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Spatial precision of a catalytic carboxylate of F_1-ATPase β subunit probed by introducing different carboxylate-containing side chains

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Abstract

Combining mutation and chemical modification, we have introduced Asp, Gln, Cys, *S*-carboxymethylcysteine (Cax) and *S*-carbamoylmethylcysteine (Cam) into the positions of Glu^{190} and Glu^{201} of the β subunit of F_1-ATPase from the thermophilic *Bacillus* PS3. The steady-state ATPase activities of $\alpha_3\beta_3\gamma$ complexes containing these changed β subunits were 12% (E190Cax), 7% (E190D), 3% (E190Cam), <1% (E190C), <1% (E190Q), and 73% (E201D), 40% (E201Cax), 25% (E201C), 20% (E201Q), 4% (E201Cam), of that of the wild-type $\alpha_3\beta_3\gamma$ complex. For the complexes containing E190C or E190Q, even the ability of single-site catalysis was lost. Thus, the presence of a carboxylate at 190 (but not at 201) is absolutely required for catalysis and its spatial precision is very strict. Analysis of inactivation of the complexes by dicyclohexylcarbodiimide suggests that Glu^{190} and Glu^{201} are interacting in the F_1-ATPase.

Key words: F_1-ATPase; Catalytic carboxylate residue; Monoiodoacetic acid; Dicyclohexylcarbodiimide

1. Introduction

F_0F_1-ATPsynthase catalyzes ATP synthesis/hydrolysis coupled with proton flow across membranes. F_1-ATPase is the soluble component of F_0F_1-ATPsynthase. It catalyzes ATP hydrolysis and has the subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$ [1–3]. Several residues that may have an essential role in catalysis have been identified on the β subunit from a study of chemical modification [4]. Among them are two glutamic acid residues of the β subunits which are labeled by dicyclohexylcarbodiimide (DCCD). When F_1-ATPase from the thermophilic *Bacillus* PS3 (TF_1) is inactivated with DCCD, Glu^{190} is selectively modified [5]. The inactivation of TF_1 by DCCD is accelerated 7-fold upon binding of ADP to a single adenine nucleotide binding site on one of three β subunits and further addition of Mg^{2+} abolishes the effect of ADP [6]. In contrast to TF_1, when F_1-ATPases from *Escherichia coli* (EF_1), bovine heart mitochondria (MF_1), and spinach chloroplasts (CF_1) are inactivated with DCCD, a different glutamic acid residue of the β subunit which corresponds to the Glu^{201} of the TF_1-β subunit, is specifically modified [7–9]. Consistently, when Glu^{190} or Glu^{201} of the TF_1-β subunit was replaced by Gln by site-directed mutagenesis, the $\alpha_3\beta_3\gamma$ complexes containing these mutant β subunits were ATPase-inactive [10]. However, this conclusion was challenged by the results obtained from the same mutants of EF_1 [11] which showed that βGlu^{190} was important but not absolutely required for catalysis and that βGlu^{201} was not essential for catalysis (numbering of residues throughout this article is according to the sequence of the β subunit of TF_1). Later, further study of mutant β subunits of EF_1 by the same group [12] and by another group [13] revealed the essential role of βGlu^{190} in catalysis.

In the present study, we have tried to obtain more information about the roles of carboxylates at Glu^{190} and Glu^{201} of the TF_1-β subunit using mutagenesis and chemical modification. We have generated mutant TF_1-β subunits; β(E190D), β(E190C), β(E201D), and β(E201C). Since the wild-type TF_1-β subunit does not contain Cys, a sulfhydryl reagent, monoiodoacetic acid, should only react with the mutationally induced Cys residue producing a *S*-carboxymethylcysteine residue (Cax) at position 190 (β(E190Cax)) and 201 (β(E201Cax)) (Fig. 1). The carboxylate of Cax is connected to the peptide main chain with a longer side chain than in the case of Glu, by an additional sulfur atom inserted between the β and γ carbon atom of Glu. As a control β subunit without carboxylate, mutant β subunits modified with *S*-carbamoylmethylcysteine (Cam) were also generated, designated as β(E190Cam) and β(E201Cam). In addition, the mutants β(E190Q) and β(E201Q) were re-examined since the presence of the $\alpha_3\beta_3$ complex had been found [14] after a previous report [10] and there was the possibility that our previous preparation of the $\alpha_3\beta_3\gamma$ complex had been seriously contaminated by $\alpha_3\beta_3$ complex which tended to dissociate into $\alpha_1\beta_1$ hetrodimers during the ATPase assay. These β subunits with changed residues at 190 or 201 were reconstituted into $\alpha_3\beta_3\gamma$ complex, and

