Volume 336, number 2, 231–235 FEBS 13416 December 1993

In vivo affinity label of a protein expressed in *Escherichia coli*

Coenzyme A occupied the AT(D)P binding site of the mutant F_1-ATPase β subunit (Y307C) through a disulfide bond

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Received 13 October 1993; revised version received 8 November 1993

When Tyr-307 of the β subunit of F_1-ATPase from a thermophilic *Bacillus* strain PS3 is replaced by cysteine and expressed in *Escherichia coli* cells, about a half population of the mutant β subunit are labeled by Coenzyme A at Cys-307 through a disulfide bond which is cleavable by reducing treatment. The mutant β subunit can be reconstituted into the $\alpha_3\beta_3$ complex of which ATPase activity is stimulated two-fold by reducing treatment either prior or after reconstitution. Since Tyr-307 has been supposed to be located at one of subdomains which form the ATP binding site of the β subunit, Coenzyme A binds to the mutant β subunit as an AT(D)P analogue in *E. coli* cells and then covalently attaches to Cys-307.

F_1-ATPase; Affinity label; CoA; ATP analogue

1. INTRODUCTION

F_1-ATPase, a peripheral sector of H^+-ATP synthase, has a subunit structure of $\alpha_3\beta_3\gamma\delta\varepsilon$ [1–4]. The isolated α and β subunit can bind AT(D)P individually but both have only trace amount of ATPase activity [5–8]. Reconstitutions of F_1-ATPase and subunit complexes with various subunit composition from isolated subunits have been demonstrated for enzymes from a thermophilic *Bacillus* strain PS3 (TF_1) [9,10] and *Escherichia coli* [5,11]. The ATPase-active complex with minimum subunit composition so far demonstrated by reconstitution is the $\alpha_3\beta_3$ complex [10,12]. The genes of the α and the β subunit of TF_1 were cloned and overexpression system in *E. coli* of each subunit was established [13,14].

Tyr-307 of the β subunit of the TF_1 is conserved in the primary sequences of the β subunits of all known F_1-ATPases. As Ferguson et al. demonstrated for the F_1-ATPase from bovine heart mitochondria (MF_1) [15], ATPase activity of TF_1 and the $\alpha_3\beta_3$ complex is inactivated when Tyr-307 of a single β subunit out of the three copies of the β subunits in the molecule is modified by 7-chloro-4-nitrobenzofurazan (Nbf-Cl) [16,17]. When the modified enzyme is incubated with sulfhydryl reducing reagents such as 2-mercaptoethanol, the bound nitrobenzofurazan (Nbf) moiety is removed regenerating the phenolic group of tyrosine and ATPase activity is recovered. Under alkaline condition, an O → N migration takes place in which the Nbf moiety is transferred directly from Tyr-307 to Lys-154 [16]. Since Lys-154 is located in the so-called 'P-loop' sequence that is assumed to be a part of binding domain for the phosphoanhydride part of ATP [18], Tyr-307 could be also located in or near the ATP binding sites. However, Tyr-307 is not directly involved in catalysis. This conclusion has been deduced from the experiments using mutant β subunits whose Tyr-307 (or the equivalent tyrosine of *E. coli* $F_1\beta$) is replaced by other amino acids [19,20].

On the process of further characterization of the mutant β subunit whose Tyr-307 was replaced by cysteine (β(Y307C)), we found that incubation of the $\alpha_3\beta$-$(Y307C)_3$ complex with dithiothreitol (DTT) resulted in permanent activation of the ATPase activity. Because the wild-type β subunit of TF_1 does not contain cysteine and the α subunit has a single cysteine which is non-reactive to sulfhydryl reagent without denaturation [21], the newly introduced cysteine at 307 of the β(Y307C) should be responsible for this activation. The pursuit of the reason of this activation ended up with the finding that Cys-307 of the β(Y307C) is covalently labeled by Coenzyme A (CoA) through disulfide bond. This is an unusual 'in vivo affinity label' of a recombinant protein expressed in *E. coli*.

