# Chapter 10

# Creation of Antigen-Dependent β-Lactamase Fusion Protein Tethered by Circularly Permuted Antibody Variable Domains

**Hiroto Iwai, Miki Kojima-Misaizu, Jinhua Dong, and Hiroshi Ueda**

## Abstract

Antibody-based molecular switches that are able to recognize a range of exogenous antigens can be highly useful as a versatile biosensor. However, regulating the catalytic activity of enzymes by antibodies is still hard to achieve. Here, we describe a design method of unique antibody variable region Fv introduced with two circular permutations, called Clampbody. By tethering the Clampbody to a circularly permuted TEM-1 β-lactamase (BLA), we successfully constructed a genetically encoded molecular switch Cbody-cpBLA that shows antigen-dependent catalytic activity.

**Key words** TEM-1 β-lactamase, Antibody variable region, Circular permutation, Allosteric regulation, Immunosensor

## 1 Introduction

Regulating the catalytic activity of enzymes by exogenous substances is potentially useful for the detection of biomarkers, environmental pollutants, and for enzyme-based prodrug therapies [1, 2]. To create the molecular switches that can recognize a wide variety of molecules, methods to control the catalytic activity of an enzyme by means of antibody domains are considered highly important [3].

To date, there have been a number of fusion proteins of antibody domains and enzymes whose catalytic activity was regulated by the binding antigen-dependent binding to their cognate antibody [4, 5]. Recently, we reported a fusion protein consisting of a circularly permuted TEM-1 β-lactamase (cpBLA) and Fv, called Fv-cpBLA [6]. This protein switch was made on a cpBLA that had been successfully used to make a maltose-dependent switch when fused with a circularly permuted maltose-binding protein (cpMBP) [7–9]. Fv-cpBLA showed antigen-dependent catalytic activation

Viktor Stein (ed.), *Synthetic Protein Switches: Methods and Protocols*, Methods in Molecular Biology, vol. 1596,
DOI 10.1007/978-1-4939-6940-1_10, 

and antigen-dependent growth-enhancement of *E. coli* expressing the fusion protein in the presence of ampicillin. However, the increase of the specific activity was only up to 25% and there was a room for improvement.

More recently, aiming to transduce the antigen-dependent binding signal more effectively to the catalytic domain, we tried to optimize Fv-cpBLA by recombining the antibody domains to cpBLA more directly instead of using commonly employed flexible linkers [10]. The distance between the N- and C-termini of antibody variable domain $V_H$ and $V_L$ is between 30 and 40 Å. This is considered too long and may disrupt the optimal orientation of protein fragments or the conformation of circularly permuted enzymes. Therefore, based on the previous report [11], we focused on the 3 and 3b loops located far from antigen-binding loops but near the domain interface between $V_H$ and $V_L$ (Fig. 1a). Thus, we