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Abbreviations: Cax, *S*-carboxymethylcysteine; Cam, *S*-carbamoylmethylcysteine; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; TNP-ATP, and TNP-ADP, the 2',3'-*O*-(2,4,6-trinitrophenyl) derivatives of ATP and ADP, respectively; TF_1, EF_1, MF_1, and CF_1, F_1-ATPase from the thermophilic *Bacillus* strain PS3, *Escherichia coli*, bovine heart mitochondria, spinach chloroplasts, respectively.

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their ATPase activities and susceptibility to DCCD inactivation were measured. The results indicated that the catalytic site of F_1-ATPase involves an essential carboxylate of Glu^{190} which possibly interacts with a non-essential carboxylate of Glu^{201}. The precision of the spatial volumes accommodating each of the two carboxylates was also evaluated from comparison of the β subunits with Asp, Glu, and Cax residues at positions 190 or 201.

2. Experimental

2.1. Materials

Monoiodoacetic acid and monoiodoacetamide were purchased from Nakarai Tesque (Kyoto). DCCD and 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) were from Sigma. ATP and ADP were from Kyowa Hakko. Rabbit muscle pyruvate kinase, hog muscle lactate dehydrogenase and NADH were from Boehringer-Mannheim GmbH.

The β(E190C), β(E190D), β(E201C) and β(E201D) mutants were generated by oligonucleotide-directed mutagenesis, expressed in *E. coli* strain DK-8 and purified as reported previously [15]. The α subunit was purified as described in [16]. Previous purification procedures of the γ subunit [15] were modified in order to improve the yield. The *E. coli* strain NM522, expressing the γ subunit as inclusion bodies, was suspended in 30 mM Tris-SO_4 (pH 7.5) containing 30 mM NaCl, and disrupted thoroughly by sonication. The suspension was subjected to a centrifugation at 8,000 rpm for 10 min and the pellet was washed with 30 mM Tris-SO_4 (pH 8.0) containing 30 mM NaCl and 1 M sucrose. The washed pellet was suspended with 2% Triton X-100 solution containing 10 mM EDTA and left at 4°C overnight. After centrifugation the pellet was solubilized in 20 mM Tris-SO_4 (pH 8.0) containing 0.5 mM EDTA and 10 M urea, and then the concentration of urea was diluted to 8 M by 20 mM Tris-SO_4 (pH 8.0). The solution was applied to a CM52 (Whatman) column (2.5 × 4.5 cm) equilibrated with 10 mM Tris-Cl (pH 8.0) containing 0.5 mM EDTA and 8 M urea, and eluted with a 0–0.3 M NaCl gradient. The γ subunit eluting at 0.25 M was dialyzed against 20 mM Tris-Cl (pH 8.0) and then against water at 4°C. The γ subunit was precipitated in the dialysis tube. The precipitate was collected, lyophylized, and stored at 4°C.

2.2. Modification of sulfhydryl residues

Purified β(E190C) and β(E201C), each 3 mg, were individually suspended in 1 ml of the dithiothreitol buffer comprising 100 mM Tris-SO_4 (pH 8.0), 1 mM EDTA and 0.29 μmol dithiothreitol (5-fold of protein sulfhydryl groups). The solution was incubated at 30°C for 3 h and was subjected to HPLC in a gel permeation column (TSK-GEL G3000SWXL; Tosoh) equilibrated and eluted with 50 mM Tris-SO_4 (pH 7.0) and 0.2 M Na_2SO_4 at room temperature. The protein fraction was concentrated to about 100 μl by a Centricon 10 microconcentrator (Amicon Corp.) and 30 μl of freshly prepared monoiodoacetic acid or monoiodoacetamide solution (2 mg/ml water) was added. The solution was incubated at 30°C for 1 h in the dark and then the protein was separated from excess reagents by gel filtration centrifugation [6]. The eluted fraction was used for the reconstitution procedure.

