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# *N*-Ethylmaleimide-sensitive mutant ($\beta$Val-153→Cys) *Escherichia coli* F1-ATPase: cross-linking of the mutant $\beta$ subunit with the $\alpha$ subunit

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**Abstract** A $\beta$ subunit mutation, $\beta$Val-153→Cys, in the glycine-rich sequence (phosphate-binding loop) of *Escherichia coli* $F_1$ was constructed. Like vacuolar-type ATPase, the mutant enzyme was inhibited by *N*-ethylmaleimide (NEM) and labeled with [$^{14}$C]NEM. The inhibition and labeling were prevented by ATP. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) (3 $\mu$M) almost completely inhibited the mutant enzyme, and cross-linked one pair of $\alpha$ and $\beta$ subunits. These results suggest that the interaction of the domain near $\beta$Val-153 with the $\alpha$ subunit is essential for catalytic cooperativity of the enzyme and that $\beta$Val-153 is within 10 Å of the $\alpha$ subunit.

*Key words:* $F_1$; $F_1$-ATPase; Vacuolar-type ATPase; Cross-linking; *N*-Ethylmaleimide; *m*-Meleimidobenzoyl-*N*-hydroxysuccinimide

## 1. Introduction

Like the mitochondrial and chloroplast enzymes, the ATP synthase or F-type ATPase ($F_oF_1$) of *Escherichia coli* synthesizes ATP coupled with an electrochemical gradient of protons (for reviews, see [1–4]). Studies on the bacterial enzyme by mutagenesis and affinity labeling revealed part of the catalytic site near ATP $\gamma$ phosphate. Two catalytic residues $\beta$Lys-155 and $\beta$Thr-156 of the enzyme are located in the $\beta$ subunit glycine-rich or phosphate-binding sequence (Gly-Gly-Ala-Gly-Val-Gly-Lys-Thr, residues 149–156 of the $\beta$ subunit) conserved in many nucleotide binding proteins (conserved residues, underlined above) [5]. Four residues ($\beta$Gly-172, $\beta$Ser-174, $\beta$Glu-192, and $\beta$Val-198) were suggested to be located near $\beta$Gly-149 by introducing the second mutations into the defective $\beta$Cys-149 mutant [6,7]. The defective coupling efficiency of the $\beta$Ser-174 → Phe mutant was suppressed by the $\alpha$Arg-296→Cys mutation in the a subunit [8], indicating the importance of interaction between the b subunit catalytic region(s) and the a subunit for energy coupling.

The catalytic *A* subunit of vacuolar-type ATPase [9,10] is homologous to the F-type ATPase $\beta$ subunit and has a similar glycine-rich sequence (Gly-Ala-Phe-Gly-Cys-Gly-Lys-Thr). In addition to the residues conserved among nucleotide-binding proteins, the cysteine residue is conserved among Vacuolar-type ATPase *A* subunits. This residue corresponds to $\beta$Val-153 of the *E. coli* $\beta$ subunit. The Vacuolar-type ATPase is sensitive to sulfhydryl reagents such as *N*-ethylmaleimide (NEM) [11,12], and Feng and Forgac [13] showed recently that NEM-modification of the cysteine residue results in inhibition of the coated vesicle Vacuolar-type ATPase. As F-type ATPase is insensitive to NEM, it was of interest to introduce a cysteine residue at position 153 of the $\beta$ subunit and study the inhibitor sensitivities of the mutant enzyme. The mutant ($\beta$Val-153→Cys) enzyme constructed in this study was sensitive to sufhydryl reagents including NEM. Furthermore, *m*-maleimidobenzoyl-*N*-hydroxyl succinimide (MBS) cross-linked the $\alpha$ and $\beta$ subunits, suggesting that $\beta$Cys-153 is near (within 10 Å) the $\alpha$ subunit.

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*Abbreviations:* DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; NEM, *N*-ethylmaleimide; [$^{14}$C]NEM, [$^{14}$C]*N*-ethylmaleimide.