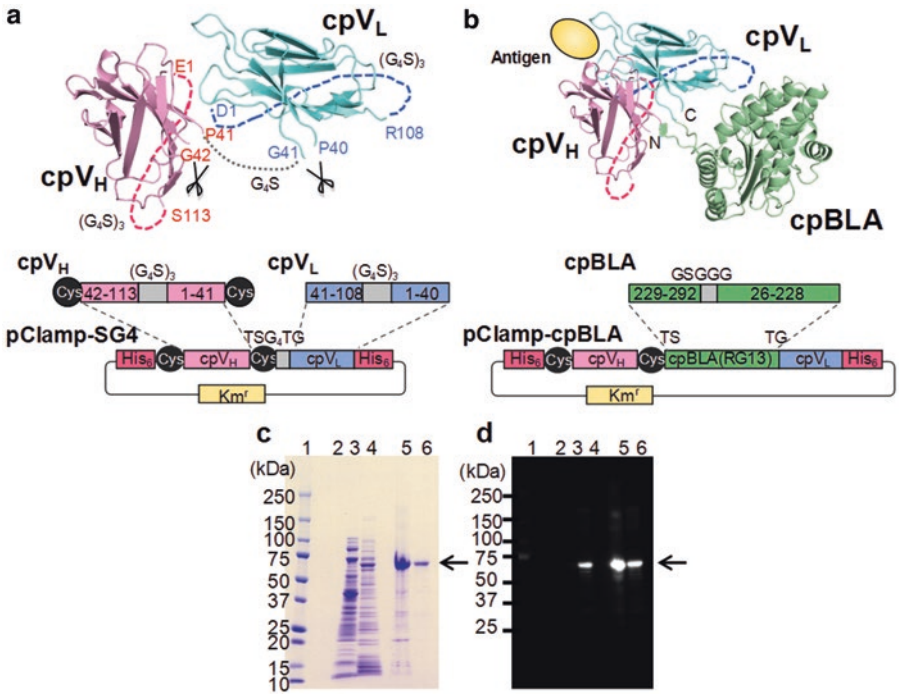


**Fig. 1** Creation of Clampbody and its fusion to cpBLA. (**a**) Schematic structure of single chain Clampbody (sc-Cbody). cpV$_H$ and cpV$_L$ chains are shown in *magenta* and *cyan*, respectively. The (G$_4$S)$_3$ linkers connecting their native N- and C-termini are drawn as *dashed lines*. Two cysteine residues were inserted at the N- and C-termini of cpV$_H$ to promote correct folding via disulfide linkage. The SG$_4$ linker connecting cpV$_H$ and cpV$_L$ is drawn as *dotted line*. (**b**) Schematic structure of Cbody-cpBLA, in which the structure of cpBLA (PDB code 4DXB) is shown in *green*. (**c**, **d**) Expression and purification of Cbody-cpBLA. (**c**) CBB-stained SDS – PAGE. (*1*) MW marker, (*2*) concentrated culture supernatant, (*3*) soluble fraction from the cell lysate, (*4*) insoluble fraction from the same, (*5*) solubilized insoluble protein, (*6*) purified and refolded protein. (**d**) Western blot to detect the His tag. The lanes are the same as in (**c**). The bands for Cbody-cpBLA are indicated by *arrows*. Reproduced from Iwai et al. [10] with permission from American Chemical Society

designed a novel antibody Fv domain format whose termini were located at these sites, and named this doubly circularly permuted antibody Fv as Clampbody. In addition, we also designed a fusion protein comprising Clampbody and cpBLA by connecting $cpV_H$ and $cpV_L$ to the N- and C-termini of cpBLA [7] with a minimum number of linker residues between them (Clampbody-cpBLA, Cbody-cpBLA) (Fig. 1b).

We demonstrated the specific antigen binding of Clampbody, and more importantly, the antigen-dependent catalytic activity of novel molecular switch Cbody-cpBLA. Interestingly, we also found that the antigen-dependency of Cbody-cpBLA became more significant in reaction buffers containing some denaturant or detergent such as urea and Triton X-100. From these results, we reasoned that Cbody-cpBLA was stabilized by the binding of the antigen peptide, and therefore showed antigen-dependent catalytic activity. This would be a good example of the allosteric regulation driven by ligand-induced stabilization or thermodynamic shift, which is collectively called "ensemble" model [12–15]. By optimizing the reaction condition, Cbody-cpBLA could detect their antigen peptide specifically at single nM level. Considering the variety of available substrates, Cbody-cpBLA would be applied to biosensors, prodrug therapies, and possibly to bacterial growth regulation. We also propose Clampbody as a useful tool in antibody-based sensing proteins such as protein complementation assay [16], fluorescence resonance energy transfer-based assay [17, 18], and transduction assay of reaction intermediate [19, 20]. Taking advantage of the proximity of their termini, Clampbody might be fused with many protein domains and fragments, and therefore be able to control the distance and orientation of fused protein(s) in an antigen-dependent manner.

## 2 Materials

Prepare all solutions using ultrapure water (made by purifying deionized water to attain resistivity of 18 MΩ cm at 25 °C). For all the reagents, use them of the highest grade available. The chemicals and other reagents, unless otherwise indicated, were obtained from Sigma (St. Lois, MO), Nacalai Tesque (Kyoto, Japan), or Wako Pure Chemical Industries (Osaka, Japan).

### 2.1 Construction of Clampbody Genes

1. Oligonucleotides (*see* Table 1) (e.g., Greiner Japan, Tokyo, Japan; Eurofins genomics, Tokyo, Japan).
2. High-fidelity thermostable DNA polymerase (e.g., KOD FX, Toyobo, Osaka, Japan; PrimeSTAR® Max DNA Polymerase, Takara-Bio, Shiga, Japan).