2.3. Reconstitution of $\alpha_3\beta_3\gamma$ complex

The previous method for reconstitution of the $\alpha_3\beta_3\gamma$ complex [17] was modified to avoid contamination of the $\alpha_3\beta_3$ complex. Since $\alpha_3\beta_3$ complex is stabilized in Na_2SO_4 and destabilized in NaCl, NaCl was included in the buffer throughout all procedures. The α, β, and γ subunits, each 1 mg, were dissolved in 1 ml of 50 mM Tris-Cl (pH 7.0) containing 0.2 M NaCl, and incubated overnight at 30°C. The mixture was passed through a Sephadex G-50 column (1.5 × 10 cm) equilibrated with 50 mM Tris-Cl (pH 7.0) containing 0.2 M NaCl to remove the insoluble materials which were mostly composed of the γ subunit, and was concentrated with a Centriprep 30 at 25°C. The solution was subjected to a G3000SWXL column equilibrated with 50 mM Tris-Cl (pH 7.0) and 0.2 M NaCl, and fractions containing $\alpha_3\beta_3\gamma$ complex were combined. PAGE in the presence of sodium dodecylsulfate of the purified $\alpha_3\beta_3\gamma$ complexes reconstituted from each of mutant and chemically modified β subunits showed the apparently natural stoichiometric presence of α, β, and γ subunits, indicating that contamination by $\alpha_3\beta_3$ complex in the purified preparation of $\alpha_3\beta_3\gamma$ complex was very minimal, if any. The purified complex was stable for 1 week at 4°C.

2.4. Analytical methods

The ATPase activity was measured at 25°C in the presence of an ATP regenerating system. The assay mixture contained 50 mM Tris-SO_4 (pH 8.0), 5 mM $MgCl_2$, 10 mM KCl, 3 mM phosphoenolpyruvate, 5 mM ATP, 20 μg/ml pyruvate kinase, 30 μg/ml lactate dehydrogenase, and 0.2 mM NADH. In general, ATP hydrolysis catalyzed by TF_1 and the $\alpha_3\beta_3\gamma$ complexes undergoes a non-linear phase (a slow phase and then an accelerated phase) at an initial period of the reaction, before reaching a steady-state phase. Therefore, we waited until the catalysis reached an apparent steady-state phase, usually at 600–800 s after initiating the reaction, and measured the rate of ATP hydrolysis. One unit of activity is defined as the activity producing 1 μmol of ADP per min. Single-site hydrolysis of substoichiometric amount of substrate was measured using 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) as a substrate. Single-site hydrolysis of TNP-ATP by TF_1 is similar to that of ATP but is slow enough to be measured easily [18]. The solution containing 1.0 μM $\alpha_3\beta_3\gamma$ complexes, 20 mM Tricine-K buffer (pH 7.8), and 2 mM $MgCl_2$ was mixed with an equal volume of the solution containing 0.6 μM TNP-ATP, 20 mM Tricine-K buffer (pH 7.8), and 2 mM $MgCl_2$, and the mixture was incubated at 25°C. At indicated times, an aliquot (100 μl) of the reaction mixture was taken out and 5 μl of 24% perchloric acid were added. After centrifugation, the amount of TNP-ATP and TNP-ADP was measured with reverse-phase HPLC [18]. Protein concentrations were determined by the method of Bradford [19]. ADP binding to the isolated β subunit was detected by difference circular dichroism (CD) spectra generated by the addition of 250 μM ADP into 20 mM Tricine-Na buffer (pH 8.0) containing 2 mM $MgCl_2$ and 12 μM β subunit [20]. The quantitative analysis of sulfhydryl groups using DTNB in the presence or absence of 6 M guanidine-HCl was carried out by the method of Glazer [21].

3. Results

3.1. Properties of the isolated mutant β subunits

Since all of the six mutant β subunits used in this study (Cys, Asp, or Gln at position 190 or 201) could be reconstituted into $\alpha_3\beta_3\gamma$ complexes and their CD spectra at 220–300 nm were very similar to that of the wild-type β subunit (data not shown), the gross structures of the mutant β subunits might not be changed significantly from that of the wild-type β subunit. When each of six mutant β subunits was mixed with ADP, a typical difference CD spectrum which indicated binding of ADP was generated (data not shown). Therefore, residues at 190 and 201 of the β subunit may not directly participate in binding of adenine nucleotide and our previous observation that isolated β(E201Q) did not bind ATP should be corrected.

