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Photochemical regulation of the activity of an endonuclease *Bam*HI using an azobenzene moiety incorporated site-selectively into the dimer interface

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Endonuclease *Bam*HI mutants having an azophenylalanine residue in the dimer interface (azoAla-*Bam*HI) were synthesized; while the activity was almost suppressed using *trans*-azoAla-*Bam*HI, the *cis*-isomer generated with photoirradiation recovered its intrinsic activity.

Communication between multiple proteins plays an important role in the signal transduction, transcription, and various cascades in the living system. The protein–protein interaction is achieved mainly through non-covalent bonding such as hydrogen bonding (H-bonding), electrostatic interactions, and hydrophobic interactions.[1–4] Since precisely arranged structures of protein complexes are supported by the several amino acids involved in these interactions, when one key amino acid is mutated, the activity of these protein complexes is drastically reduced or abolished.[5,6] We have previously controlled the activity of a restriction enzyme *Bam*HI, which is activated through dimer formation,[7–9] by arranging the salt-bridge network in the dimer interface by introducing the photoremovable 6-nitroveratryl groups into the side chains of the specific amino acids.[10]

In this report, we intended to control the association mode of the *Bam*HI dimer by introducing an azobenzene moiety into specific positions in the dimer interface. *trans–cis* Photoisomerization of the azobenzene moiety is well-characterized and is employed to regulate the conformation and subsequent properties of macromolecules with irradiation of the specific wavelengths.[11–15] To photochemically regulate the enzymatic activity, we replaced the key amino acids in the dimer interface of *Bam*HI with a phenylazophenylalanine (azoAla) residue for photochemical control of the local structure and subsequent activation through the mechanical movement of the azobenzene moiety. In the dimer interface, side chains of four amino acids K132, H133, E167, and E170 consist of a salt bridge network to maintain the conformation of the *Bam*HI dimer in an active form (Fig. 1).[7–9] Thus, we used the three positions (132, 167, and 170) for introduction of the azoAla residue, since these sites involve the key amino acid K132 which shares H-bonding with two glutamate residues E167 and E170 in the dimer interface.[10]

Site-selective incorporation of azoAla was achieved by an *in vitro* translation system with mRNA containing a four base codon (CGGG) and azoAla-tRNA$_{CCCG}$ (Fig. 2).[10,15–17] The full-length azoAla-incorporated *Bam*HI mutants were obtained only in the presence of the azoAla-tRNA$_{CCCG}$ [compare absence (−) and presence (+) of azoAla-tRNA$_{CCCG}$], indicating that the azoAla residue was site-selectively incorporated into these positions. The efficiencies for the incorporation of the K132, E167, and E170 sites were 52, 64, and 43%, respectively.

To examine the properties of the azoAla functionalized *Bam*HI mutants, photoirradiation was performed at 0 °C using a high pressure mercury lamp (500 W) equipped with a monochromator for extraction of 366 nm light.[18] The photoirradiated enzymes were incubated with a substrate DNA to examine the activity. As shown in Fig. 3, K132A^{azo} mutant without irradiation almost suppressed the activity (3.0% cleavage of a substrate DNA) (lane 3). In addition, the K132A^{azo} mutant recovered the intrinsic activity (98% cleavage) after photoirradiation (lane 4).[19] This activity and

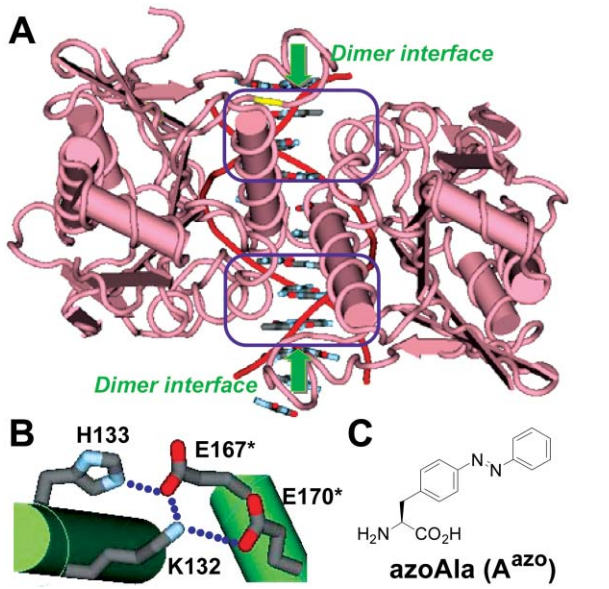

Fig. 1 *Bam*HI dimer structure. (A) Side view of the *Bam*HI dimer–DNA complex (PDB 1BHM). (B) The detailed interaction of amino acid side chains in the dimer interface as shown in the boxes in Fig. 1A. K132 and H133 are located on the same α-helix and E167* and E170* are on the counterpart α-helix. (C) Photofunctionalized amino acid phenylazophenylalanine (azoAla) employed in this experiment.