## 2. Experimental

### 2.1. Plasmids and mutagenesis

The $\beta$ subunit gene fragment (*StuI–SacI*) of pBWU13 (a plasmid carrying the entire *unc* operon for F-type ATPase) [6] was replaced by a synthetic double-stranded DNA of the same fragment carrying $\beta$Val-153→Cys (codon change, GTA → TGT) mutation [7]. The resulting plasmid pBMUD13-V153C was introduced into *E. coli* strain DK8 (*Δunc B-C, ilv*::Tn10, *thi*) [14] lacking the *unc* operon. Growth conditions for DK8 carrying mutant or wild-type plasmids were described previously [6,7]

### 2.2. Chemical modification and cross-linking of the enzyme

After passage through a Sephadex G-50 (Pharmacia) column equilibrated with 50 mM HEPES-NaOH (pH 8.0), wild-type or mutant $F_1$ (1.0 $\mu$M) was incubated at 25°C for 10 min with various concentrations of NEM (or [$^{14}$C]NEM) or other sulfhydryl reagents in 50 mM HEPES-NaOH (pH 8.0). DTT (1 mM ) was added to terminate NEM-binding, and aliquots were promptly assayed for ATPase activity. The NEM-modified $F_1$ was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [15]. Essentially the same conditions were used for cross-linking the enzyme with MBS except that 1 mM DTT and 1 mM ethanol amine were added to terminate MBS-binding.

### 2.3. Other procedures and chemicals

Published methods were used for preparations of membranes and EDTA extract, protein measurement, ATPase assay, and purification of $F_1$ [5–8]. Enzymes for DNA manipulation were from Takara Shuzo Co., and [$\alpha$-$^{32}$P]dCTP and [$^{14}$C]NEM (*N*-ethyl-1-[$^{14}$C]maleimide) were obtained from Amersham. MBS and DTNB were products from Pierce Chem. Co. and Nacalai Tesque Inc., respectively. All other chemicals used were of the highest grade commercially available.

## 3. Results and discussion

### 3.1. Construction and properties of the $\beta$Cys-153 enzyme

A recombinant plasmid pBMUD13-V153C carrying the *unc* operon ($F_oF_1$ genes) with the $\beta$ subunit mutation ($\beta$Val-153→Cys) was constructed and transformed into strain DK8 (*unc* operon deletion). The DK8/pBMUD13-V153C showed essentially the same growth (rate and yield) on succinate by oxidative phosphorylation as strain DK8 harboring pBWU13

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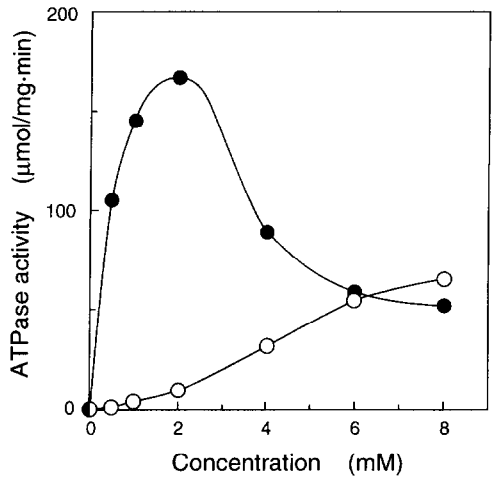


Fig. 1. Effects of $Ca^{2+}$ and $Mg^{2+}$ on ATPase activity of the mutant ($\beta$Cys-153) $F_1$-ATPase. The mutant ($\beta$Cys-153) $F_1$-ATPase was assayed with 4 mM ATP in the presence of various concentrations of $CaCl_2$ (open circles) or $MgCl_2$ (closed circles). A different titration curve for $Ca^{2+}$-dependent ATPase was obtained with the wild-type enzyme as reported previously [6].