3.2. Modification of Cys of β(E190C) and β(E201C) by sulfhydryl reagents

Numbers of free sulfhydryl groups of β(E190C) and β(E201C) were measured by DTNB titration. Values around 1.0 were obtained for these mutants and these values were not changed by denaturation in 6 M guanidine-HCl, indicating both residues are easily accessible from the water phase (Table 1). This single sulfhydryl group of β(E190C) and β(E201C) disappeared after

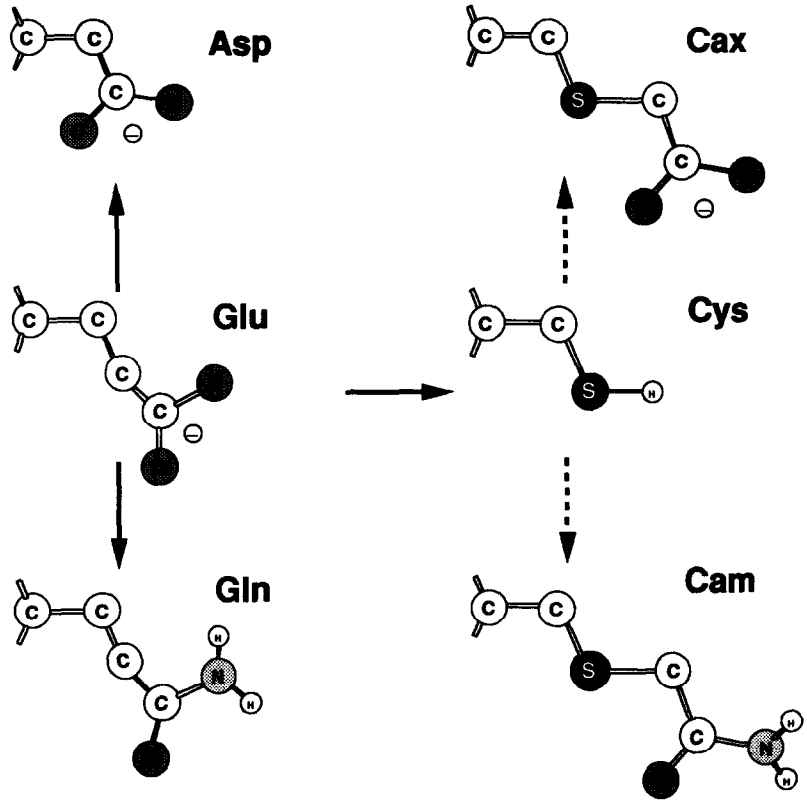

Fig. 1. Mutation and chemical modification of Glu^{190} and Glu^{201} of the TF_1-β subunit. Solid and dashed arrows indicate mutagenesis and chemical modification, respectively. Hydrogen atoms bonded to carbon atoms are not shown.

treatment with monoiodoacetic acid or monoiodoacetamide, ensuring that the modification by these reagents proceeded to completion. Four kinds of chemically modified β subunits, β(E190Cax), β(E201Cax), β(E190Cam), and β(E201Cam) (Fig. 1), used for the following experiments were prepared by modification in the absence of guanidine-HCl. As to the wild-type β subunit in which Cys is absent, DTNB titration always gave a low background value. DTNB reacted with the α subunit only after denaturation since it has a single Cys which is buried inside the protein [22].

3.3. *Effect of replacement of $bGlu^{190}$ by different carboxylates and amides on ATPase activities*

Some small steady-state ATPase activity (7%) was retained by the $\alpha_3\beta_3\gamma$ complex containing β(E190D) (Fig. 2A). In contrast, replacement of βGlu^{190} by Cys or Gln resulted in almost complete loss of the steady-state ATPase activities of $\alpha_3\beta_3\gamma$ complex. These complexes even lost the ability to catalyze single-site hydrolysis of TNP-ATP, indicating a direct role for βGlu^{190} in catalysis (Fig. 3). When carboxylate was recovered at position 190 of β(E190C) by monoiodoacetic acid modification, the re-

Table 1
Number of free SH groups in isolated α and β subunits from DTNB titration

	Native (mol/mol)	Denatured[a] (mol/mol)	IAA-modified[b] subunit (mol/mol)	IAM-modified[b] subunit (mol/mol)
α	0.04	1.28	n.d.	n.d.
β(wild)	0.15	0.20	0.22	0.22
β(E190C)	1.07	1.16	0.19	0.02
β(E201C)	0.99	1.14	0.24	0.03

IAA, monoiodoacetic acid; IAM, monoiodoacetiamide; n.d. not determined.
[a] In the presence of 6 M guanidine-HCl.
[b] Subunits were modified by IAA or IAM in the absence of guanidine-HCl, and titration with DTNB was carried out in the presence of 6 M guanidine-HCl.

