

# Design and Synthesis of Photochemically Controllable Restriction Endonuclease *Bam*HI by Manipulating the Salt-Bridge Network in the Dimer Interface

Masayuki Endo, Koji Nakayama, and Tetsuro Majima\*

*Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan*

*majima@sanken.osaka-u.ac.jp*

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The strategy for the design of photochemically controllable enzymes by manipulating the dimer interface is described. Employing a restriction endonuclease *Bam*HI, the selective incorporation of amino acids having a photoremovable 6-nitroveratryl group into the specific position (Lys132) in the dimer interface of the *Bam*HI mutant (H133A) was performed. The activity of the photofunctionalized *Bam*HI mutant was significantly suppressed, and the following photoirradiation induced the recovery of the activity. In addition, uncaging of the 6-nitroveratryl group introduced to Lys132 did not seriously reduce the catalytic activity and affinity for the substrate. These results indicate that the activity of the enzyme can be effectively regulated by caging and uncaging of the specific amino acid in the dimer interface using the photoremovable group.

## Introduction

Complex formation of proteins is a key phenomenon for expressing specific activities and functions in vitro and in vivo. The higher order structures of proteins to form active complexes need precise assembly through several key amino acids in the protein–protein interface.<sup>1</sup> A salt-bridge network involving these amino acids often seen in the dimer interface stabilizes the formation of the complex and contributes to the precise alignment of the dimer. Substitution of these amino acids, for example, alanine scanning, results in a significant decrease or inactivation of the functions and formation of the complex.<sup>2</sup> This means that if chemically controllable functional groups, especially photoreactive residues, can be selectively introduced into these amino acids, the association of the proteins and the enzymatic activity can be regulated by photoirradiation from the outside without the addition of reagents for activation. Numbers of studies have been made by caging of a target protein with

a photoremovable group for inactivation followed by uncaging it with photoirradiation for activation. These studies have revealed that the photoregulation using a caged protein is practically advantageous for the initiation of the protein activity in vitro and in vivo.<sup>3</sup>

To control the activity of the enzyme, we planned to manipulate the amino acids involved in a salt-bridge network in a protein–protein interface by introduction of photoreactive molecules. We employed a restriction endonuclease *Bam*HI which has a dimer interface including hydrogen bondings (H-bondings) with salt bridges and requires dimerization to exhibit the activity.<sup>4</sup> The salt-bridge network in the dimer interface of *Bam*HI consists of four amino acids: two basic amino acids, Lys132 and His133, in the  $\alpha$  helix specifically interact with two acidic amino acids, Glu167 and Glu170, in the counterpart  $\alpha$  helix (Figure 1).<sup>4</sup>

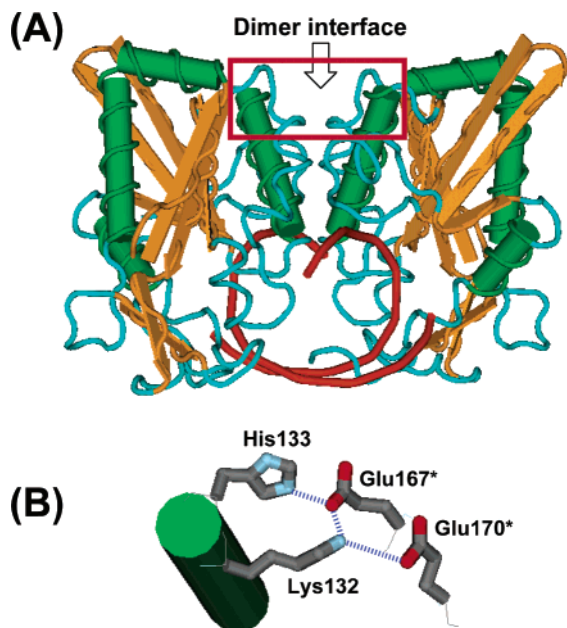
For labeling of proteins, a promising method for site-selective incorporation of unnatural amino acids has been developed using an in vitro translation system with special codons and the corresponding aminoacyl-tRNA.<sup>5–7</sup> The incorporation of a photoremovable group has been achieved by these methods.<sup>8</sup> To regulate the enzymatic

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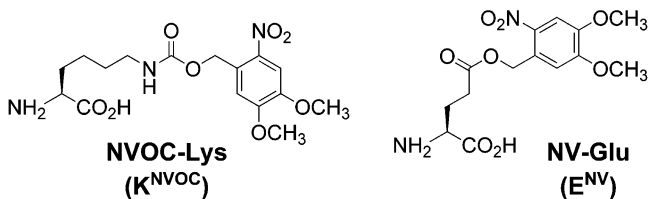
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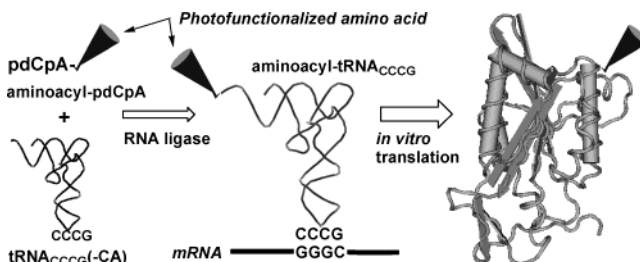
**FIGURE 1.** Crystal structure of endonuclease *Bam*HI (PDB, 1BHM). (A) The complex of *Bam*HI dimer and DNA (red rope). (B) The salt-bridge network with the amino acid side chains in the *Bam*HI dimer interface. Dashed lines indicate the H-bondings with salt-bridges. Asterisks denote the counterpart *Bam*HI monomer in the dimer interface. Figures were created by a Cn3D program distributed by the National Center for Biotechnology Information.