(wild-type *unc* operon). The $F_1$-ATPase of $\beta$Cys-153 mutant enzyme could be solubilized from the membranes and purified in the presence of 1 mM DTT by the procedure used for the wild-type enzyme. The mutant $F_1$-ATPase had essentially the same subunit composition as the wild type, but about 1.3-fold higher specific activity ($Mg^{2+}$-dependent activity). These results suggest that the $\beta$Cys-153 enzyme had essentially no defect in ATP synthesis or hydrolysis. However, mutant $F_1$ had altered $Ca^{2+}$-dependent ATPase activity and gave a sigmoidal titration curve ($Ca^{2+}$ vs. activity): the mutant activity was very low ($\leq 5\%$ of the $Mg^{2+}$-dependent activity) in the presence of 0.5–2 mM $CaCl_2$, but increased with increase in $CaCl_2$, to about the same activity as the $Mg^{2+}$-dependent activity (Fig. 1). On the other hand, the $Ca^{2+}$-dependent ATPase activity of the wild-type enzyme was about half the $Mg^{2+}$-dependent activity in the presence of 2 mM $CaCl_2$ and gave a hyperbolic titration curve with increase in $MgCl_2$, as shown previously [6]. It is interesting that a single amino acid replacement had such a dramatic effect on the $Ca^{2+}$-dependency. These results suggest that $Ca^{2+}$ -dependent activity is not responsible for ATP synthesis and that the region including $\beta$Val-153 may be related to divalent cation binding. In this regard, it is noteworthy that $F_1$-ATPase with a $\beta$Gly-149 → Ser mutation had similar $Ca^{2+}$-dependent activity [6] to the present mutant enzyme.

### 3.2. *Sensitivity of the βCys-153 enzyme to sulfhydryl reagents*

The $\beta$Cys-153 mutant $F_1$-ATPase became sensitive to NEM: 3 $\mu$M NEM inhibited the mutant enzyme (multisite or steady state catalysis) about 50%, whereas even 100 $\mu$M NEM did not inhibit the wild-type enzyme significantly (Fig. 2a). The inhibition of the mutant enzyme was saturated by 10 $\mu$M NEM, about 30% of the activity remaining. Thus the mutant $F_1$-ATPase with a cysteine residue at the corresponding position of Vacuolar-type ATPase became sensitive to NEM. The enzyme was protected from NEM inhibition by ATP: essentially no inhibition was observed when it was incubated with 3 $\mu$M NEM and 4 mM ATP + 2 mM $MgCl_2$ (Fig. 2b). 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) was a slightly stronger inhibitor than NEM, but had no effect on the wild-type $F_1$-ATPase at 10 $\mu$M (data not shown). Iodoacetate at up to 100 $\mu$M did not inhibit the mutant or wild-type enzyme suggesting that the $\beta$Cys-153 residue is not exposed to the medium.

### 3.3. *[$^{14}$C]NEM binding to the β Cys-153 enzyme*

Consistent with the inhibition of ATPase activity, the $\beta$ subunit of the $\beta$Cys-153 mutant enzyme was labeled with 1–100 $\mu$M [$^{14}$C]NEM (Fig. 3a, lanes 1–4), whereas the wild-type $\beta$ subunit was not labeled even with 100 $\mu$M [$^{14}$C]NEM (Fig. 3a, lane 5).