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Abbreviations: AMEDA, P^1-(5′-adenosyl)-P^2N-(2-mercaptoethyl) diphosphoramidate; CD, circular dichroism; CoA, Coenzyme A; DTT, dithiothreitol; the β(Y307C), the mutant β subunit whose tyrosine 307 is replaced by cysteine; the $\beta(Y307C)^r$, the β(Y307C) treated with a reducing sulfhydryl reagent; Nbf-Cl, 7-chloro-4-nitrobenzofurazan; MF_1, F_1-ATPase from mitochondria; TF_1, F_1-ATPase from a thermophilic *Bacillus* PS3; PyDHase, pyruvate dehydrogenase; HPLC, high performance liquid chromatography; SDS, sodium dodecylsulfate.

2. MATERIALS AND METHODS

2.1. *Materials*

All adenine nucleotides and *Lactobacillus delbrueckii* pyruvate dehydrogenase (PyDHase) were purchased from Sigma. CoA was further purified with HPLC on a reverse phase column SUPERCOSIL LC-18T. The concentration of CoA was determined using the absorption coefficient, $\varepsilon_{259.5} = 16{,}800\ M^{-1} \cdot cm^{-1}$. Calf intestine alkaline phosphatase was obtained from Boehringer. TF_1 was prepared as described previously [22]. The α, β(WILD), and β(Y307C) subunits of TF_1 were individually expressed in *E. coli* and purified as described previously [14,20]. It should be noted that during purification procedures proteins did not contact with reducing sulfhydryl reagents. The purified preparations were stored as a suspension in 75% saturated ammonium sulfate at 4°C. The $\alpha_3\beta_3$ complex was reconstituted from isolated subunits and purified with HPLC on a Tosoh G3000SWXL column as described [10,17].

2.2. *Reduction of the β(Y307C) with sulfhydryl reagents*

The β(Y307C) stored as ammonium sulfate suspension was precipitated and dissolved in a minimum volume of 50 mM triethanolamine-sulfate (pH 7.5). DTT was added at final concentration 2 mM and the mixture was incubated at room temperature for 30 min. After incubation, solid ammonium sulfate was added to the solution, and the proteins were precipitated by centrifugation. The reduced β(Y307C) subunit ($\beta(Y307C)^r$) was stored as a precipitate at 4°C and the supernatant fraction was stored at −20°C for analysis. The $\alpha_3\beta(Y307C)^r_3$ complex was reconstituted from the α subunit and the $\beta(Y307C)^r$ subunit.

2.3. *Difference UV spectra*

The UV spectra were measured with *Ubest-30* spectrophotometer (JASCO, Tokyo). The β(Y307C) was dissolved in 100 mM Tris-sulfate (pH 8.0). DTT was added at a final concentration of 2 mM to a half of the β(Y307C) solution and an equal volume of water was added to the other half of the solution. Both solutions were incubated for 1 h at room temperature and perchloric acid was added at a final concentration of 2% at 0°C. The denatured proteins in both solutions were precipitated by centrifugation and were dissolved in equal volume of 100 mM Tris-sulfate (pH 8.0) containing 1% sodium dodecylsulfate. The spectrum of each solution was measured and the difference was calculated using a computer.

2.4. *Circular dichroism spectra*

Circular dichroism (CD) spectra were measured at 25°C with a J-500 CD spectrophotometer (JASCO, Tokyo) equipped with a computerized data processor using a quartz cuvette with a 2-mm light path. Eight scans from 300 to 250 nm were averaged. The sample solution contained 40 μM of the β subunit, 20 mM Tricine-NaOH (pH 8.0), 10 μM cyclohexyldiaminetetraacetic acid and, when indicated, 250 μM ADP. The difference CD spectrum of the β(Y307C) before and after reduction was obtained with a data processor. Difference CD spectra induced by binding of ADP to the β subunit were obtained by subtracting the CD spectrum of the protein solution and that of ADP solution from the CD spectrum of the protein plus ADP solution.