**Table 1**
**Oligonucleotides used in this study**

| Name | DNA sequence |
|---|---|
| NcoVHrev | 5′-GATGGCCATGGCCGAGG ACAGCTGGAG-3′ |
| VHSpefor | 5′-TGTTACTAGTTGAGGAGACGGTGACCGT-3′ |
| AgeVLrev | 5′-CAAAACCGGTGATATTGTGCTGACCCAA-3′ |
| VLNotfor | 5′-CGTGCGGCCGCCCGTTTTATTTCCAGC-3′ |
| NcoCcpVHrev | 5′-GATGGCCATGGCCGGACAAGGCCTTGAATGGATC-3′ |
| VHG4Sfor | 5′-CGACCCGCCACCGCCGCTGCCACCTCCGCCTGAACCGCCTCCACCGCTGGAGACGGTGACCGTGGTC-3′ |
| G4S3VHrev | 5′-GGTGGAGGCGGTTCAGGCGGAGGTGGCAGCGGCGGTGGCGGGTCGGAGGTACAGCTGGAGGAG-3′ |
| cpVHCSpefor | 5′-CGGACTAGTACATGGGCTCTGTTTGACCCAGTG-3′ |
| AgecpVLrev | 5′-GATACCGGTGGCCAGTCTCCAAAGCTCCTG-3′ |
| VLG4Sfor | 5′-AGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCCCGTTTTATTTCCAGCTTGG-3′ |
| G4S3VLrev | 5′-GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCTGATATTGTGCTGACCCAATCT-3′ |
| cpVLNotfor | 5′-CGTGCGGCCGCTGGCTTCTGCAGGTACCAATG-3′ |
| MCScpBLArev | 5′-CCTCCATGGTACGAGATCTCAATCTCGAGGCAAACTAGTAACAATGAAGCCATACCAAACG-3′ |
| cpBLAMCSfor | 5′-GGGTGCGGCCGCATTGGTCGACATGGGATATCAGAGACCGGTTTTGGCTTCATTCAGCTCCGGT-3′ |
| InfVHSpeRG13rev | 5′-GTCTCCTCAACTAGTTGGTTTATTGCTGATAAATC-3′ |
| RG13OL1for | 5′-GACCACCTCCTGAACCCCAATGCTTAATCAGTGA-3′ |
| RG13OL2rev | 5′-GGTTCAGGAGGTGGTCACCCAGAAACGCTGGTG-3′ |
| RG13AgeVLInffor | 5′-GCACAATATCACCGGTGCCAGCCGGAAGGGCCGA-3′ |
| VHBGPEAAAK2Spefor | 5′-CGGACTAGTCTTGGCAGCCGCCTCTTTAGCCGCGGCTTCGCTCGAGACGGTGACCGTG-3′ |
| AgeEAAAK2EcoVLrev | 5′-GATACCGGTGAAGCCGCGGCTAAAGAGGCGGCTGCCAAGGATATCGTGCTGACCCAATC-3′ |
| TSG4TGrev | 5′-CTAGTGGAGGTGGCGGTA-3′ |
| TSG4TGfor | 5′-CCGGTACCGCCACCTCCA-3′ |

3. DNA purification kits (e.g., Wizard® SV Gel and PCR Clean-Up System, Promega, WI).
4. Restriction enzymes (e.g., New England Biolabs, Beverly, MA).
5. DNA ligation kit (e.g., Ligation high ver. 2, Toyobo).
6. BGP C-terminal peptides (e.g., Genscript, Piscataway, NJ).
7. pET26/Fv-cpBLA (previously described [6]).
8. pIT2(13CG2) (previously described [21]).
9. pET30b(+) (Novagen, Darmstadt, Germany).
10. pCR4Blunt-TOPO (Zero Blunt TOPO PCR Cloning Kit for Sequencing, Thermo Fisher Scientific, Waltham, MA).
11. In-Fusion® HD cloning kit (Clontech, CA).
12. Tris-EDTA buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.4.