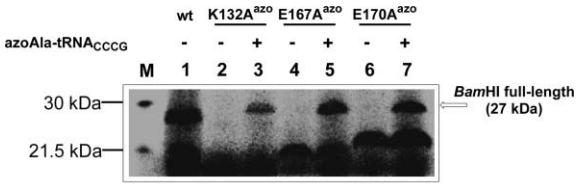

Fig. 2 *In vitro* translation of azo-*Bam*HI mutants labelled with [^{35}S]-methionine. Lane 1, wild-type *Bam*HI; lanes 2 and 3, translation with K132 mutant mRNA in the absence and presence of azoAla-tRNA$_{CCCG}$, respectively; lanes 4 and 5, translation with E167 mutant mRNA in the absence and presence of azoAla-tRNA$_{CCCG}$, respectively; lanes 6 and 7, translation with E170 mutant mRNA in the absence and presence of azoAla-tRNA$_{CCCG}$, respectively.

sequence selectivity were similar to those of the wild-type *Bam*HI (lane 2). The results show that the activity of the K132A^{azo} mutant can be regulated through the photoisomerization of the azobenzene moiety. In the previous study, we identified that the key amino acid to control the activity is the K132 residue using the protection and deprotection of the ε-amino group with a photoremovable group.[10] We also showed that one H-bonding between H133 and E167 is sufficient for exhibition of the activity of *Bam*HI.[10] The result obtained in this experiment indicates that the *trans*-isomer of the azobenzene moiety would change the local structure of the dimer interface for an inactive dimer complex.

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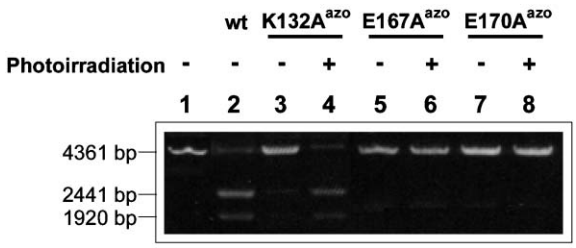

Fig. 3 Cleavage of a substrate DNA (pBR322 digested by *Nde*I) with wild type and photofunctionalized *Bam*HI mutants before and after photoirradiation at 366 nm. Lane 1, substrate DNA (4361 bp); lane 2, DNA fragments (1920 and 2441 bp) cleaved by wild type *Bam*HI; lanes 3 and 4, DNA cleavage with K132A^{azo} before (−) and after (+) photoirradiation, respectively; lanes 5 and 6, DNA cleavage with E167 A^{azo} before (−) and after (+) photoirradiation, respectively; lanes 7 and 8, DNA cleavage with E170 A^{azo} before (−) and after (+) photoirradiation, respectively.

Previous study revealed that the *Bam*HI mutant having a 6-nitroveratryl group at the K132 position showed the activity.[10] The results suggest that the long butyl side chain of lysine would be easily excluded to outside of the dimer interface because of its flexibility, while the short methylene chain of azoAla constrains the direction of the bulky azobenzene moiety, resulting in the generation of steric hindrance against the counterpart α-helix and consequent abolishment of the activity. Recovery of the activity after the photoirradiation means the correct *Bam*HI dimer formation with the *cis*-isomer. The compact *cis*-azobenzene moiety would not perturb the dimer formation, and permits the correct *Bam*HI dimer formation to exhibit the activity.

Incorporation of the azoAla into the 167 and 170 positions abolished the activity, indicating that these glutamate residues cannot be displaced to other amino acids or serious misfolding caused by introduction of the bulky azoAla residue into these positions.

