data at lower p^2H render the obtained value ambiguous. The change in chemical shift obtained from the fitting was 2.4 ppm. This is smaller than that observed for iodoacetic acid (3.2 ppm). The titration curves of all carboxyl carbon signals of aspartic acid (Asp) and Glu residues of basic pancreatic trypsin inhibitor [13] and RNase HI [14] have been reported. The chemical shift change was in the range of 2.7–3.5 ppm with one exception for Asp residues and 3.5–4.6 ppm for Glu residues. On the other hand, the chemical shift changes of the free carboxyl carbons on ionization of Asp and Glu residues in oligopeptides were 3.5 and 4.6 ppm, respectively [15]. Therefore, 2.4 ppm is not unusually small. However, it would be safe to consider the chemical shift change of CmCys-190 to be in the range of 2.4–4.6 ppm. The pK_a value of the carboxylate of CmCys-190 of the modified β subunit can then be estimated to be 5.6 ± 0.4 .

To determine the normal pK_a value of this type of carboxylate, pH titration of the ^1H -NMR signals of *S*-carboxymethylcysteine was carried out. The chemical shift of the δ methylene proton signal is plotted as a function of p^2H in Fig. 2B. Non-linear least-squares fitting gave a pK_a value of 3.20 ± 0.05 . Therefore, the pK_a of the carboxymethyl group of $\text{Cm}\beta$ is clearly higher than the normal value. The higher pK_a value of the carboxylate in the protein shows that the protonated state is stabilized by the environment around the

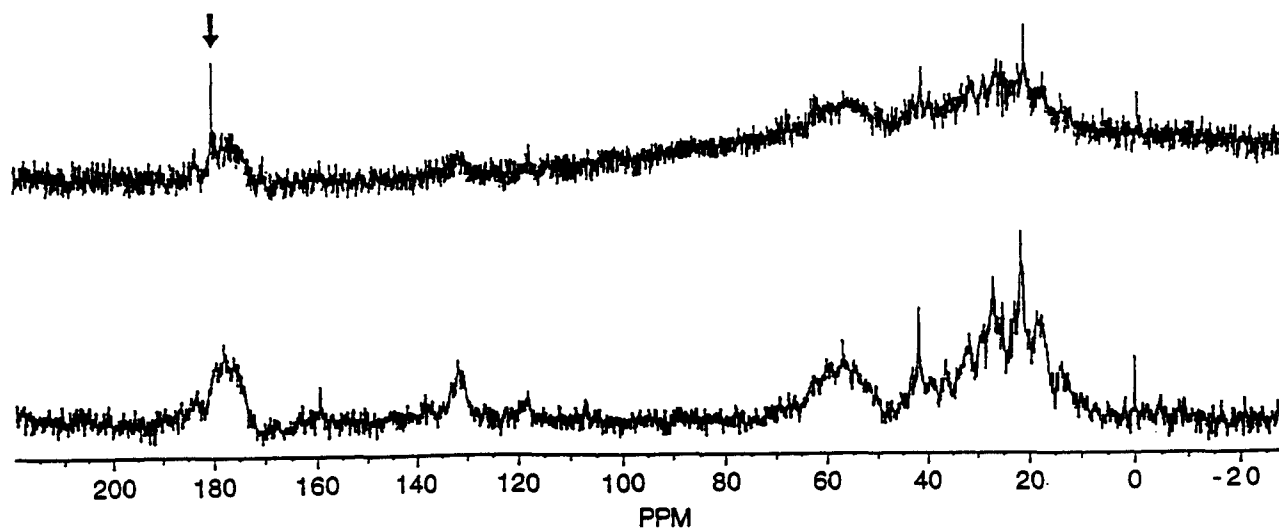


Fig. 1. ^{13}C -NMR spectra of carboxymethylated TF_1 - $\beta(\text{E190C})$. Top trace: 0.66 mM $\text{Cm}[1\text{-}^{13}\text{C}]\beta$ at p^2H 7.42; 1768 transients accumulated. Bottom trace: 0.65 mM $\text{Cm}\beta$ at p^2H 7.15; 8920 transients accumulated. The signal assigned to the ^{13}C -labeled carboxylate is denoted by an arrow.