activity by photoirradiation, we individually introduced a photoremovable 6-nitroveratryloxycarbonyl (NVOC) group to Lys132 and a 6-nitroveratryl (NV) group to Glu167 and Glu170 (Chart 1). We expected to control the following three important changes in the salt-bridge network of the dimer interface by protection of the amino and carboxylate groups with 6-nitroveratryl derivatives: (1) inhibition of H-bond formations between the amino acid side chains in the salt-bridge network; (2)

#### CHART 1. Photofunctionalized Amino Acids Employed in This Experiment: NVOC-Lys ( $K^{NVOC}$ ) and NV-Glu ( $E^{NV}$ )



#### SCHEME 1. Synthetic Scheme for Site-Selective Incorporation of Photofunctionalized Amino Acids into *Bam*HI<sup>a</sup>



<sup>a</sup> In the first step, a synthetic aminoacyl-pdCpA was coupled with  $tRNA^{CCCG}(-CA)$  to give a photofunctionalized aminoacyl-tRNA. In the second step, mRNA with CCGG codon at the specific position was translated with the aminoacyl-tRNA using the in vitro translation system.

reduction of electrostatic interaction by introduction of the photoremovable groups to the side chains of the basic and acidic amino acids; (3) steric effect caused by the bulkiness of the 6-nitroveratryl groups to prevent correct positioning of amino acid side chains in the dimer interface. Based on this strategy, we designed and synthesized the photofunctionalized *Bam*HI and investigated for photochemical control of the correct dimer formation of *Bam*HI and subsequent recovery of the enzymatic activity.

## Results and Discussion

The synthesis of the photofunctionalized *Bam*HI has been performed by the in vitro translation system. The biochemical method using four-base codon–anticodon interaction was employed for site-selective incorporation of photofunctionalized amino acids.<sup>7</sup> As shown in Scheme 1, first, a photofunctionalized aminoacyl-tRNA with a four-base anticodon was obtained from the coupling of a photofunctionalized aminoacyl-pdCpA and  $tRNA^{CCCG}$  lacking a 3'-terminal CA nucleotide [ $tRNA^{CCCG}(-CA)$ ]. Second, site-selective incorporation of photofunctionalized amino acids was performed by in vitro translation with mRNA containing the four-base codon (CGGG) and the photofunctionalized aminoacyl-tRNA<sup>CCCG</sup>.

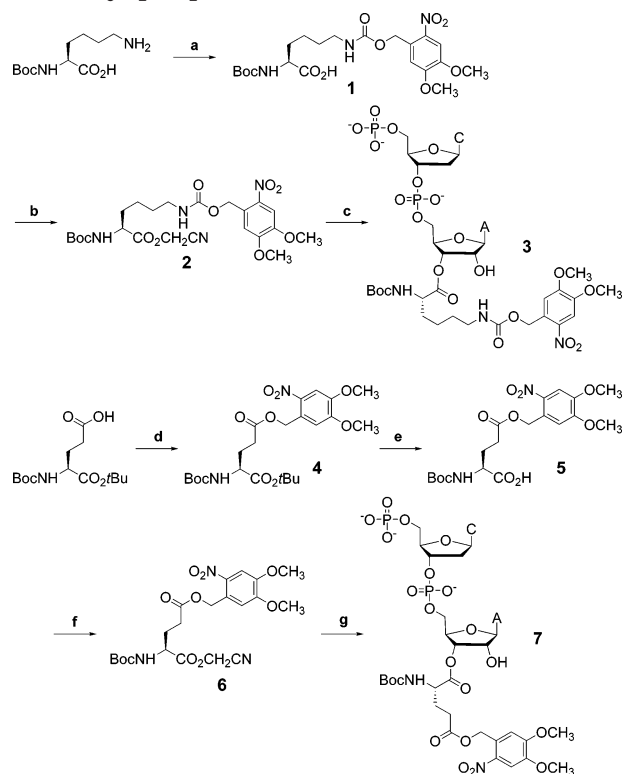
**Synthesis of Photofunctionalized Amino Acids and Aminoacyl-tRNA<sup>CCCG</sup>.** The synthesis of active esters of lysine and glutamic acid with a 6-nitroveratryl group was carried out according to Scheme 2. The 6-nitroveratryl group to the  $N^\epsilon$  position of lysine (**1**) was introduced by a reaction of  $N^\epsilon$ -(*tert*-butoxycarbonyl)-L-lysine with 6-nitroveratryl chloroformate. Esterification of **1** with chloroacetonitrile gave  $N^\epsilon$ -Boc- $N^\epsilon$ -NVOC-Lys cyanomethyl ester **2**. Introduction of the 6-nitroveratryl