Using the K132A^{azo} mutant, the dependence of the photoirradiation time on the DNA cleaving activity was examined (Fig. 4). During photoirradiation for 5 min, the activity of the enzymes gradually increased, and the *trans* to *cis* photoisomerization in the K132A^{azo} mutant was completed in 5 min. In the cases of irradiation for more than 5 min, the activity reached the saturated level. Photoisomerization of *trans*-isomer of an azoAla monomer to *cis*-isomer occurs rapidly, and the production of 80% *cis*-form is accomplished within 20 s under the same photoirradiation conditions.[20] The result suggests that the movement of the azobenzene moiety at the 132 position would be restrained under the environment in the dimer interface, which affects the rate controlling of the photoisomerization. Interestingly, the activity level of this azobenzene functionalized enzyme can be adjusted depending on the irradiation time. The wavelength used here can selectively induce n–π* transition of the azobenzene moiety where the *trans* to *cis* photoisomerization proceeds through the ultra-fast inversion process.[21] This means that the population of the active (*cis*-isomer) and inactive (*trans*-isomer) *Bam*HI can be purposely controlled by photoirradiation time.

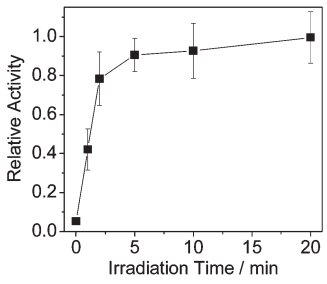

Fig. 4 Recovery of the activity of the K132A^{azo} mutant with photoirradiation (366 nm). Photoirradiation was carried out at 0 °C for 0, 1, 2, 5, 10, and 20 min, and then the samples were incubated with a substrate DNA at 30 °C for 2 h.

We also tried the *cis* to *trans* photoisomerization with irradiation at 436 nm. No substantial change of the activity was observed even after the irradiation. Once the enzyme acquires the activity after the correct dimer formation, it may be difficult for the regenerated *trans*-azobenzene moiety to change the conformation of the dimer interface to an inactive conformation.

In conclusion, we have successfully regulated the activity of *Bam*HI by incorporating the azoAla residue into the dimer interface. This strategy can be extended to control the association of multiple proteins in the signal transduction and transcription.

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17 Translation reaction was carried out using *E. coli* S30 Extract system (Promega) in 10 μL of reaction mixture containing mRNA (2–3 μg), azoAla-tRNA$_{CCCG}$ (1 μg), 0.10 mM amino acid mixture (lacking methionine and arginine), 0.01 mM arginine, 4 μL of premix, 3 μL of *E. coli* S30 extract, and [^{35}S]-methionine (3 μCi) at 30 °C for 3 h. Wild-type *Bam*HI was prepared in the same fashion without azoAla-tRNA$_{CCCG}$. The mixtures of proteins were denatured in a solution containing 50 mM Tris-HCl (pH 6.8), 0.1 M DTT, and 2% SDS, and loaded onto 18% SDS-polyacrylamide gel for electrophoresis. The SDS-PAGE gels were visualized and quantified by an imaging analyzer (Fujix BAS1000 analyzer).
18 Photoirradiation was carried out using a 500 W ultra high pressure mercury lamp (Ushio USH-500D; 500 W) equipped with a monochromator (Ritsuoyo Kogaku MC-10N) which can control specific wavelength within 4 nm full-width at half maximum. *Trans* to *cis* photoisomerization was carried out using the translation mixture prepared above with irradiation of 366 nm light on ice for 20 min. The translation mixtures (1 μL) before or after photoirradiation were diluted by a reaction buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, and 0.01% BSA, and then linearized pBR322 (18 μM) digested by *Nde*I was added. The reaction mixtures were incubated at 30 °C for 2 h. The reaction mixtures were loaded onto a 1.0% agarose gel in TBE buffer. The gels were visualized by ethidium bromide staining and quantified by ImageJ program (NIH).
19 The activity of wild-type *Bam*HI was not affected by addition of azoAla-tRNA and following photoirradiation, indicating that trace of azoAla derivatives does not work as an inhibitor of *Bam*HI.
20 *Trans to cis* photoisomerization of an azoAla monomer was performed in a 50% methanolic solution containing 0.05 mM azoAla and 10 mM Tris-HCl (pH 8.0) at 0 °C using the same equipment (366 nm). After photoirradiation, the samples were characterized by a UV/vis spectrometer.
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