2.5. *HPLC analysis of the unknown compound*

The unknown compound released from the β(Y307C) by reducing treatment as described in 2.2 was analyzed with three HPLC systems; reverse phase HPLC and ion pair HPLC on a SUPERCOSIL LC-18-T column (3 μm, 4.6 mm × 250 mm), and anion exchange HPLC on a Tosoh DEAE-2SW column (10 μm, 4.6 mm × 250 mm). The elutions were monitored with absorbance at 260 nm. The eluting conditions of HPLC were as follows. Reverse phase HPLC: solvent A, 100 mM potassium phosphate (pH 6.0); solvent B, 70% of solvent A + 30% of methanol. The column was equilibrated with 100% solvent A and content of solvent B was increased as; 0 to 9 min, 0%; 9 to 15min, 0 to 25%; 15 to 17.5 min, 25 to 90%; 17.5 to 25 min, 90%. The flow rate was 1.3 ml/min. Ion pair HPLC: solvent A, 100 mM potassium phosphate (pH 6.0) containing 4 mM tetrabutyl ammonium hydrogen sulfate; solvent B, 70% of solvent A + 30% of methanol. The column was equilibrated with 100% solvent A and content of solvent B was increased as follows: 0 to 2.5 min, 0%; 2.5 to 5 min, 0 to 30%; 5 to 10 min, 30 to 60%; 10 to 13 min, 60%; 13 to 20 min, 100%. The flow rate was 1.5 ml/min. Ion exchange HPLC: the column was equilibrated and eluted isocratically with 350 mM sodium phosphate (pH 6.0) at 0.8 ml/min. The dephospholylation of CoA and the unknown compound was performed using calf intestine alkaline phosphatase in 50 mM Tris-HCl (pH 9.0) containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine at 30°C for 30 min.

2.6. *Assay of CoA by pyruvate dehydrogenase*

The unknown compound was assayed by pyruvate dehydrogenase (PyDHase) at 25°C in 50 mM triethanolamine sulfate (pH 7.6) containing 5 mM sodium pyruvate, 1 mM $MgSO_4$, 0.5 mM NAD^+, and 0.5 mM thiamine pyrophosphate [23]. The reaction was started by the addition of PyDHase, and the reaction was monitored by the increase of the absorbance at 340 nm.

2.7. *Other procedures*

ATP hydrolysis was assayed at 25°C in 50 mM Tris-sulfate (pH 8.0) containing 5 mM $MgCl_2$, 5 mM phospho*enol*pyruvate, 10 mM KCl, 200 mM Na_2SO_4, 10 μg/ml pyruvate kinase, 10 μg/ml lactate dehydrogenase, 0.2 mM NADH, and 2 mM ATP. The rate of ATP hydrolysis was measured as the oxidation rate of NADH, which was monitored by the increase of the absorbance at 340 nm. Since ATP hydrolysis catalyzed by the $\alpha_3\beta_3$ complex, in general, is non-linear in terms of time and ATP concentrations [20], the rates of ATP hydrolysis at 2 mM ATP at 6 min after initiation of the reaction were taken as ATPase activity. The activity necessary to hydrolyze 1 μmol of ATP per min was defined as 1 unit. Protein concentrations were measured using Coomassie brilliant blue dye reagents [24].

3. RESULTS

3.1. *Activation of the $\alpha_3\beta(Y307C)_3$ complex by reducing treatment*

It is interesting that the $\alpha_3\beta(Y307C)_3$ complex has a higher ATPase activity than the $\alpha_3\beta(WILD)_3$ complex. When the $\alpha_3\beta(Y307C)_3$ complex was preincubated with DTT, ATPase activity was activated more than two fold (Table I, line B). The preincubation with 2-mercaptoethanol also resulted in similar activation of the $\alpha_3\beta(Y307C)_3$ complex (data not shown). Such an activation was observed only for the complex containing the β(Y307C) subunit and the same procedures did not cause activation of the complexes containing β(WILD) (Table I, line A) or other mutant β subunits such as β(Y341C) and β(Y364C) (data not shown). Activation of the $\alpha_3\beta(Y307C)_3$ complex was irreversible since, once ATPase activity was activated, it did not return to the original activity after reducing reagent was removed by a gel filtration procedure. Then, the isolated β(Y307C) subunit was treated with DTT ($\beta(Y307C)^r$) at first and the $\alpha_3\beta_3$ complex was reconstituted using the $\beta(Y307C)^r$. The thus obtained $\alpha_3\beta(Y307C)^r_3$ complex showed activated ATPase activity and further activation was no longer observed by preincubation of this complex with DTT (Table I, line C). Since the β(WILD) subunit contains no cysteine, sulfhydryl reducing rea-