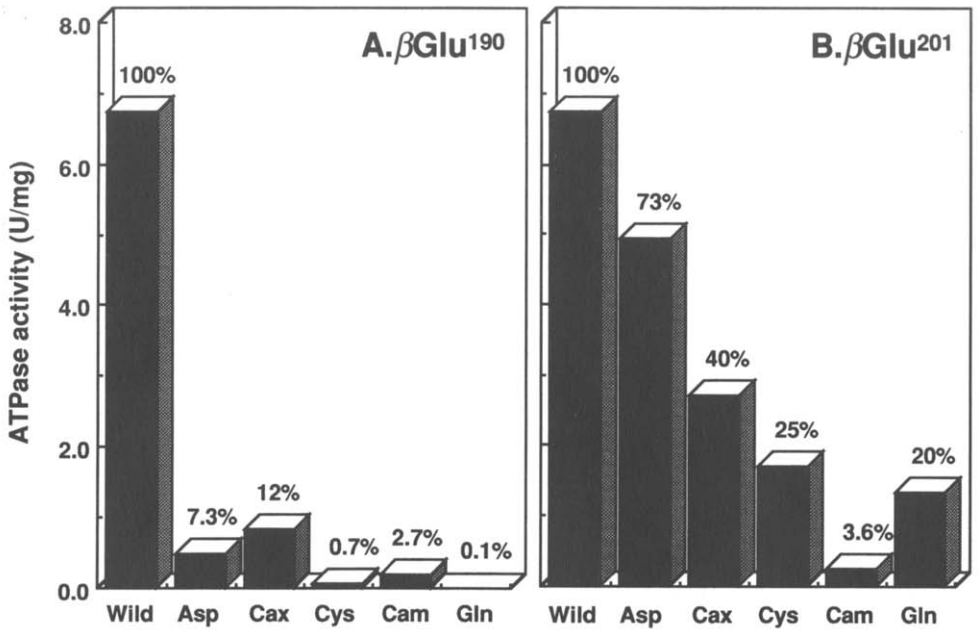

Fig. 2. Effect of replacement of (A) βGlu^{190} and (B) βGlu^{201} by other residues on steady-state ATPase activity of the reconstituted $\alpha_3\beta_3\gamma$ complexes. Detailed experimental conditions are described in section 2.

sultant β(E190Cax) was able to reconstitute the complex with significant steady-state ATPase activity (12%) (Fig. 2A). However, when an amide group was attached to β(E190C) by monoiodoacetamide modification, the ATPase activity of the complex containing the resultant β(E190Cam) was very little, which was possibly due to deamidation of the amide group of these β subunits. Besides the magnitude of the ATPase activities, the kinetic behavior of the $\alpha_3\beta_3\gamma$ complexes containing β(E190D) and β(E190Cax), such as the shape of the kinetic curves of activities vs. ATP concentration and the initial non-linear phase of ATP hydrolysis, did not differ drastically from those of the wild-type $\alpha_3\beta_3\gamma$ complex (data not shown). To summarize, carboxylation at position 190 is absolutely required for catalysis and cannot be replaced by amides without loss of activity. Among carboxylate-containing residues with different side chain length, Glu (wild-type) is superior to Cax and Asp, indi-