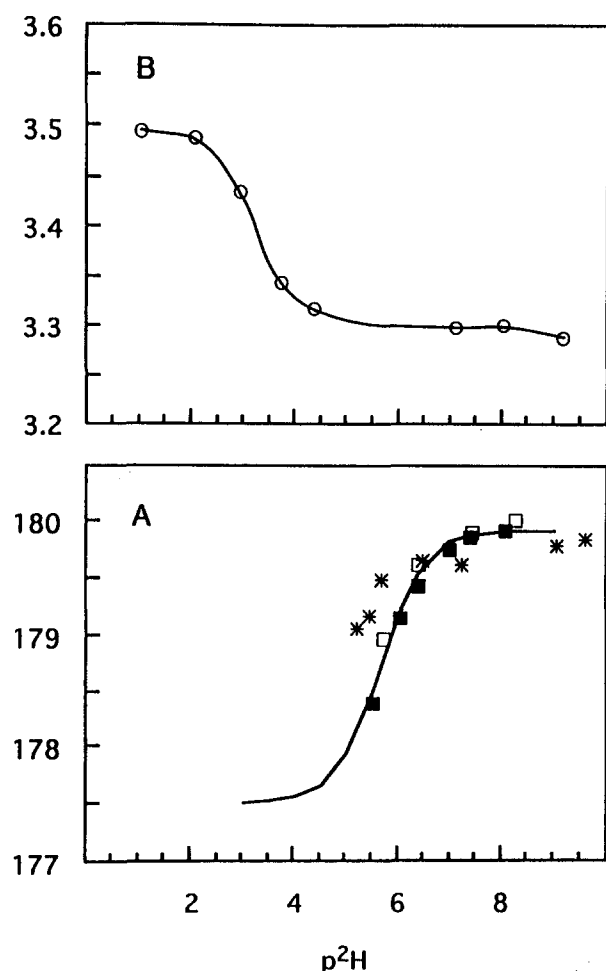


Fig. 2. pH titration curves of the carboxyl groups of *S*-carboxymethylcysteine and $\text{TF}_1 \text{ Cm}[1-^{13}\text{C}]\beta$. (A) Titration curves of the carboxyl ^{13}C signal of $\text{TF}_1 \text{ Cm}[1-^{13}\text{C}]\beta$ at 25°C . The open and closed squares correspond to the data obtained in two independent experiments, respectively. The solid line represents the best-fit curve obtained by the non-linear least-squares regression method. The asterisks indicate the results in the presence of 5.3 mM ADP. (B) Titration curve of the C_δH signal of *S*-carboxymethylcysteine at 25°C .

carboxylate of interest. The following situations can increase the pK_a : (1) the presence of a negative charge in the vicinity; (2) involvement of the carboxylate in the hydrogen bonding; and (3) the location of the carboxylate in a hydrophobic region.

pH titration of $\text{Cm}[1-^{13}\text{C}]\beta$ was also carried out in the presence of an excess amount of ADP (molar ratio 10:1) (Fig. 2A). Since the K_d has been reported to be $24 \mu\text{M}$ [16], all of the β subunits should be bound by ADP under these conditions. The titration curve shifted to a lower pH, suggesting the pK_a value is lower than that in the absence of ADP. In addition, the chemical shift value of the dissociated form of the carboxylate was also different from that in the absence of ADP. This suggests that the binding of ADP changes the environment of the carboxyl group. The pK_a value of the carboxylate decreased on binding of ADP in spite of its negative charge, suggesting that the phosphate portion of ADP is not located near the carboxylate or that the appearance of a positive charge would compensate for the negative charge of the phosphate.