### 2.2 Protein Expression

1. SHuffle T7 express lysY competent cells (New England Biolabs).
2. LB medium: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0.
3. LBK medium: LB containing antibiotics (50 μg/mL kanamycin).
4. Agar.
5. 1 M Isopropyl-β-d-thiogalactopyranoside (IPTG).
6. Supersonic homogenizer (e.g., VP-15S Taitec, Saitama, Japan).
7. 2-mercaptoethanol.
8. 0.5 M EDTA, pH 8.0.
9. Guanidium hydrochloride (Gdn-HCl).
10. TALON extraction buffer: 300 mM NaCl, 50 mM sodium phosphate, adjusted to pH 7.0.
11. TALON metal affinity resin and TALON disposable gravity column (Clontech, CA).
12. TALON elution buffer: TALON extraction buffer supplement with 200 mM imidazole.
13. Pierce™ BCA protein assay kit for determining protein concentrations (Thermo Fisher Scientific, Waltham, MA).

### 2.3 Refolding of Clampbodies

1. Slide-A-Lyzer Dialysis Cassettes, 3.5 K MWCO, 3 mL (Thermo Fisher Scientific).
2. Dialysis buffer: 50 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 8.0.

### 2.4 Western Blot of Clampbodies

1. SDS-PAGE gradient gels (5 ~ 20%) (e.g., e-PAGEL, ATTO, Tokyo, Japan).
2. SDS running buffer: 25 mM Tris–HCl, 192 mM l-glycine, 1 g/L sodium dodecyl sulfate.
3. PVDF membrane (e.g., Clear Blot Membrane –P plus, ATTO).

4. Transfer buffer: 25 mM Tris–HCl, 192 mM l-glycine.
5. Tris buffered saline (TBS): 50 mM Tris–HCl, 150 mM NaCl, pH 7.4.
6. TBS with Tween (TBST): TBS supplemented with 0.05% of Tween 20.
7. TBST with skim milk (TBST-S): TBS-T supplemented with 5% skim milk.
8. HRP-conjugated anti-$His_6$ antibody (e.g., Roche Diagnostics Japan, Tokyo, Japan).
9. Amersham ECL Prime (GE Healthcare, UK).
10. Chemiluminescence imager (e.g., LAS-4000 mini Luminoimager, Fujifilm, Tokyo, Japan).

### 2.5 ELISA for Clampbodies

1. Transparent 96-well microplates (e.g., Greiner 655,061).
2. 10 μg/mL streptavidin (e.g., type II, Wako, Osaka, Japan).
3. Phosphate buffered saline (PBS): 1.47 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, 2.7 mM KCl, 137 mM NaCl, pH 7.4.
4. PBST: PBS containing 0.1% Tween 20.
5. Blocking solution (e.g., Immunoblock, DS Pharma, Osaka, Japan).
6. 1 μg/mL N-terminally biotinylated BGP-C11 peptide in PBST.
7. TMBZ reaction buffer: 100 μg/mL TMBZ (3,3′,5,5′-tetramethylbenzidine), 0.006% $H_2O_2$, 100 mM NaOAc, pH 6.0.
8. 10% $H_2SO_4$ to stop the reaction.

### 2.6 Enzyme Assays for Clampbodies

1. Black 96-well microplates (e.g., Corning Costar 3693).
2. Transparent 96-well microplates (e.g., Greiner 655061).
3. Dimethyl sulfoxide (DMSO).
4. Fluorocillin™ Green 495/525 β-Lactamase Substrate, soluble product (Thermo Fisher Scientific, Waltham, MA). 1 mM stock solution in DMSO can be stored for several months at −30 °C.
5. Nitrocefin (e.g., Calbiochem, Darmstadt, Germany). 20 mM stock solution in DMSO can be stored for several months at −30 °C.
6. PBS containing 1% Triton X-100.
7. PBS containing 3 M urea.
8. PBS containing 20 mg/mL bovine serum albumin (BSA) (e.g., Sigma-Aldrich, St. Lois, MO).
9. A fluorescence microplate reader (e.g., GENios Pro Tecan, San Jose, CA).
10. A microplate reader (e.g., SH-1000, Corona Electric, Hitachi, Ibaraki, Japan).

## 3 Methods

### 3.1 Construction of the $V_H$ and $V_L$ genes for Anti-BGP C-Terminal Peptide Antibody

To create a Clampbody as an antigen-dependent switch, any antibody for small molecule antigen, whose variable region (Fv = $V_H$ + $V_L$) is stabilized by the bound antigen, will suffice. Here, we employ the $V_H$ and $V_L$ domains of antibody KTM219 [22], which recognizes the C-terminal peptide of the human bone Gla protein (BGP) as a model. First, clone the $V_H$ and $V_L$ genes by the following procedure.