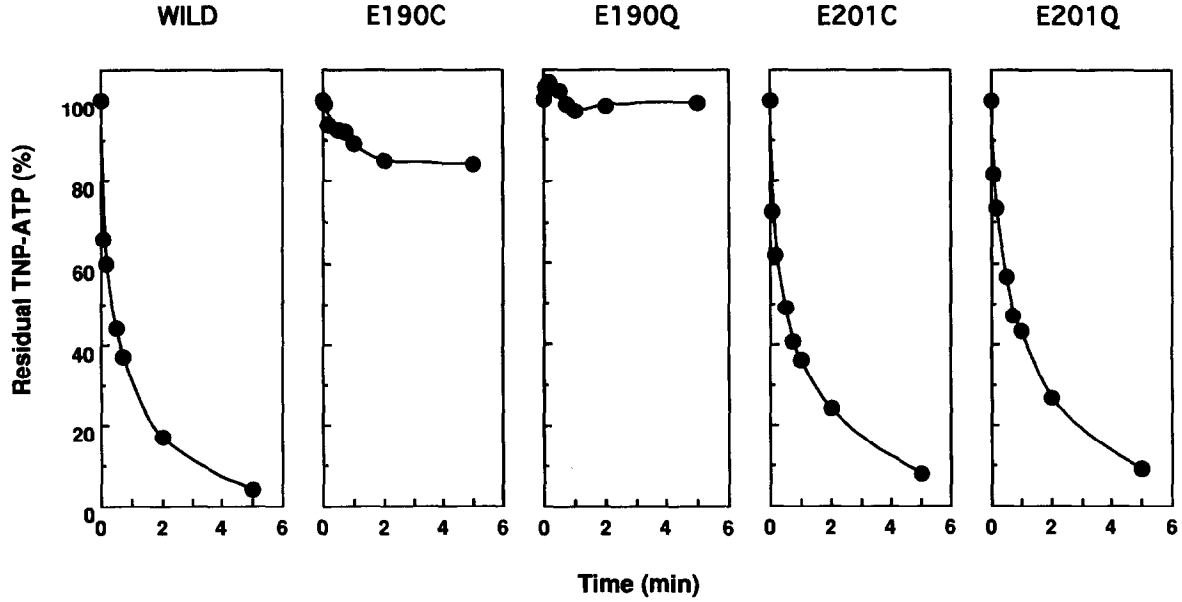

Fig. 3. Single-site hydrolysis of TNP-ATP catalyzed by the $\alpha_3\beta_3\gamma$ complexes containing β(E190C), β(E190C), β(E201C), or β(E201Q). Substoichiometric amount of TNP-ATP (0.3 μM) was incubated with 0.5 μM of the complexes and the amount of residual TNP-ATP was measured. Detailed experimental conditions are described in section 2.

cating that the spatial precision required for the catalytic carboxylate is rather strict. When Cax and Asp are compared, Cax is better than Asp, probably because the precise spatial location of the carboxylate can be more easily attained by a carboxylate with a longer side chain than a carboxylate with a shorter side-chain.

3.4. Effect of replacement of βGlu[201] by different carboxylates and amides on ATPase activities

The $\alpha_3\beta_3\gamma$ complexes reconstituted from any one of the changed β subunits retained their ATPase activity (Fig. 2B). The order of ATPase activity is: β(wild)>β(E201D)>β(E201Cax)>β(E201C)>β(E201Q)>β(E201Cam). The kinetic behavior, including single-site hydrolysis of TNP-ATP (Fig. 3), of the $\alpha_3\beta_3\gamma$ complexes containing β subunits with replaced residues at 201 did not differ drastically from those of the wild-type $\alpha_3\beta_3\gamma$ complex, besides the magnitude of ATPase activities. Therefore, a carboxylate at position 201 of the β subunit is not essential for catalysis. This conclusion agrees with the results obtained for EF_1 but is in apparent contradiction with our previous result [10]. Probably, our previous preparation of $\alpha_3\beta_3\gamma$ complex reconstituted from β(E201Q) had been seriously contaminated by $\alpha_3\beta_3$ complex, the presence of which was noticed later [14]. Since $\alpha_3\beta_3$ complex containing β(E201Q) is inactive in ATP hydrolysis (Amano and Yoshida, unpublished observation), the preparation containing large amounts of $\alpha_3\beta_3$ complex should show only minimal activity.

3.5. DCCD inactivation of $\alpha_3\beta_3\gamma$ complexes containing β subunits changed at 190 or 201

DCCD inactivates the ATPase activity of TF_1 and the $\alpha_3\beta_3\gamma$ complex in the presence of ADP by labeling βGlu[190] [5,17]. As observed for TF_1, all the $\alpha_3\beta_3\gamma$ complexes containing changed β subunits were only slightly inactivated by DCCD when ADP was absent (data not shown). In the presence of ADP, the complexes containing β(E190Cax) were inactivated by DCCD at a comparable rate with the complex containing β(wild) (Fig. 4). In contrast, DCCD inactivation of the complex containing βGlu[201] mutants (β(E201D), β(E201Q), and β(E201Cax)) proceeded at a much slower rate (Fig. 4). Thus, reactivity of carboxylate 190 to DCCD is affected by the residue present at position 201.