The modification of $\alpha_3\beta(\text{E190C})_3\gamma$ with IAA was achieved by dissociation of the complex in the presence of urea. The complex was reconstituted by the removal of urea after carboxymethylation. In this experiment, we used a complex containing $\alpha(\text{C193S/W463F})$ to avoid the carboxymethylation of Cys-193 of the α subunit. This complex was reported to have the same level of ATPase activity as that of the wild-type $\alpha_3\beta_3\gamma$ complex at 25°C [8]. The number of free sulfhydryl groups of the complex was estimated to be 3.2 by DTNB titration. These sulfhydryl groups disappeared on treatment with IAA, confirming that the modification proceeded to completion. The reconstitution was further confirmed by polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate after purification (data not shown). The specific activity of the IAA-modified $\alpha_3\beta(\text{E190C})_3\gamma$ was approx. 0.6 units/mg protein, which is 10% of the activity of the wild-type $\alpha_3\beta_3\gamma$ complex. The ^{13}C -NMR spectrum of $[1-^{13}\text{C}]$ -carboxymethylated $\alpha_3\beta(\text{E190C})_3\gamma$ ($\alpha_3\text{Cm}[1-^{13}\text{C}]\beta_3\gamma$) is shown in Fig. 3.

In spite of its very high molecular mass ($\sim 370 \text{ kDa}$), a reasonable spectrum could be obtained. Since the ^{13}C -NMR spectrum of carbonyl carbons in the flexible backbone of the dimer of IgG2a (300 kDa) has been reported [17], it is not surprising that carboxyl carbon signal is obtained for the side chain in a protein with similar molecular mass. This is another piece of evidence for the usefulness of ^{13}C -NMR in studying a very large protein molecule. On comparison of this spectrum with that of the unlabeled β subunit (Fig. 1), the signal at 180 ppm can be assigned to the labeled carboxylate. This result shows that the chemical environment of at least one CmCys-190 in the $\alpha_3\text{Cm}\beta_3\gamma$ complex should be similar to that in the isolated $\text{Cm}\beta$.

4. Discussion

The error range of the determined pK_a values of $\text{Cm}\beta$ was relatively large because of its incomplete titration curve. However, it is important to note that the pK_a of the carboxylate at the putative active site of $\text{Cm}\beta$ is much higher than the normal value, even when taking the lowest value in the estimated range. Since the $\alpha_3\text{Cm}\beta_3\gamma$ complex retains ATPase activity, this unusual pK_a could have something to do with the catalytic mechanism as will be discussed later.

Furthermore, we can discuss the chemical nature of Glu-190 of the $\text{TF}_1\beta$ subunit on the basis of the results for $\text{Cm}\beta$. Two factors contribute to the pK_a value of a carboxylate in a protein: one is the intrinsic pK_a in aqueous solution, the other being the change (ΔpK_a) induced by the transfer of the carboxylate from aqueous medium to a protein [18]. The difference in chemical structure would affect both the intrinsic pK_a and ΔpK_a of the carboxylate. Namely, the pK_a of the γ -carboxylate of glutamic acid is 4.3 (4.4–4.6 in a polypeptide chain) [18] which is 1.1 pH units higher than that of *S*-carboxymethylcysteine (CmCys). The intrinsic pK_a should be defined as that in a polypeptide chain. Since the difference between the pK_a values of a free amino acid and that in a polypeptide chain mainly results from the effect of charged groups at the C_α position, the difference becomes smaller with longer side chains. For example, it amounts to 0.6 and 0.2 for aspartic acid and glutamic acid, respectively [18], suggesting that 0.2 is the largest pK_a difference expected for CmCys . In total, the correction value for the intrinsic pK_a