Table I

ATPase activities of the complexes preincubated with or without DTT

	Complex	Activity (unit/mg)	
		-DTT	+DTT
A	$\alpha_3\beta$(WILD)$_3$	0.71	0.68
B	$\alpha_3\beta$(Y307C)$_3$	1.2	2.7
C	$\alpha_3\beta$(Y307C)$_3^r$	2.5	2.6

The ammonium sulfate suspension of the complexes were precipitated, dissolved in a minimum volume of 50 mM triethanolamine-sulfate (pH 7.5), preincubated in the absence or presence of 2 mM DTT for 30 min at room temperature, and the ATPase activity of each complex was measured. The $\alpha_3\beta$(Y307C)$_3^r$ complex was reconstituted from the α subunit and the β(Y307C)^r. Other experimental details were described in Section 2.

gents should react with the newly introduced cysteine at 307 of the β(Y307C) subunit.

3.2. *The unknown compound with adenine nucleotide-like spectrum is bound at Cys-307*

We noticed that the UV spectra of the β(Y307C) and the β(Y307C)^r differed significantly at the region of 260 nm and this difference was observed even after the proteins were denatured (Fig. 1). The shape of difference UV absorbance spectrum has a peak at around 260 nm and is very similar to the spectrum of adenine nucleotide. When the β(Y307C) was treated with DTT, precipitated by perchloric acid, and UV absorbance of the supernatant fraction was measured, the spectrum was very similar to the difference spectrum (Fig. 1, *inset*). These results indicate that some unknown compound whose spectrum is very similar to adenine nucleotide is bound to Cys-307 through a disulfide bond.

3.3. *The unknown compound was identified as CoA*

Since no adenine nucleotide was added throughout all the purification procedures, the unknown compound must have originated from the host *E. coli* cells. We compared the unknown compound with the several typical adenine nucleotides which are abundantly present in *E. coli* cells using three types of HPLC analysis; reverse phase, ion pair, and anion exchange. The compound which was released from the β(Y307C) by reducing treatment was always eluted as a single peak at exactly the same retention time as the authentic CoA (Fig. 2A,B). When the unknown compound and CoA were treated with calf intestine alkaline phosphatase, the retention times of both compounds were shifted to 21.2 min (Fig. 2C,D). In addition, when they were degraded in alkaline conditions (50 mM KOH, 90°C, 30 min), elution profiles of the unknown compound and the authentic CoA were changed in the same way (data not shown). When analyzed with ion pair HPLC and with anion exchange HPLC as described in Section 2.5, the unknown compound was eluted as a single peak at

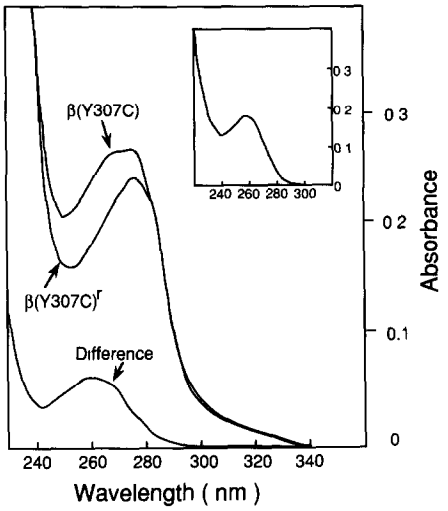

Fig. 1. The UV absorbance spectra of the β(Y307C), the β(Y307C)^r, and their difference spectrum. The proteins were denatured and precipitated by perchloric acid in the absence or the presence of DTT. The precipitated proteins were dissolved in the buffer containing SDS and UV spectra were measured. (Inset) The UV absorbance spectrum of the material released from the β(Y307C) by DTT-treatment. Detailed experimental conditions were described in Section 2.