1. Amplify the DNA fragment encoding $V_H$ fragment of the antibody by PCR using the primers NcoVHrev and VHSpefor, pET26/Fv-cpBLA as a template, and KOD FX DNA polymerase and the DNA fragment encoding $V_L$ fragment of the antibody by PCR with AgeVLrev and VLNotfor as primers.
2. Purify the resulting DNA fragments by Wizard® SV Gel and PCR Clean-Up System as per manufacturer's instructions.
3. Ligate these fragments into pCR4Blunt-TOPO as per manufacturer's instructions.

### 3.2 Construction of the $cpV_H$ Gene

Make a construct for a circularly permuted $V_H$ ($cpV_H$), whose new termini are generated between Pro41 and Gly42 (as described according to Kabat numbering) while the native N- and C-termini are connected via a flexible linker $(G_4S)_3$. Considering the possible lesser stability of $V_H$ fragment, two Cys residues are added at the N- and C-termini of $cpV_H$ to promote the correct folding and increased stability through the disulfide bridge.

1. Amplify the DNA fragment encoding Gly42 to Ser113 of $V_H$ with a linker peptide $(G_4S)_3$ at the C-terminus by PCR using the primers NcoCcpVHrev and VHG4Sfor, pET26/Fv-cpBLA as a template, and KOD FX DNA polymerase.
2. Amplify the DNA fragment encoding Glu1 to Pro41, described according to Kabat numbering, with the linker at the N-terminus by PCR using G4S3VHrev and cpVHCSpefor as primers.
3. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.
4. Perform overlap-extension PCR with the gel-purified PCR products (20 ng each) and KOD FX for 15 cycles without primers and 35 cycles with NcoCcpVHrev and cpVHCSpefor primers.
5. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.
6. Ligate into pCR4Blunt-TOPO.

### 3.3 Construction of the cpV$_L$ Gene

Similarly, make a construct for the circularly permuted $V_L$ (cp$V_L$) with new termini between Pro40 and Gly41. In this case, two Cys residues at the N- and C-termini of cp$V_L$ are not added to avoid unfavorable interdomain crosslinking at the later stage.

1. Amplify the DNA fragment encoding Gly41 to Arg108 of $V_L$ with a linker peptide $(G_4S)_3$ at the C-terminus by PCR using the primers AgecpVLrev and VLG4Sfor, pET26/Fv-cpBLA as a template, and KOD FX DNA polymerase.
2. Amplify the DNA fragment encoding Asp1 to Pro40 with the linker at the N-terminus by PCR with G4S3VLrev and cpVL-Notfor as primers.
3. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.
4. Perform overlap-extension PCR with the gel-purified PCR products (20 ng each) and KOD FX DNA polymerase for 15 cycles without primers and 35 cycles with AgecpVLrev and cpVLNotfor primers.
5. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.
6. Ligate into pCR4Blunt-TOPO.

### 3.4 Construction of the cpBLA(RG13) Gene

To make a fusion protein comprising Clampbody and cpBLA, first make a construct for the cpBLA with known 3D structure that Guntas et al. reported [23, 24].

1. Amplify the DNA fragment encoding Trp227 to Trp286 of TEM-1 β-lactamase with a short peptide linker (GSGGS) at the C-terminus by PCR using the primers InfVHSpeRG13rev and RG13OL1for, pIT2/31IJ3 as a template, and PrimeSTAR® Max DNA Polymerase.
2. Amplify the fragment encoding His24 to Gly226 with the linker at the N-terminus by PCR with RG13OL2rev and RG13AgeVLInffor as primers.
3. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.
4. With the gel-purified PCR products (20 ng each), perform overlap-extension PCR with PrimeSTAR® Max DNA Polymerase for 15 cycles without primers and 35 cycles with InfVHSpeRG13rev and RG13AgeVLInffor primers.
5. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.

### 3.5 Construction of Cbody-cpBLA Fusion Protein Expression Vectors

Make a construct for a fusion protein comprising Clampbody and cpBLA by connecting cp$V_H$ and cp$V_L$ to the N- and C-termini of cpBLA [7] with a minimum number of amino acid residues (Clampbody-cpBLA, Cbody-cpBLA) (Fig. 1b, c).