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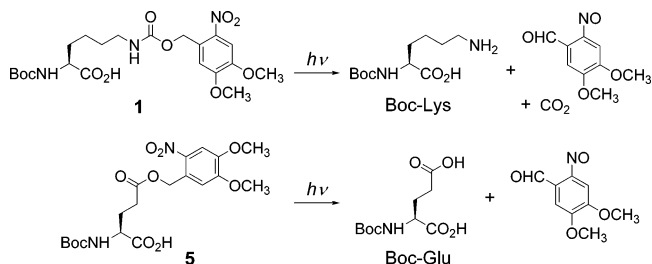
**SCHEME 2. Synthetic Scheme for Functionalized Aminoacyl-pdCpA 3 and 7<sup>a</sup>**


<sup>a</sup> (a) 6-Nitroveratryl chloroformate; (b) chloroacetonitrile; (c) pdCpA Bu<sub>4</sub>N<sup>+</sup> salt; (d) 6-nitroveratryl alcohol, DCC; (e) (i) TFA, (ii) 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile; (f) chloroacetonitrile; (g) pdCpA Bu<sub>4</sub>N<sup>+</sup> salt.

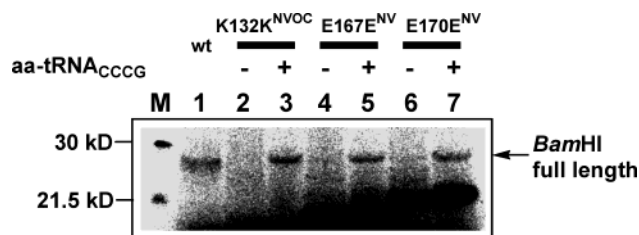
group to the  $\gamma$ -carboxylate of glutamic acid was performed by the coupling of *N*<sup>t</sup>-*tert*-butoxycarbonylglutamic acid- $\alpha$ -*tert*-butyl ester and 6-nitroveratryl alcohol with dicyclohexyl carbodiimide (DCC) to give **4**. *tert*-Butoxycarbonyl and *tert*-butyl groups of **4** were removed by treatment with trifluoroacetic acid (TFA), and then the *N*<sup>t</sup>-amino group was protected with the Boc group using 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile to afford **5**. Esterification of **5** with chloroacetonitrile gave *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu cyanomethyl ester **6**.

The incorporation of the modified amino acids was performed using a four-base codon–anticodon method according to the previously reported method.<sup>7b</sup> At first, aminoacyl-tRNAs were obtained by a reaction of the *N*<sup>t</sup>-Boc-*N*<sup>t</sup>-NVOC-Lys cyanomethyl ester (**2**) or *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu cyanomethyl ester (**6**) with a nucleoside dimer 5'-phospho-2'-deoxycytidyl-(3'-5')-adenosine (pdCpA) to give *N*<sup>t</sup>-Boc-*N*<sup>t</sup>-NVOC-Lys-pdCpA **3** (75% yield) and *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu-pdCpA **7** (93% yield). The Boc-protected aminoacyl-pdCpA was treated with TFA followed by ligation onto a tRNA<sub>CCCG</sub>(–CA) using T4 RNA ligase to afford *N*<sup>t</sup>-NVOC-Lys-tRNA<sub>CCCG</sub> and  $\gamma$ -NV-Glu-tRNA<sub>CCCG</sub>.

The photoreactivities of the *N*<sup>t</sup>-Boc-*N*<sup>t</sup>-NVOC-Lys (**1**) and *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu (**5**) were examined (Scheme 3). The photoirradiation was carried out in a 1 mM methanolic solution of the nitroveratryl derivatives at 0 °C by a transilluminator at 365 nm. The products were analyzed by a reversed-phase HPLC. The nitroveratryl groups were easily deprotected, and no other byproduct was detected. Like the photodeprotection for caged peptides

**SCHEME 3. Photochemical Reactions of *N*<sup>t</sup>-Boc-*N*<sup>t</sup>-NVOC-Lys **1** and *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu **5** with Photoirradiation at 365 nm**


and proteins previously studied,<sup>3,9</sup> the removal of the NVOC and NV gave *N*<sup>t</sup>-Boc-Lys and *N*<sup>t</sup>-Boc-Glu during the photoirradiation of *N*<sup>t</sup>-Boc-*N*<sup>t</sup>-NVOC-Lys **1** and *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu **5**, respectively. The half-lives for degradation of the Boc-NVOC-Lys **1** and Boc-NV-Glu **5** were 1.2 and 3.5 min, respectively, under these experimental conditions.