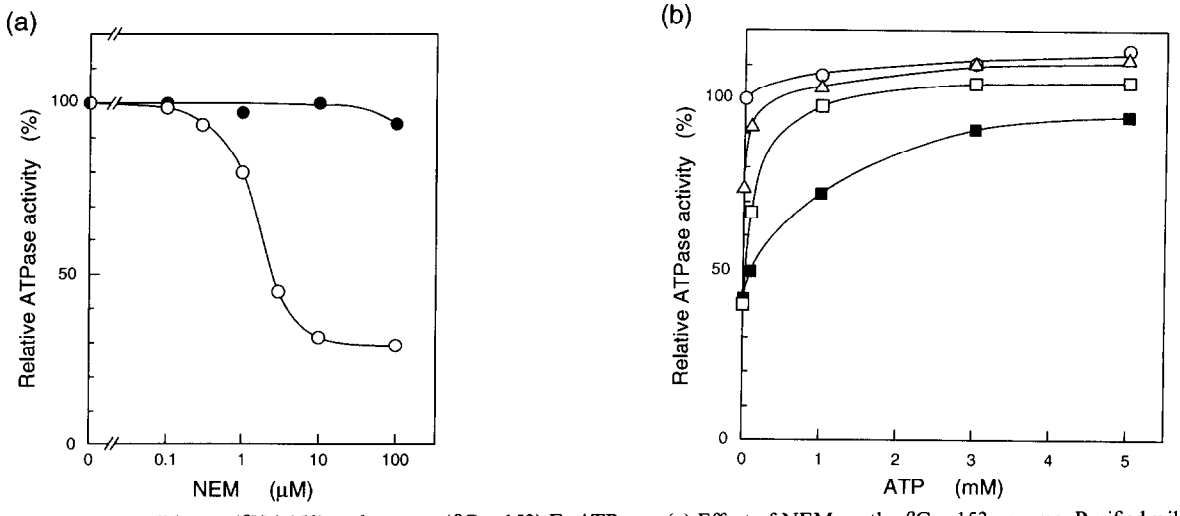


Fig. 2. Effects of NEM on wild-type ($\beta$Val-153) and mutant ($\beta$Cys-153) $F_1$-ATPases. (a) Effect of NEM on the $\beta$Cys-153 enzyme. Purified wild-type ($\beta$Val-153) (closed circle) and mutant ($\beta$Cys-153) (open circle) (1.0 $\mu$M) $F_1$-ATPases were incubated at 25 °C with various concentrations of NEM in 50 mM HEPES-NaOH (pH 8.0) for 10 min, and aliquots were assayed for ATPase activity immediately after addition of 1 mM DTT. Results are expressed as relative activities. The control activities without NEM treatment were wild-type $F_1$, 129 and $\beta$Cys-153 mutant $F_1$, 172, expressed as units /mg protein. (b) Effects of ATP on NEM sensitivity of the $\beta$Cys-153 enzyme. The wild-type and mutant enzymes were incubated with various concentrations of ATP in the pesence of 2 mM $MgCl_2$ (open symbols) or 0.5 mM EDTA (closed symbols) for 20 s and then for 10 min after addition of NEM. Aliquots were assayed for ATPase activity, without NEM, open circles; with 1 $\mu$M NEM, open triangles; with 3 $\mu$M NEM, open or closed squares. Other conditions were as described above.