1. Amplify the DNA fragment encoding *Nco*I, *Spe*I, *Age*I, and *Not*I sites by PCR using the primers MCScpBLArev and cpBLAMCSfor, pET26/Fv-cpBLA as a template, and KOD FX DNA polymerase.
2. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System.
3. Digest the gel-purified PCR product (2 μg) with *Nco*I HF and *Not*I HF.
4. Purify the resulting DNA fragments using the Wizard® SV Gel and PCR Clean-Up System and insert into pIT2(13CG2) digested with the same, resulting in pIT2/MCS.
5. Digest the $cpV_H$ gene with *Nco* I and *Spe* I.
6. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System and inserted into pIT2/MCS digested with the same.
7. After the $cpV_H$ insertion, digest the $cpV_L$ gene with *Age* I and *Not* I.
8. Purify these fragments using Wizard® SV Gel and PCR Clean-Up System and inserted into $cpV_H$-inserted pIT2/MCS.
9. Digest these plasmids with *Nco*I and *Not*I.
10. Purify these fragments by Wizard® SV Gel and PCR Clean-Up System and insert into pET30b(+) digested with the same, resulting in pET30b/cpVH-cpVL.
11. Insert the cpBLA(RG13) gene into pET30b/cpVH-cpVL with In-Fusion® HD Cloning Kit according to the manufacturer, resulting in pClamp-cpBLA.

### 3.6 Construction of scCb Expression Vectors

Prepare a single-chain protein construct where $cpV_H$ and $cpV_L$ are connected by a short linker $G_4S$ (single-chain Clampbody, scCb) to evaluate the antigen-binding activity of the Clampbody.

1. Anneal TSG4TGrev and TSG4TGfor in Tris-EDTA buffer to prepare the DNA fragment encoding the $SG_4$ linker.
2. Insert the fragment into pClamp-cpBLA digested with *Age*I and *Spe*I, resulting in pClamp-SG4.

### 3.7 Expression and Purification of Clampbodies [6, 25]

When Clampbody and Cbody-cpBLA are expressed in *E. coli*, the majority of the expressed proteins will accumulate in inclusion body even if cultured at lower temperature. In such cases, perform the following procedures to solubilize the insoluble Clampbodies.

1. Transform SHuffle T7 Express lysY competent cells with expression vectors (*see* **Note 1**).

2. Incubate the transformed *E. coli* on a LB medium containing antibiotics (50 μg/mL kanamycin) (LBK) and 1.5% agar at 37 °C for 24 h.
3. Pick one colony up and culture it in 4 mL LBK medium at 37 °C.
4. Inoculate the 1 mL of cultured cells to 250 mL of LBK medium in a 1000 mL flask, and culture in 37 °C.
5. When the $OD_{600}$ reaches 0.4–0.6, add to a final concentration of 0.4 mM of IPTG, and culture the cells at 16 °C overnight.
6. Centrifuge at 10,000 × *g* for 15 min at 4 °C to collect the cells (*see* **Note 2**).
7. Resuspend the pellet with 30 mL of TALON extraction buffer and sonicate the *E. coli* on ice using a supersonic homogenizer VP-15S for 2 min (50% interval) three times.
8. Centrifuge the sonicated solution at 10,000 × *g* for 60 min at 4 °C (*see* **Note 3**).
9. Resuspend the pellet with 25 mL of distilled water containing 1% Triton-X100 and 1 mM EDTA.
10. Centrifuge at 75,000 × *g* for 15 min at 4 °C. Resuspend pellet with 25 mL of distilled water containing 1% Triton-X100 and 1 mM EDTA.
11. Repeat **step 11** to wash the pellet.
12. Resuspend the pellet with 10 mL of TALON extraction buffer containing 6 M guanidium hydrochloride (Gdn-HCl) to solubilize it.
13. Centrifuge at 75,000 × *g* for 15 min at 4 °C and collect the supernatant carefully.
14. Add 200 μL of TALON Metal Affinity Resin to the supernatant, and rotate or shake gently for 20 min (*see* **Note 4**).
15. Centrifuge at 700 × *g* for 2 min, and resuspend the resin with 10 mL of TALON extraction buffer containing 6 M Gdn-HCl.
16. Rotate or shake gently for 10 min.
17. Repeat **steps 11** and **12** twice.
18. Centrifuge at 700 × *g* for 2 min, and collect the resin.
19. Suspend the resin with 3 mL of TALON extraction buffer containing 6 M Gdn-HCl, and put the suspension into a TALON 2-mL Disposable Gravity Column.
20. Allow to drain until it reaches the top of the resin bed.
21. Wash the column with 2 mL of TALON extraction buffer containing 6 M Gdn-HCl.
22. Elute the protein with 1.5 mL of TALON elution buffer containing 5.4 M Gdn-HCl.