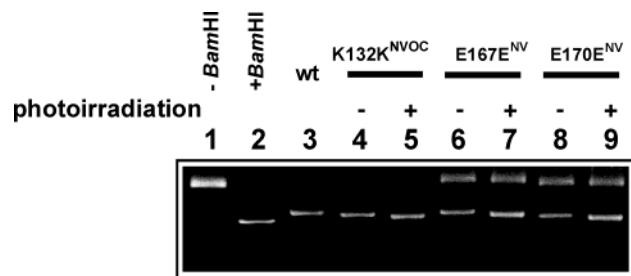


**FIGURE 2.** In vitro translation of *Bam*HI mutants with unnatural aminoacyl-tRNA<sub>CCCG</sub>: lane 1, translation with mRNA of wild-type *Bam*HI; lanes 2 and 3, translation with K132K<sup>NVOC</sup> mutant mRNA without and with NVOC-Lys-tRNA<sub>CCCG</sub>, respectively; lanes 4 and 5, translation with E167E<sup>NV</sup> mutant mRNA without and with NV-Glu-tRNA<sub>CCCG</sub>, respectively; lanes 6 and 7, translation with E170E<sup>NV</sup> mutant mRNA without and with NV-Glu-tRNA<sub>CCCG</sub>, respectively.

**Synthesis of Site-Selectively Photofunctionalized *Bam*HI Mutants.** The photofunctionalized amino acids previously synthesized were incorporated into the specific positions of *Bam*HI. The introduction of the modified amino acids into the specific positions of *Bam*HI was carried out by employing the in vitro translation system with mRNA containing a four-base codon and the synthesized aminoacyl-tRNA<sub>CCCG</sub>. In vitro translation was carried out in a mixture containing mRNA, unnatural aminoacyl-tRNA<sub>CCCG</sub>, *E. coli* S30 extract, and amino acid with [<sup>35</sup>S]-methionine at 30 °C for 3 h. After the reaction, the mixtures were loaded onto an 18% SDS–PAGE, and the gel was visualized by an imaging analyzer (Figure 2). Wild-type *Bam*HI was expressed as a full-length form at 27 kDa (lane 1). In the cases of translation with mRNA containing a four-base codon mutation, incomplete length of *Bam*HI was observed in the absence of aminoacyl-tRNA (lanes 2, 4, and 6). In contrast, full-length *Bam*HI was obtained in the presence of the specific aminoacyl-tRNA (lanes 3, 5, and 7). The efficiency of incorporation of NVOC-Lys at position 132 (K132K<sup>NVOC</sup>) was 60% quantified by the radioactivity of [<sup>35</sup>S]-methionine. On the other hand, the incorporation efficiencies of the NV-Glu

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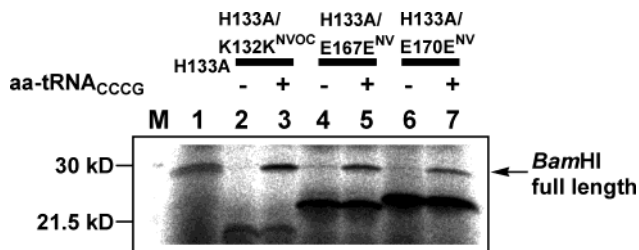




**FIGURE 3.** Cleavage of a substrate DNA (pBR322 restriction fragment) with a wild-type and mutant *Bam*HI without and with photoirradiation: lane 1, substrate DNA only; lane 2, DNA cleavage with a commercially available *Bam*HI (20 units); lane 3, a wild type *Bam*HI prepared by the in vitro translation system; lanes 4 and 5, DNA cleavage with a K132K<sup>NVOC</sup> *Bam*HI mutant without (–) and with (+) photoirradiation, respectively; lanes 6 and 7, DNA cleavage with an E167E<sup>NV</sup> *Bam*HI mutant without (–) and with (+) photoirradiation, respectively; lanes 8 and 9, DNA cleavage with an E170E<sup>NV</sup> *Bam*HI mutant without (–) and with (+) photoirradiation, respectively. The photoirradiation was carried out at 0 °C for 20 min, and then the cleavage of substrate DNA was performed at 30 °C for 2 h. The retardation of cleavage bands in lanes 3–9 may originate from the binding of proteins in the in vitro translation mixtures.

at positions 167 and 170 (E167E<sup>NV</sup> and E170E<sup>NV</sup>, respectively) were 11 and 10%, respectively. Incorporation efficiencies for NV-Glu were obviously lower than that of NVOC-Lys. Since the efficiencies of incorporation depend on the shape and bulkiness of the side chains of the amino acids,<sup>7b</sup> steric hindrance would exist to some extent in the 6-nitroveratryl ester on glutamate as compared to the longer methylene chain of NVOC-Lys when incorporated into the proteins. We employed these *Bam*HI mutants for the investigation of the photochemical control of enzymatic activities without further purification.