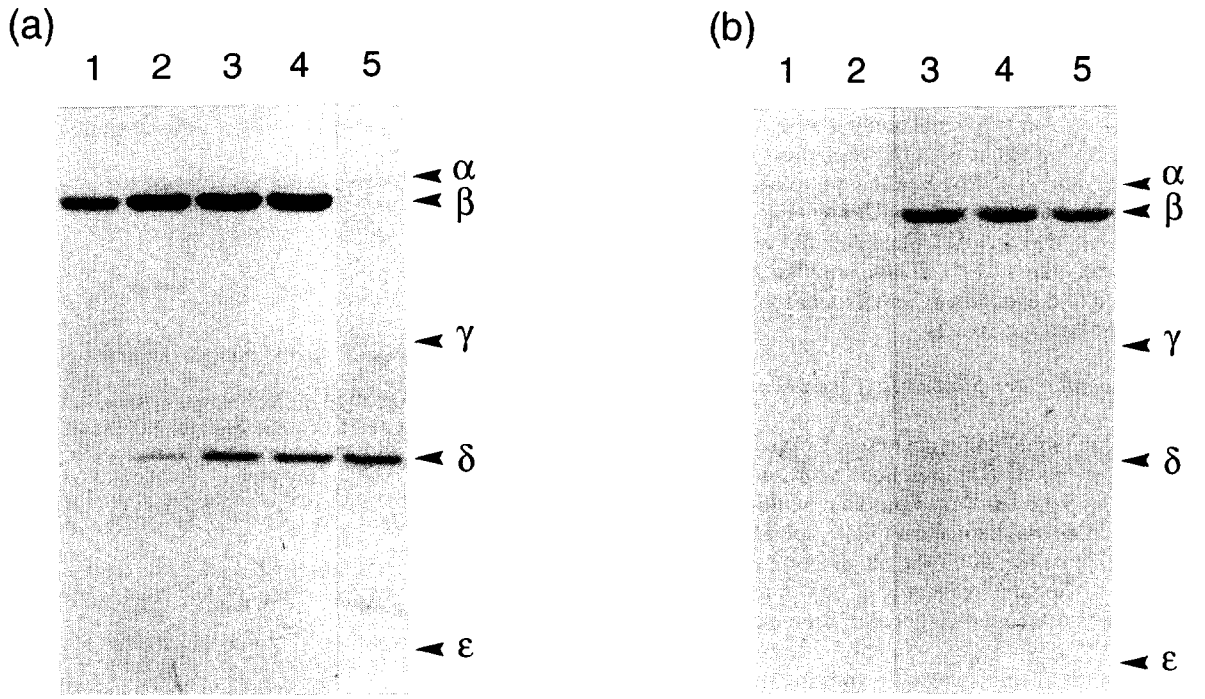


Fig. 3. [$^{14}$C]NEM binding to βCys-153 $F_1$-ATPase (a) [$^{14}$C]NEM binding to the βCys-153 enzyme. Purified mutant (βCys-153) $F_1$-ATPase was incubated with concentrations of 1, 3, 10, and 100 μM [$^{14}$C]NEM (lanes 1–4, respectively). The wild-type (βVal-153) enzyme was incubated with 100 μM [$^{14}$C]NEM (lane 5). Aliquots (6.4 μg) were applied to 12.5% polyacrylamide gel after treatment with 1 mM DTT. Gels were dried and autoradiographed by standard procedures. The positions of $F_1$ subunits (α, β, γ, δ, and ε) were determined in the same gel stained with Coomassie brilliant blue. (b) Effect of ATP on [$^{14}$C]NEM binding to the βCys-153 enzyme. Mutant enzymes were incubated with 4 mM ATP + 2 mM $MgCl_2$ (lane 1), 4 mM ATP + 0.5 mM EDTA (lane 2), 2 mM $MgCl_2$ (lane 3), or 0.5 mM EDTA (lane 4) for 20 s and incubated for further 10 min with 1 μM [$^{14}$C]NEM. The enzyme was incubated with only [$^{14}$C]NEM as a control (lane 5).