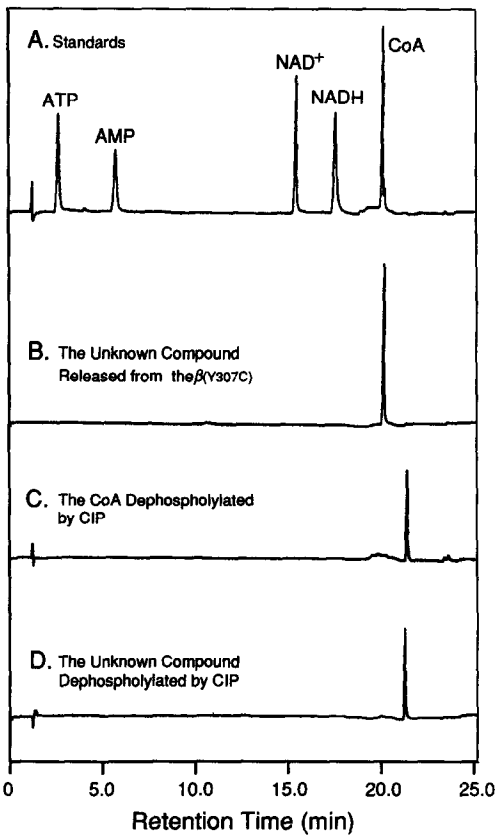

Fig. 2. Reverse phase HPLC analysis of the unknown compound released from the β(Y307C) by DTT-treatment. (A) 1 nmol of each of adenine nucleotides; (B) the unknown compound released from the β(Y307C); (C) 0.4 nmol of the CoA dephospholylated by calf intestine phosphatase (CIP); (D) the unknown compound dephospholylated by CIP. Detailed experimental conditions were described in Section 2.

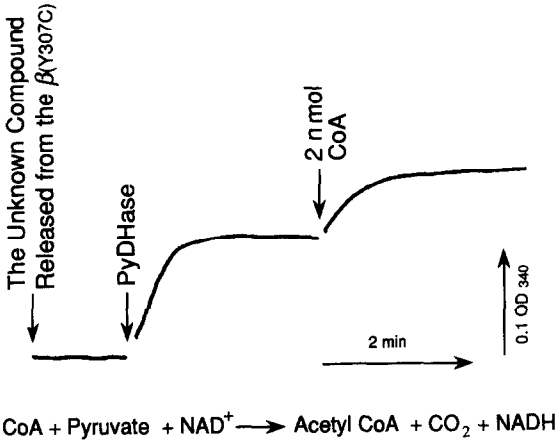

Fig. 3. The reaction catalyzed by PyDHase using the unknown compound as a substrate. The reaction was monitored with the absorbance at 340 nm. The reaction was started by the addition of 1.8 μl of 6.3 unit/ml PyDHase to the solution (200 μl) containing the unknown compound released from 7 nmol of the β(Y307C). After all the substrate in the solution was consumed, 2 n mol of the authentic CoA was added. Detailed experimental conditions were described in Section 2.

18.0 min and 10.4 min, respectively, which were the same retention times of authentic CoA (data not shown). Finally, as shown in Fig. 3, the unknown compound can be a substrate of PyDHase which catalyzes an acetylation of CoA [23]. From the results above described, we concluded that the unknown compound that occupied the adenine nucleotide binding site of the β(Y307C) was CoA.