**Activity of Photofunctionalized *Bam*HI with Photoirradiation.** Enzymatic activities of the wild-type and mutant *Bam*HI were identified by double-strand cleavage of the pBR322 restriction fragment digested by *Eco*RI as a substrate (Figure 3). The wild-type *Bam*HI prepared by in vitro translation showed sufficient activity and sequence selectivity (lane 3). Photoreaction for the functionalized *Bam*HI was carried out in a solution containing the previous *Bam*HI. For uncaging of the photoremovable groups, photoirradiation was performed by a transilluminator (365 nm) at 0 °C for 20 min. On the basis of the results of deprotection of the photoremovable groups of the amino acid monomers (NVOC-Lys and NV-Glu), over 90% of the protecting groups of photofunctionalized *Bam*HI could be removed under these experimental conditions. After the photoirradiation, the mixtures were diluted with a buffer containing 10 mM tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0), 10 mM MgCl<sub>2</sub>, 100 mM NaCl, and 1 mM DTT, and the solutions were incubated with the substrate DNA fragment at 30 °C for 2 h for examining enzymatic activities. Unexpectedly, all the mutants (K132K<sup>NVOC</sup>, E167E<sup>NV</sup>, and E170E<sup>NV</sup>) exhibited the activities without photoirradiation (lanes 4, 6, and 8) (Figure 3). After photoirradiation and subsequent enzymatic reaction, the mutants exhibited the same activities as those of the mutants without photoirradiation (lanes 5, 7, and 9). These results suggest that the 6-nitroveratryl groups

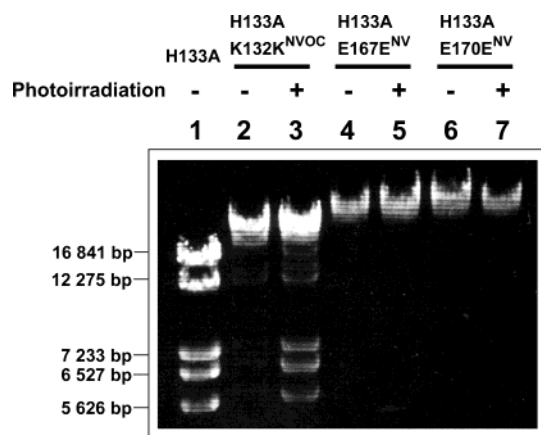


**FIGURE 4.** In vitro translation of *Bam*HI H133A mutants with unnatural aminoacyl-tRNA<sup>CCCG</sup>: lane 1, translation with an mRNA of H133A mutant; lanes 2 and 3, translation with an H133A/K132K<sup>NVOC</sup> mutant mRNA without and with NVOC-Lys-tRNA<sup>CCCG</sup>, respectively; lanes 4 and 5, translation with an H133A/E167E<sup>NV</sup> mutant mRNA without and with NV-Glu-tRNA<sup>CCCG</sup>, respectively; lanes 6 and 7, translation with an H133A/E170E<sup>NV</sup> mutant mRNA without and with NV-Glu-tRNA<sup>CCCG</sup>, respectively.

introduced to the amino acid side chains cannot prevent the dimer formation for the *Bam*HI activity. Because of the long methylene chains of Lys and Glu, these would facilitate the exclusion of the bulky 6-nitroveratryl groups outside of the dimer interface. In addition, one or two H-bondings in the salt-bridge network are still preserved in the *Bam*HI mutants even when the photoremovable groups are introduced. These results suggest that one H-bonding in the dimer interface would be sufficient to exhibit the activity and can maintain the correct dimer formation. The NV-Glu *Bam*HI mutants showed poor activities (40–60% of DNA cleavage), which may indicate some distortion or failure of the interaction with other amino acids by some misfolding. Further manipulation of the salt-bridge network in the dimer interface is required to prevent the dimer formation using the photoremovable groups.

To suppress the *Bam*HI activity before photoirradiation, we replaced histidine at position 133 to alanine (H133A mutant) to reduce the number of H-bondings (Figure 1b). When the NVOC group is introduced to the Lys132 of the H133A mutant (H133A/K132K<sup>NVOC</sup>), all the H-bondings are excluded from the salt-bridge network, meaning that the dimer formation should be completely prohibited. In the cases of Glu167 and Glu170 of the H133A mutants with NV groups (H133A/E167E<sup>NV</sup> and H133A/E170E<sup>NV</sup>, respectively), one H-bonding is still preserved between the Lys132 and glutamates. If the activity of the H133A/K132K<sup>NVOC</sup> mutant is compared with those of the H133A/E167E<sup>NV</sup> and H133A/E170E<sup>NV</sup> mutants, the effect of the H-bonding for the dimer formation could be estimated.

**Suppression and Recovery of the Activity of Photofunctionalized *Bam*HI Mutants with Photoirradiation.** We prepared the mRNA of the H133A mutant and those with a four-base codon in positions 132, 167, and 170 individually. Incorporation of the NVOC-Lys into position 132 and NV-Glu into positions 167 and 170 was carried out by the same method as previously described. As shown in Figure 4, when the NVOC group was incorporated into position 132 and the NV groups into positions 167 and 170, the full-length proteins were obtained in the presence of the specific aminoacyl-tRNA (compare lanes 3, 5, and 7 with the corresponding lanes 2, 4, and 6, respectively in Figure 4). The efficiency of incorporation of NVOC-Lys at position 132 was 55% and