### 3.8 Refolding of the Solubilized Proteins

Refold the purified denatured Clampbodies by stepwise dialysis procedure as below.

1. Add 1.05 μL of 2-mercaptoethanol and 3 μL of 0.5 M EDTA to 1.5 mL of purified solubilized protein.
2. Rotate gently at room temperature for 30 min to reduce disulfide bonds.
3. Inject protein solution into Slide-A-Lyzer Dialysis Cassettes, 3.5 K MWCO, 3 mL.
4. Dialyze protein solution by putting the Slide-A-Lyzer Dialysis Cassettes into 1 L of Dialysis buffer containing 3 M Gdn-HCl. Incubate at 4 °C for overnight (*see* **Note 5**).
5. Dialyze protein against Dialysis buffer containing 2 M Gdn-HCl at 4 °C overnight.
6. Dialyze protein against Dialysis buffer containing 1 M Gdn-HCl at 4 °C overnight.
7. Dialyze protein against Dialysis buffer containing 0.5 M Gdn-HCl at 4 °C overnight.
8. Dialyze protein against Dialysis buffer without Gdn-HCl at 4 °C overnight.
9. Collect the contents in the cassette and centrifuge at 20,000 × *g* for 5 min at 4 °C.
10. Collect the supernatant (*see* **Note 6**). Measure the concentration of purified protein with a Pierce™ BCA protein assay kit as per manufacturer's instructions.

### 3.9 Western Blotting

After the preparation of Clampbodies, perform SDS-PAGE and Western blotting to confirm the purity and the amount of the proteins (Fig. 1c, d).

1. Analyze the proteins by SDS-PAGE using a 5–20% gradient gel and SDS running buffer.
2. Transfer the protein from the gel to a PVDF membrane with Transfer buffer.
3. Incubate the membrane in TBST-S at 4 °C overnight.
4. Wash membrane three times with TBST.
5. Incubate the membrane with HRP-conjugated anti-$His_6$ antibody 4000-times diluted in TBST at 4 °C for 1 h.
6. Wash membrane three times with TBST.
7. Soak membrane in Amersham ECL Prime as per manufacturer's instructions.
8. Detect the luminescence using a LAS-4000 mini Luminoimager.

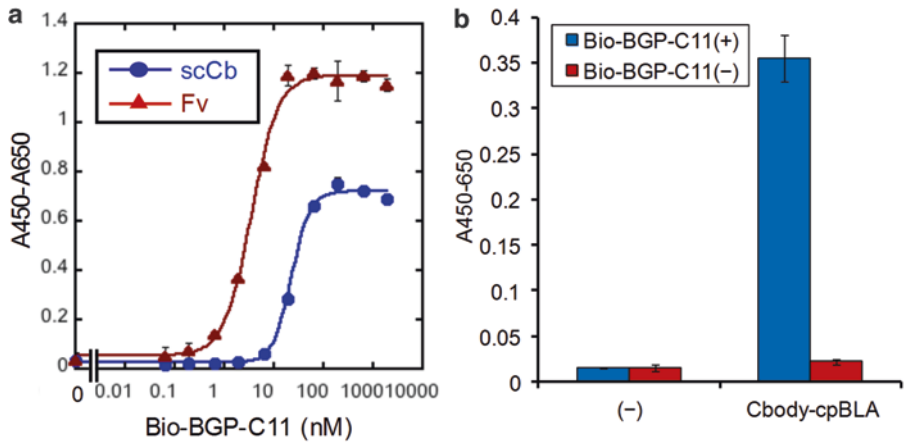


**Fig. 2** Antigen-binding activity of Fv, sc-Cbody, and Cbody-cpBLA. (**a**) Binding of parental Fv (*red triangle*) and sc-Cbody (*blue circle*) to the biotinylated BGP-C11 antigen immobilized at the indicated concentrations. (**b**) Binding of Cbody-cpBLA to the biotinylated BGP-C11 immobilized at 0.9 μM. *Blue* and *red bars* indicate the binding signals to antigen-positive and negative wells, respectively. Averages of three samples with an error bar of 1 SD are shown. Reproduced from Iwai et al. [10] with permission from American Chemical Society

### *3.10 ELISAs of Clampbodies*

Perform following ELISAs to confirm that the refolded Clampbodies retain the antigen-binding activity derived of the parental $V_H$ and $V_L$ domains (Fig. 2a, b).