4. Discussion

This results described in this report provide some insight into Glu[190]. Consistent with previous observations by us [10] and by others [12,13], the presence of a carboxylate-containing residue at position 190 of the β subunit is an absolute requirement for catalysis. Since a residue at 190 is not involved in AT(D)P binding, as judged from difference CD spectra, this residue most likely plays an essential role in catalysis. Indeed, the $\alpha_3\beta_3\gamma$ complexes containing β(E190C) or β(E190Q) cannot even catalyze single-site catalysis (Fig. 3). The critical role of Glu[190] should be further emphasized since a precise spatial location of the carboxylate is required at position 190 for efficient catalysis; a change of side chain length of the residue resulted in significantly decreased ATPase activity (Fig. 2). Nonetheless, it is worth noting that a change to a longer side chain (Cax) is less harmful to ATPase activity than a change to a shorter one (Asp). The side

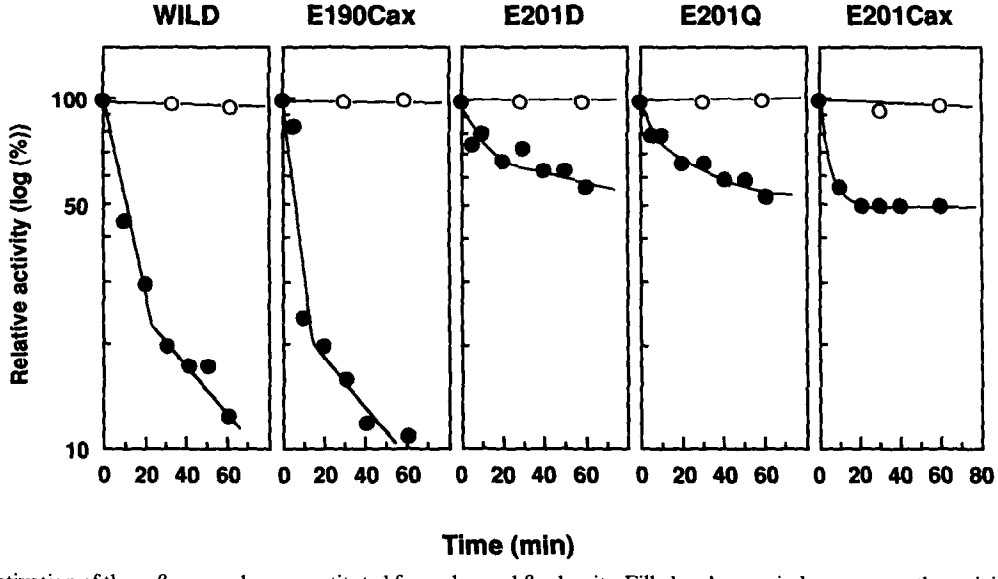

Fig. 4. DCCD inactivation of the $\alpha_3\beta_3\gamma$ complex reconstituted from changed β subunits. Filled and open circles represent the activities of the complexes incubated with or without DCCD, respectively. The reaction mixture (100 μl) contained 50 mM Tris-SO_4 (pH 7.3), 1.0 mM EDTA, 1.0 mM ADP, and 100 μg of each of the $\alpha_3\beta_3\gamma$ complexes [6]. The reaction was initiated by the addition of 1 μl of 15 mM DCCD in ethanol, and the mixtures were incubated at 30°C. Aliquots (5 μl) were taken out from the mixtures at indicated times, and residual ATPase activities were assayed at 25°C.

chain volume of Asp, Glu and Cax residues is approximately 111 Å^3 [23], 138 Å^3 [24], and 159 Å^3 [25], respectively. Probably, the carboxylate of Cax can occupy a spatial position close to that of Glu by bending its side chain: the carboxylate of Asp, however, cannot reach the right place because of a short side chain and hence ATP hydrolysis becomes less efficient.