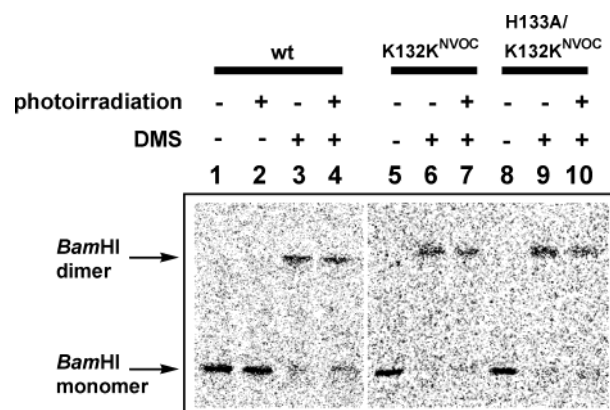


**FIGURE 5.** Cleavage of a substrate DNA ( $\lambda$ DNA) with *Bam*HI H133A mutants without and with photoirradiation: lane 1, cleavage with a *Bam*HI H133A mutant; lanes 2 and 3, cleavage with an H133A/K132K<sup>NVOC</sup> *Bam*HI mutant without (–) and with (+) photoirradiation, respectively; lanes 4 and 5, cleavage with an H133A/E167E<sup>NV</sup> *Bam*HI mutant without (–) and with (+) photoirradiation, respectively; lanes 6 and 7, cleavage with an H133A/E170E<sup>NV</sup> *Bam*HI mutant without (–) and with (+) photoirradiation, respectively. Photoirradiation was carried out at 0 °C for 20 min, and then the cleavage of substrate DNA was performed at 30 °C for 2 h.

those of the NV-Glu at positions 167 and 170 were 23 and 17%, respectively.

To detect the activities and specificities of these mutant proteins, we performed the cleavage of  $\lambda$ DNA as a substrate (Figure 5). The H133A mutant of *Bam*HI prepared by in vitro translation showed sufficient activity and sequence selectivity (lane 1); however, the catalytic activity of the H133A mutant was significantly decreased as compared to that of the wild-type *Bam*HI (vide infra). Photoirradiation to the *Bam*HI mutants (H133A/K132K<sup>NVOC</sup>, H133A/E167E<sup>NV</sup>, and H133A/E170E<sup>NV</sup>) was carried out at 0 °C for 20 min. In the case of H133A/K132K<sup>NVOC</sup> (lanes 2 and 3), the activity of the H133A/K132K<sup>NVOC</sup> mutant was almost suppressed without photoirradiation (2.7% of cleavage product), and after photoirradiation and incubation, the mutant recovered the activity and specificity, and 23% of the DNA was cleaved (lane 3). This suggests that the reconstitution of the salt-bridge network in the dimer interface by removal of the NVOC group. In contrast, the H133A/E167E<sup>NV</sup> and H133A/E170E<sup>NV</sup> mutants did not show the activities even after photoirradiation, suggesting that serious misfolding would abolish the enzymatic activities by introduction of the NV groups into these glutamates.

The activity of H133A/K132K<sup>NVOC</sup> mutant after photoirradiation was evaluated by kinetic parameters according to the previously reported method.<sup>10</sup> Using the pBR322 restriction fragment as a substrate, kinetic parameters were obtained as  $K_M = 2.9$  nM and  $V_{max} = 5.4 \times 10^{-2}$  nM min<sup>-1</sup> for the photoirradiated H133A/K132K<sup>NVOC</sup> mutant and  $K_M = 2.3$  nM and  $V_{max} = 6.5 \times 10^{-2}$  nM min<sup>-1</sup> for the H133A mutant. The caging and uncaging of H133A/K132K<sup>NVOC</sup> mutant lowered both affinity and catalytic activity, but still preserved the same



**FIGURE 6.** DMS chemical cross-linking of the wild-type *Bam*HI, K132K<sup>NVOC</sup>, and H133A/K132K<sup>NVOC</sup> mutants without and with photoirradiation: lanes 1 and 2, wild-type *Bam*HI without (–) and with (+) photoirradiation, respectively; lanes 3 and 4, DMS-treated wild-type *Bam*HI without (–) and with (+) photoirradiation, respectively; lane 5, K132K<sup>NVOC</sup> mutant; lanes 6 and 7, DMS-treated K132K<sup>NVOC</sup> mutants without (–) and with (+) photoirradiation, respectively; lane 8, H133A/K132K<sup>NVOC</sup> mutant; lanes 9 and 10, DMS-treated H133A/K132K<sup>NVOC</sup> mutants without (–) and with (+) photoirradiation, respectively. For the negative control of DMS cross-linking, wild-type *Bam*HI treated with 0.1% SDS for inhibition of the dimer formation was detected as a monomer.

level of the affinity and activity as compared to the H133A *Bam*HI mutant. These results indicate that caging and following uncaging of the NVOC group attached to the K132 of the H133A mutant do not seriously affect the activity and affinity for the substrate. The effect of the H133A mutation is critical for the activity ( $K_M = 1.7$  nM and  $V_{max} = 8.8 \times 10^{-1}$  nM min<sup>-1</sup> for the wild-type *Bam*HI), suggesting that the H-bonding between H133 and E167 would largely contribute to stabilizing the dimer structure. Because of the decrease of the number of the H-bondings involved in the salt-bridge network, elimination of the H133 would weaken the interaction of the two *Bam*HI monomers, which may facilitate to control the dimer formation by protection and deprotection using the photoremovable group.