3.4. *CoA has occupied the AT(D)P binding site of the β(Y307C)*

In order to know if CoA occupies the normal AT(D)P binding site or the some other new site of the β subunit, CD spectrum was examined since it has been established that the binding of AT(D)P to the isolated β subunit of TF_1 induces characteristic change of CD spectrum [6]. The CD spectrum of the β(Y307C) was obviously different from that of the β(WILD) (Fig. 4A,D) whereas the CD spectrum of the $\beta(Y307C)^r$ was essentially the same as that of the β(WILD) (Fig. 4B,D). Difference of the CD spectrum between the β(Y307C) and the $\beta(Y307C)^r$ should be due to the bound CoA of the $\beta(Y307C)^r$. When the spectrum of the $\beta(Y307C)^r$ is subtracted from that of the β(Y307C) (Fig. 4C), the resultant difference CD spectrum is very similar to the difference CD spectrum induced by binding of ADP to the β(WILD) (Fig. 4E). This means that similar change of CD spectra are induced by binding of CoA to the β(Y307C) and by binding of ADP to the β(WILD). Furthermore, the difference CD spectrum induced by the binding of ADP to the $\beta(Y307C)^r$ (Fig. 4F) is similar to that induced by binding of ADP to the β(WILD) (Fig. 4E), suggesting that the $\beta(Y307C)^r$ can bind ADP in a similar manner to the binding of ADP to the β(WILD). Resemblance of the spectra Fig. 4C,E,F indicates that CoA has occupied the normal ADP binding site of the β(Y307C). When ATP was used in stead of ADP, the same results were obtained (data not shown). Thus, CoA has occupied the AT(D)P binding site of the β(Y307C) as an AT(D)P analogue.

3.5. *About a half population of the β(Y307C) has a bound CoA*

Occupation of the AT(D)P binding site of the β(Y307C) by CoA is not complete. The magnitude of the UV absorbance spectra shown in Fig. 1 and the *inset* indicates that one mol of the β(Y307C) contains approximately 0.4 mol of CoA. Similarly, it is calculated from the peak area of three HPLC analyses that one mol of the β(Y307C) released about 0.5 mol of CoA (Fig. 2). Estimation of the amount of CoA from the PyDHase assay also gives a similar value; 7 nmol of the β(Y307C) released about 3 nmol of CoA (Fig. 3). Thus, about a half population of the β(Y307C) molecules have a bound CoA. If so, the rest half population of the β(Y307C) should have an empty AT(D)P binding site. Indeed, a difference CD spectrum induced by the binding of ADP to the β(Y307C) and the β(WILD) showed almost the same shape but the magnitude of the former was roughly half of the latter (data not shown). On the contrary, the $\beta(Y307C)^r$ showed an ADP-induced difference CD spectrum which was very similar in magnitude and shape to that of the β(WILD) (Fig. 4E,F). Five preparations of the β(Y307C) from different cultures of *E. coli* gave the similar stoichiometry of CoA/β(Y307C) and the reason for this incomplete label is not known.

3.6. *The effect of free CoA on the $\alpha_3\beta(Y307C)^r_3$ complex*

Free CoA itself is a weak inhibitor of ATPase activity of the $\alpha_3\beta(WILD)_3$ and $\alpha 3\beta(Y307C)^r_3$ complexes. Only

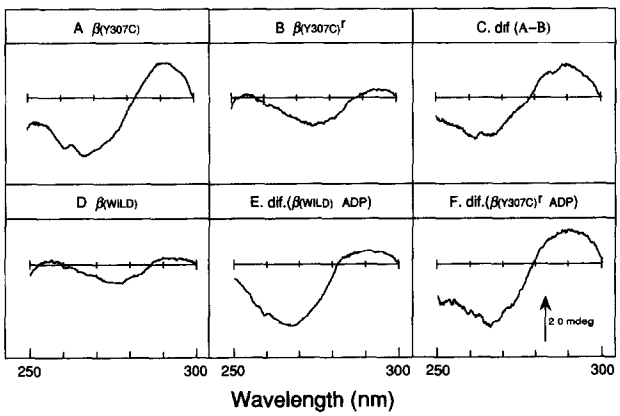

Fig. 4. Circular dichroism spectra of the β(Y307C). The protein concentrations were 40 μM. (A) CD spectrum of the β(Y307C); (B) CD spectrum of the $\beta(Y307C)^r$; (C) the difference CD spectrum between the β(Y307C) and the $\beta(Y307C)^r$; (D) CD spectrum of the β(WILD); (E) the ADP-induced difference CD spectrum of the β(WILD); (F) the ADP-induced difference CD spectrum of the $\beta(Y307C)^r$. Detailed experimental conditions were described in Section 2.