From the observation that the reactivity of Glu^{190} with DCCD was reduced when Glu^{201} was replaced by Asp, Gln, or Cax (Fig. 4), it is suggested that Glu^{190} interacts with Glu^{201} at the tertiary structure level of the β subunit. Since, at neutral or slightly acidic pH, DCCD is supposed to react with carboxylate, which has an unusually high p*K*a value [4], Glu^{201} possibly interacts with the carboxylate of Glu^{190} so as to keep the p*K*a of Glu^{190} high. Another explanation is that DCCD reacts first with Glu^{201} of TF_1 and then transferred to Glu^{190}. The primary product formed when DCCD reacts with carboxylate is *O*-acylisourea [26]. This reactive species has several possible fates and transfer to a neighboring carboxylate could be one of them. This explanation is attractive since Glu^{201} is indeed labeled by DCCD when EF_1, MF_1, and CF_1 are inactivated by DCCD. For some unknown reason, only in the case of TF_1 is *O*-acylisourea at Glu^{201} transferred to Glu^{190} and, simultaneously or successively, rearrangement to a stable *N*-acylurea derivative might occur. The unusual dependency of DCCD inactivation of TF_1 on ADP can be understood in this context; the transfer could occur only when ADP occupies the adenine nucleotide binding site of the β subunit. The nearby location of Glu^{190} and Glu^{201} is also consistent with the result of limited proteolysis of the β subunit which indicates that these two residues are in the same loop region in the tertiary structure of the β subunit [15].

References

[1] Futai, M., Noumi, T. and Maeda, M. (1989) Annu. Rev. Biochem. 58, 111–136.

[2] Penefsky, H.S. and Cross, R.L. (1991) Adv. Enzymol. Relat. Areas Mol. Biol. 64, 173–214.

[3] Boyer, P.D. (1993) Biochim. Biophys. Acta 1140, 215–250.

[4] Allison, W.S., Bullough, D.A. and Andrews, W.W. (1986) Methods in Enzymol. 126, 741–761.

[5] Yoshida, M., Poser, J.W., Allison, W.S. and Esch, F.S. (1981) J. Biol. Chem. 256, 148–153.

[6] Yoshida, M. and Allison, W.S. (1983) J. Biol. Chem. 258, 14407–14412.

[7] Esch, F.S., Bohlen, P., Otsuka, A.S., Yoshida, M. and Allison, W.S. (1981) J. Biol. Chem. 256, 9084–9089.

[8] Yoshida, M., Allison, W.S., Esch, F.S. and Futai, M. (1982) J. Biol. Chem. 257, 10033–10037.

[9] Sakurai, H., Iwashima, H., Hosoda, T. and Hisabori, T. (1989) Seikagaku (in Japanese), 61, 1098.

[10] Ohtsubo, M., Yoshida, M., Ohta, S., Kagawa, Y., Yohda, M. and Date, T. (1987) Biochem. Biophys. Res. Commun. 146, 705–710.

[11] Parsonage, K., Wilke-Mounts, S. and Senior, A.E. (1988) Arch. Biochem. Biophys. 261, 222–225.

[12] Senior, A.E. and Al-Shawi, M.K. (1992) J. Biol. Chem. 267, 21471–21478.

[13] Futai, M., Park, M.-Y., Iwamoto, A., Omote, H. and Maeda, M. (1994) Biochim. Biophys. Acta in press.

[14] Miwa, K. and Yoshida, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6484–6487.

[15] Tozawa, K., Odaka, M., Date, T. and Yoshida, M. (1992) J. Biol. Chem. 267, 16484–16490.

[16] Tozawa, K., Miyauchi, M. and Yoshida, M. (1993) J. Biol. Chem. 268, 19044–19054.

[17] Yokoyama, K., Hisabori, T. and Yoshida, M. (1989) J. Biol. Chem. 264, 21837–21841.

[18] Hisabori, T., Muneyuki, E., Odaka, M., Yokoyama, K., Mochizuki, K. and Yoshida, M. (1992) J. Biol. Chem. 267, 4551–4556.

[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.

[20] Ohta, S., Tsuboi, M., Oshima, T., Yoshida, M. and Kagawa, Y. (1980) J. Biochem 87, 1609–1617.

[21] Glazer, A.N., Delange, R.J. and Sigman, D.S. (1975) in Chemical Modification of Proteins, American Elsevier, North-Holand, pp. 21–25.

[22] Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1979) J. Biol. Chem. 254, 9525–9533.

[23] Eggleston, D.S., Valente, E.J. and Hodgston, D.J. (1981) Acta Crystallogr. B37, 1428–1430.

[24] Eggleston, D.S., Valente, E.J. and Hodgston, D.J. (1981) Acta Crystallogr. B37, 1430–1433.

[25] Mighell, A.D., Hubbard, C.R., Harris, J., Staffa, J.A. and Zervos, C. (1979) Acta Crystallogr. B35, 1258–1261.

[26] Kurzer, K. and Dowaghi-Zadeh, K. (1967) Chem. Rev. 67, 107–151.