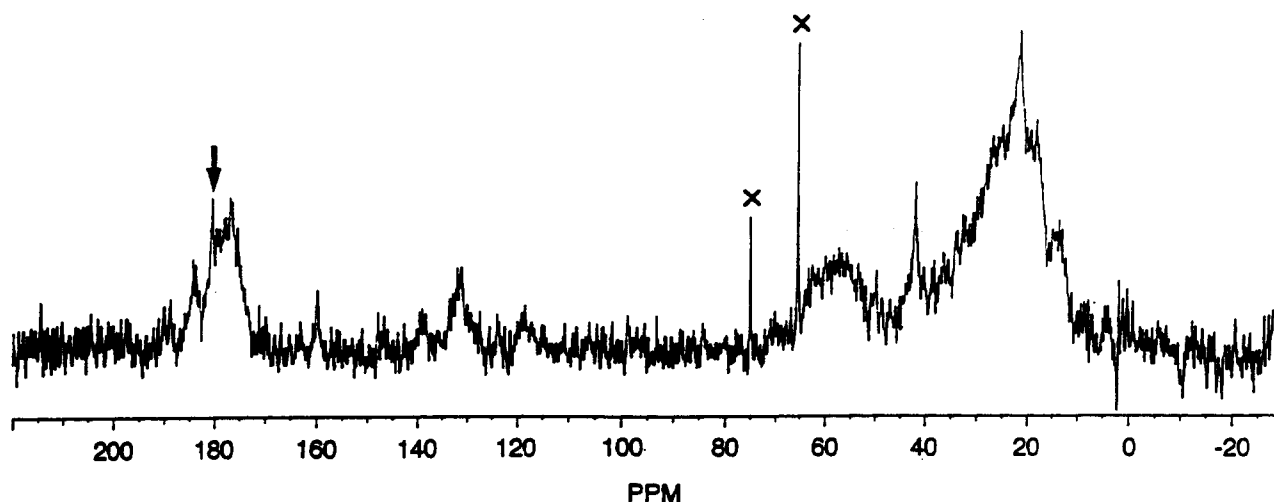


Fig. 3. ^{13}C -NMR spectrum of $\text{TF}_1 \alpha(\text{C193S/W463F})_3\text{Cm}[1-^{13}\text{C}]\beta_3\gamma$ at p^2H 8.1 and 25°C . 5200 transients were accumulated. The signal assigned to the ^{13}C -labeled carboxylate is denoted by an arrow. The sharp signals indicated by crosses are due to contaminating small molecules from the buffer.

should be 1.2 ± 0.1 . Therefore, the pK_a of the carboxylate of Glu-190 should be 6.8 ± 0.5 , provided that its ΔpK_a is the same as that of CmCys.

The polarity of the inside of a protein is generally lower than that of water. The presence of sulfur in the side chain should affect the ΔpK_a . However, the contribution should be small, since it retains hydrophobic nature in spite of its high electronegativity [18]. Furthermore, the length of the side chain of *S*-carboxymethylcysteine is greater than that of glutamic acid because of the additional sulfur. Since $\alpha_3\text{Cm}\beta_3\gamma$ is still catalytically active [9], the environment of the carboxylate of CmCys-190 should not be significantly different from that of Glu-190. Consequently, the ΔpK_a of CmCys-190 should be quite similar to that of Glu-190. Now, the pK_a of Glu-190 can be estimated to be 6.8 ± 0.5 to a first approximation. On the basis of the results on basic pancreatic trypsin inhibitor, RNase HI and mouse epidermal growth factor [19], the pK_a values of normal Glu residues in proteins fall within the range 3–5. This shows that the pK_a of Glu-190 in $\text{TF}_1\beta$ is unusually high, even taking into account its large error range. Actually, Asp-10 of RNase HI was also reported to have an unusually high pK_a of 6.1 [14]. This residue is also located at the active site.

Since the chemical shift of the carboxylate signal of CmCys-190 in the $\alpha_3\text{Cm}\beta_3\gamma$ complex is similar to that of the Cm β subunit, we can infer the role of the carboxylate at the 190 position in the F_1 -ATPase on the basis of the results of this work. In the crystal structure of mitochondrial F_1 , this carboxyl group forms a hydrogen bond with a water molecule in the $\text{Mg}\cdot\text{AMP}\cdot\text{PNP}$ binding form. The oxygen of the water molecule is located 4.4 Å from the γ -phosphate of AMP-PNP. The unusually high pK_a estimated in the β subunit suggests that the carboxylate can act as a conjugate base to activate the water to attack the γ -phosphate of ATP. If this is the case, the lower activity of the $\alpha_3\text{Cm}\beta_3\gamma$ complex should be associated with the lower pK_a of CmCys-190 as well as with the structural difference around the side chain. It is also well known that the rate of ATP hydrolysis decreases with lower pH in the acidic region [20–22]. The carboxylate

of Glu-190 must be one of the factors regulating this pH-dependent activity.

This work has shown that the introduction of ^{13}C -labeled *S*-carboxymethylcysteine at the glutamic acid position of a protein is a powerful means of monitoring the chemical nature and the microenvironment of a carboxylate of a glutamate residue in such a large protein as F_1 -ATPase, as far as the carboxymethylated protein retains its activity.

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