15% inhibition of ATPase activity was observed when 1 mM CoA was included in the reaction mixture. Consistently, the binding affinity of the β(Y307C)^r to CoA should be weak since 0.25 mM CoA did not induce the difference CD spectra of the β(WILD) and the β(Y307C) (data not shown). Covalent label by CoA was not observed even after incubation of the β(Y307C)^r with 1 mM CoA at 25°C for 10 h with gentle stirring. The formation of the disulfide bond between CoA and Cys-307 should be accelerated in *E. coli* cells by some reason, for example, possible involvement of peptidyl disulfide isomerase, possible existence of an incomplete folding species or an assembled form of the β(Y307C) which has a higher affinity to CoA, etc.

4. DISCUSSION

The message of this report is that, when the β(Y307C) of a thermophilic *Bacillus* PS3 is expressed in *E. coli* as a recombinant protein, chemical labeling by CoA through a disulfide bond at the newly introduced Cys-307 occurred to about half the population of the β(Y307C). This labeling occurs in vivo, that is, in the cytoplasm of *E. coli* cells. Although this kind of in vivo affinity label should be very rare, the example given in this report is a warning that one should keep in mind that it could happen when a cysteine residue(s) is mutationally manipulated. Taniyama et al. reported that a recombinant mutant human lysozyme, in which Cys-77 was replaced by Ala, was secreted by yeast with a glutathione bound at Cys-95 [25]. Similar to the β(Y307C), the labeling of this mutant lysozyme with glutathione was not complete. The molar ratio of glutathione/lysozyme was about 0.3.

The labeling by CoA of the β(Y307C) appears to have only little effect on the ability to reconstitute the $\alpha_3\beta_3$ complex with the α subunit. Probably the $\alpha_3\beta$(Y307C)$_3$ complex was the mixture of the $\alpha_3\beta_3$ complexes containing 1, 2, or 3 copies of the β(Y307C·CoA). Although quantitative estimation of the ATPase activity of each specie of the complexes is not possible, the fact that the ATPase activity of the $\alpha_3\beta$(Y307C)$_3$ complex is about 50% of that of the $\alpha_3\beta$(Y307C)$^r_3$ complex (Table I) suggests that the complex containing the β(Y307C · CoA) may be less active (or not at all active) than the complex without CoA.

Difference CD spectra indicate that CoA occupies the normal AT(D)P binding site of the isolated β(Y307C). CoA has a long pantothenic acid-mercaptoethylamine 'tail' from 3′-phospho ADP moiety. Therefore, the binding of the CoA 'tail' to Cys-307 does not necessarily mean that the adenosine moiety of CoA binds to the site at 307. In this respect, the report by Wu et al. is indicative [26]. They reported that when MF_1, previously inactivated by Nbf-Cl, was incubated with an ATP analogue, P^1-(5′-adenosyl)-P^2N-(2-mercaptoethyl) diphosphoramidate (AMEDA), the sulfhydryl group of AMEDA reacted with Nbf moiety of MF_1 to form the AMEDA-Nbf conjugate which then dissociated from MF_1 and, as a consequence, MF_1 was reactivated. Since the MF_1, whose β-Tyr-311 (corresponding to β-Tyr-307 of TF_1) was chemically labeled by the Nbf moiety, can still interact with AMEDA and reactivation of Nbf-MF_1 by AMEDA was inhibited by ADP or ATP, a speculation is possible that the adenosine moiety of ATP is fixed by different residues from β-Tyr-311 but the phosphate group of the ATP is extending to reach the Tyr-311 site. In the case of the $TF_1\beta$ subunit, we are currently obtaining the results indicating that ATP binds to the β subunit with its adenosine moiety at Tyr-341 and its phosphate group at Tyr-307.

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