**Direct Observation of the Dimer formation by Chemical Cross-Linking.** To directly examine the dimer formation of the photofunctionalized *Bam*HI mutants, the wild-type, K132K<sup>NVOC</sup>, and H133A/K132K<sup>NVOC</sup> mutants were investigated by a chemical cross-linking method using dimethyl suberimidate dihydrochloride (DMS) (Figure 6).<sup>11</sup> In this experiment, we used the purified *Bam*HI for cross-linking the proteins. In the case of the wild-type *Bam*HI, both proteins before and after photoirradiation were detected as the dimer on SDS-PAGE (lanes 3 and 4). The K132K<sup>NVOC</sup> and H133A/K132K<sup>NVOC</sup> mutants before photoirradiation were detected as the dimer (lanes 6 and 9), indicating that the NVOC does not prevent the dimer formation. After photoirradiation, both mutants were also detected as the dimer. In the case of the K132K<sup>NVOC</sup> mutant, both mutants without and with photoirradiation had the activities, and the cross-linking results are consistent with the cleavage results (see Figure 3). In contrast, the

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H133A/K132K<sup>NVOC</sup> mutant only showed the activity after photoirradiation (Figure 5), meaning that the H133A/K132K<sup>NVOC</sup> forms an inactive dimer before photoirradiation. This result is similar to the case of photofunctionalized HIV-1 protease, which forms a dimer without photoirradiation as an inactive form.<sup>8f</sup> The results we obtained may suggest that the photoremovable NVOC group in the dimer interface would prevent the correct dimer formation, and the removal of the NVOC group from the dimer interface would reconstitute the salt-bridge network and rearrange the *Bam*HI dimer as an active form.

## Conclusions

We have demonstrated the direct photochemical control of the enzymatic activity and function by precise manipulation of the salt-bridge network in the dimer interface employing the photofunctionalized *Bam*HI mutants. In our experiment, the key amino acid in *Bam*HI for control of the enzymatic activity is the Lys132, and this residue can be available for the potential control of the *Bam*HI activity. Using the method as we have demonstrated here, the photochemical activation of the enzymes and proteins can be achieved by introducing photoreactive molecules into the key amino acids in the dimer interface based on precise and rational modification of the proteins with respect to the structure. The strategy of the photochemical regulation of the enzymatic activities by the control of protein–protein interactions can be applicable to various enzymes and proteins possessing the salt-bridge network in the dimer interface.

## Experimental Section

**Synthesis of Aminoacyl-tRNA<sup>CCCG</sup>.** Deprotection of *N*<sup>t</sup>-Boc-*N*<sup>t</sup>-NVOC-Lys-pdCpA **3** and *N*<sup>t</sup>-Boc- $\gamma$ -NV-Glu-pdCpA **7** was performed with trifluoroacetic acid according to a previous method.<sup>7b</sup> The deprotected aminoacyl-pdCpA was dissolved in DMSO to a concentration of 5 mM and stored at –20 °C. A tRNA<sup>CCCG</sup> (–CA) was obtained by in vitro transcription from a *Fok*I treated pUC19 plasmid containing a T7 promoter and a synthetic yeast tRNA<sup>Phe</sup> sequence.<sup>12</sup> Ligation of aminoacyl-pdCpA to tRNA<sup>CCCG</sup> (–CA) was carried out in a 30  $\mu$ L solution containing 5  $\mu$ g of tRNA<sup>CCCG</sup> (–CA), 0.5 mM aminoacyl-pdCpA, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM ATP, 0.01% BSA, 10% DMSO, and T4 RNA ligase (40 units; Takara Shuzo, Kyoto, Japan). The reaction mixture was incubated at 10 °C for 2 h and then quenched by the addition of 3  $\mu$ L of 3 M sodium acetate (pH 5.2). The aminoacyl-tRNA<sup>CCCG</sup> was desalted by ethanol precipitation, dried, and dissolved in RNase-free water to a final concentration of 1  $\mu$ g/ $\mu$ L.

**Construction of Expression Plasmids for Wild-Type and Mutant *Bam*HI.** Wild-type *Bam*HI gene (642 bp) was prepared by assembling six short fragments of double-strand DNA (about 110 base pairs each) by T4 DNA ligase. Synthetic wild-type *Bam*HI gene was subcloned into a pUC19 plasmid, and the sequence was confirmed by dideoxy DNA sequencing. The full length *Bam*HI gene amplified by PCR with primers having *Nde*I and *Xho*I restriction sites was then inserted into a pET26b expression plasmid (Novagen, Madison, WI). Mutant *Bam*HI genes substituted by a CGGG four base codon at

positions 132, 167, and 170 were prepared by the QuikChange site-directed mutagenesis method (Stratagene, LaJolla, CA) with *Pfu* DNA polymerase. Mutant *Bam*HI gene substituted by alanine at position 133 was also prepared by the same mutagenesis method. The sequences for all the plasmids prepared here were confirmed by automated dideoxy DNA sequencing.