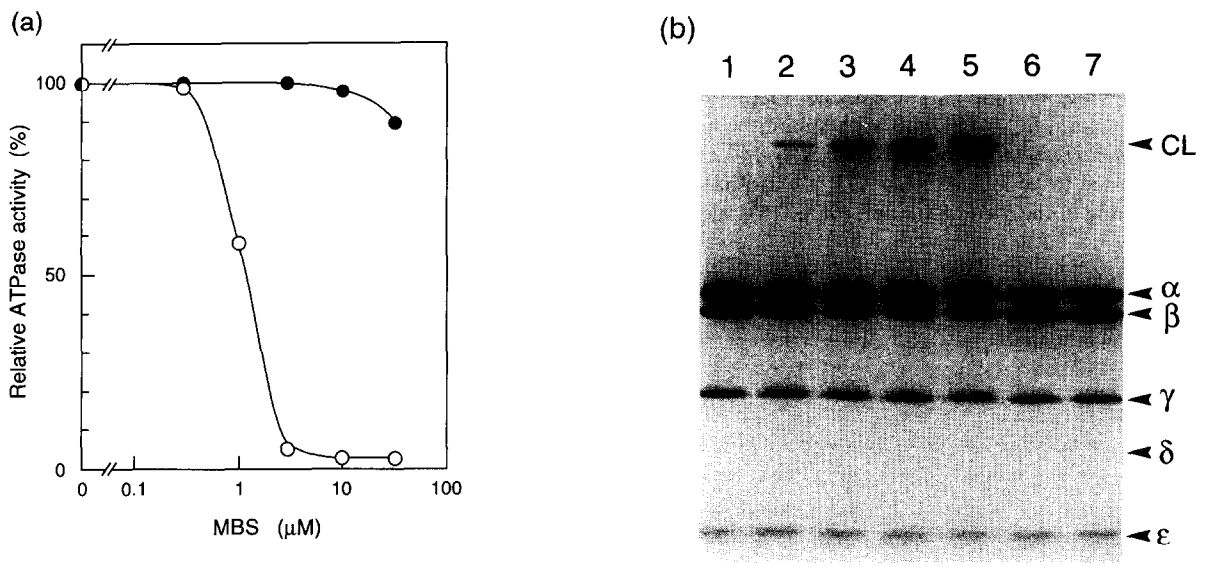


Fig. 4. Effects of MBS on wild type (βVal-153) and mutant (βCys-153) $F_1$ ATPases (a) Inhibition of the βCys-153 enzyme by MBS. Purified wild-type (βVal-153) (closed circles) or mutant (βCys-153) (open circles) $F_1$-ATPase (1.0 μM) was incubated for 10 min with various concentrations of MBS in 50 mM HEPES-NaOH (pH 8.0). Aliquots were assayed for ATPase activity immediately after addition of 1 mM DTT and 1 mM ehthanolamine. (b) Cross-linking of the βCys-153 enzyme with MBS. Purified mutant (βCys-153) $F_1$ was incubated with concentrations of 0, 1, 3, 10, and 100 μM MBS (for lanes 1–5, respectively). Aliquots were subjected to linear gradient (7–21%) polyacrylamide gel electrophoresis, and stained with Coomassie brilliant blue. Two control experiments were carried out: mutant $F_1$ was treated with 100 mM NEM at 25° for 10 min before incubation with 100 μM MBS (lane 6); wild-type $F_1$ was incubated with 100 μM MBS (lane 7). The mobility of the cross-linked product (CL) was less than expected, possibly because of the gradient system used and the properties of the product. Similar experiments were repeated with various concentrations of the mutant enzyme. The results of gel scanning suggested 1/3 decrease of the α and β subunits and formation of the cross-linked product at 3 μM MBS (lane 3). Essentially no decreases of other subunits were observed after treatment with MBS. Furthermore, no intermolecular cross-linked product was observed by sucrose gradient centrifugation of the mutant $F_1$ treated with MBS.

On the other hand, the wild-type and mutant $\delta$ subunits were labeled similarly when incubated with 10–100 $\mu$M [$^{14}$C]NEM. No other mutant or wild-type subunits were labeled under the same conditions.

About 50% inhibition and 1 mol binding were observed with 3 $\mu$M [$^{14}$C]NEM. Consistent with the results on inhibition of ATPase activity, no [$^{14}$C]NEM-binding was observed in the presence of 4 mM ATP (+ $Mg^{2+}$ or EDTA) (Fig. 3b, lanes 1, 2 and 5). However, $Mg^{2+}$ or EDTA had no effect on [$^{14}$C]NEM-binding (Fig. 3b, lanes 3–5). These results suggest that [$^{14}$C]NEM bound to the mutant $\beta$Cys-153 near the catalytic site and inhibited the enzyme activity.

### 3.4. *Cross-linking of $\alpha$ and $\beta$ subunits of the mutant $F_1$ by MBS*

Of several bifunctional sulfhydryl cross-linkers tested, only MBS (9.9 Å cross-linker) [16] inhibited the $\beta$Cys-153 enzyme and produced a major cross-linked product. Multisite catalysis of the mutant enzyme was almost completely inhibited by 3 $\mu$M MBS, whereas that of the wild-type enzyme was not inhibited even by 10 $\mu$M of the same reagent (Fig. 4a). A mutant cross-linked product was detected together with decrease of both the $\alpha$ and $\beta$ subunits (Fig. 4b, lanes 1–5), but the amounts of other subunits were essentially unchanged, suggesting that the $\alpha$ and $\beta$ subunits were cross-linked. Consistent with these results, the cross-linked product reacted with antibodies against the $\alpha$ and $\beta$ subunits but not with those of the $\gamma$ and $\varepsilon$ subunits (data not shown). Densitmetric scanning demonstrated that the equivalent amounts of $\alpha$ and $\beta$ subunits were decreased and the cross-linked product was formed after incubation with various concentrations of MBS: about 30% of both the $\alpha$ and $\beta$ subunits cross-linked with 3 $\mu$M MBS. The cross-linked product was decreased significantly when $F_1$ was incubated with NEM before addition of MBS (Fig. 4b, lane 6). Furthermore, no cross-linking was observed in wild-type $F_1$ ATPase incubated with 100 $\mu$M MBS (Fig. 4b, lane 7). These results suggest that MBS cross-linked mutant $\beta$Cys-153 with an $\alpha$ subunit residue.

As the glycine-rich sequence (between $\beta$Gly-149 and $\beta$Thr-156) is suggested to be located near ATP $\gamma$-phosphate [17,18], the cross-linking of the $\alpha$ and $\beta$ subunit ($\beta$Cys-153) suggest that the interaction of the catalytic site with the $\alpha$ subunit is important for the enzyme activity and support the model locating the catalytic site at the interface of the two subunits. From the length of the actual cross-linking by MBS, $\beta$Val-153 is suggested to be located within 10Å of the $\alpha$ subunit residue.

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