### In Vitro Translation of Wild-Type and Mutant *Bam*HI.

Preparation of mRNA was carried out in a solution containing template DNA (5  $\mu$ g), T7 RNA polymerase (110 units), 2.5 mM NTP, 40 mM Tris-HCl (pH 7.5), 20 mM MgCl<sub>2</sub>, and 5 mM DTT at 37 °C for 6 h. Prepared mRNA was purified by an RNeasy Mini Kit (Qiagen, Hilden, Germany), and approximately 50–150  $\mu$ g was obtained. *E. coli* S30 Extract System (Promega, Madison, WI) was employed for in vitro protein synthesis following the manufacturer's protocol. The translation reaction was carried out in 10  $\mu$ L of reaction mixture containing mRNA (2–3  $\mu$ g), aminoacyl-tRNA<sup>CCCG</sup> (1  $\mu$ g), 0.10 mM amino acid mixture (lacking methionine and arginine), 0.01 mM arginine, 4  $\mu$ L of premix, 3  $\mu$ L of *E. coli* S30 extract, and L-[<sup>35</sup>S] methionine (3  $\mu$ Ci) at 30 °C for 3 h. Wild-type and H133A *Bam*HI were prepared by the same method without aminoacyl-tRNA. The mixtures of proteins were denatured in a solution containing 50 mM Tris-HCl (pH 6.8), 0.1 M DTT, 2% SDS, and 10% glycerol, and loaded onto an 18% SDS-polyacrylamide gel for electrophoresis. The SDS–PAGE gels were visualized and quantified by an imaging analyzer (Fujix BAS1000 analyzer, Tokyo, Japan). For cold samples, the same procedure previously described was employed by just replacing the [<sup>35</sup>S] methionine to 0.1 mM methionine, and the concentration was quantified by Western blotting using a hexahistidine antibody as a primary antibody. Generation of the full-length proteins was about 10 ng from a 10  $\mu$ L scale synthesis of the in vitro translation system.

**Photoirradiation and Measurements of the *Bam*HI Activities.** Photoirradiation for the *Bam*HI mutants was carried out with a UV transilluminator (LMS-20E, 40 W; UVP, Inc., Upland, CA) with 365 nm light on ice for 20 min. The reaction mixtures of *Bam*HI (3  $\mu$ L) with or without photoirradiation were diluted by a solution containing 10 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 100 mM NaCl, and 1 mM DTT, and then the pBR322 *Eco*RI restriction fragment (2.0  $\mu$ g) or  $\lambda$ DNA (1.7  $\mu$ g) was added. The reaction mixtures were incubated at 30 °C for 2 h. The digested mixtures were loaded onto a 0.7% agarose gel and electrophoresed in TBE (Tris-Borate-EDTA) buffer. The gels were visualized by ethidium bromide staining. The gels were photographed, and the bands were quantified by the ImageJ program distributed by NIH. The kinetic parameters of the enzymes were calculated according to the previously reported method.<sup>10</sup> The reactions were carried out at 30 °C in the same reaction solutions containing the in vitro translation mixture of H133A or photoirradiated H133A/K132K<sup>NVOC</sup> mutant (1 ng) and pBR322 restriction fragment (3–18 nM).

**Chemical Cross-Linking of *Bam*HI.** The *Bam*HI wild-type, K132K<sup>NVOC</sup>, and H133A/K132K<sup>NVOC</sup> mutants were purified by a Ni–NTA spin column (Qiagen) for chemical cross-linking. A reaction mixture of [<sup>35</sup>S]-labeled *Bam*HI (100  $\mu$ L) was centrifuged at 10000*g* for 5 min to obtain a clear supernatant. To the supernatant was added 500  $\mu$ L of a buffer [10 mM HEPES (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.3 M NaCl, 1 mM EDTA, and 2 mM 2-mercaptoethanol] with 10 mM imidazole. The mixture was loaded onto a Ni–NTA spin column equilibrated with the same buffer as above, and the column was washed twice with 500  $\mu$ L of wash buffer (the same buffer with 20 mM imidazole). The purified protein was eluted with 150  $\mu$ L of an elution buffer (the same buffer with 250 mM imidazole). Photoirradiation for the purified *Bam*HI was carried out on ice for 20 min at 365 nm. For chemical cross-linking, the *Bam*HI was treated in a solution containing 3.7 mM DMS, 10 mM HEPES (pH 8.0), 0.3 M NaCl, 10 mM MgCl<sub>2</sub>,

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1 mM EDTA, and 2 mM 2-mercaptoethanol. After incubation at 30 °C for 30 min, the reaction was quenched by the addition of  $1/10$  volume of 1 M Tris-HCl (pH 7.6). The reaction mixtures were analyzed by an 18% SDS-PAGE, and the gel was visualized by an imaging analyzer.

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**Supporting Information Available:** Experimental procedures for the synthesis of the compounds **1–7**,  $^1\text{H}$  NMR and mass spectra of selected compounds, and the sequences of *Bam*HI and tRNA<sub>CCCG</sub>. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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