1. Apply 50 μL per well of 10 μg/mL streptavidin in microplate wells at 4 °C overnight.
2. Discard the supernatant carefully, and then apply 200 μL/well of PBST supplemented with 20% Immunoblock at room temperature for 1 h.
3. Discard the supernatant carefully, and wash wells with PBST three times.
4. Apply 50 μL per well of 1 μg/mL biotinylated BGP-C11 peptide in PBST containing 5% Immunoblock at room temperature for 1 h.
5. Discard the supernatant carefully, and wash wells with PBST three times.
6. Apply 50 μL per well of 100 nM protein samples in PBST containing 5% Immunoblock at room temperature for 1 h. Use approximately 1 μM protein stock and dilute it in PBST containing 5% Immunoblock to make 100 nM working solution immediately before the assay.
7. Discard the supernatant carefully, and wash wells with PBST three times.
8. Apply 50 μL per well of HRP-conjugated anti-$His_6$ antibody 4000-times diluted in PBST containing 5% Immunoblock at room temperature for 1 h.

9. Discard the supernatant carefully, and wash wells with PBST three times.
10. Apply 50 μL per well of TMBZ reaction buffer at room temperature. After several minutes, add 50 μL per well of 10% $H_2SO_4$ to stop the reaction.
11. Detect the absorbance at 450 nm with a reference absorbance at 650 nm.

### 3.11 Clampbody Activity Assay with Fluorocillin

Measure catalytic activity of Cbody-cpBLA using a fluorogenic substrate fluorocillin (Fig. 3). The antigen-dependency of Cbody-cpBLA becomes more significant in reaction buffers containing appropriate amount of denaturant or detergent such as urea and Triton X-100.

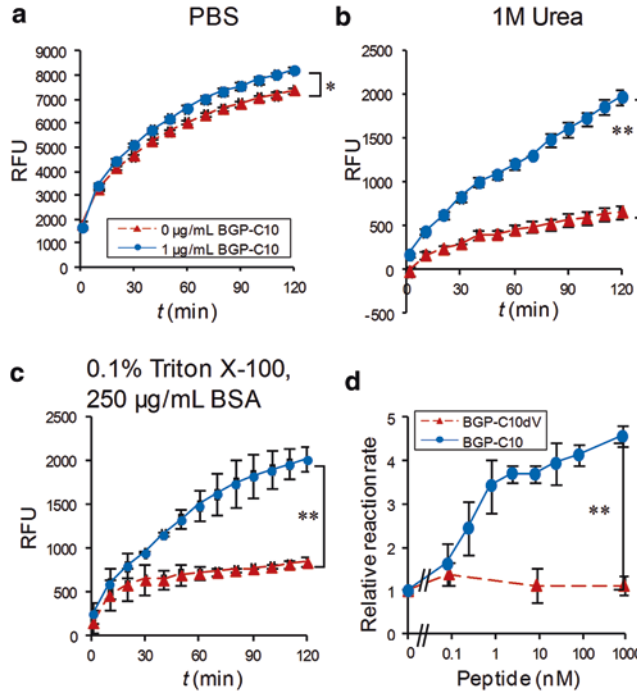


**Fig. 3** Antigen-dependent catalytic activity of Cbody-cpBLA. The solution of 70 nM Cbody-cpBLA, 1 μg/mL BGP-C10, and 1 μM fluorocillin was mixed in (**a**) PBS, (**b**) PBS containing 1 M urea, and (**c**) PBS containing 0.1% Triton X-100 and 250 μg/mL BSA, incubated at 30 °C, and read for fluorescence. Background fluorescence of the samples without Cbody-cpBLA was subtracted for each condition. Averages of three samples with an error bar of 1 SD are shown. *Blue circle* indicates the catalytic activity in the presence of 1 μg/mL BGP-C10, and *red triangle* indicates in the absence of peptide. (**d**) Dose – response curves for BGP-C10 and BGP-C10dV peptides in the reaction buffer as in (**c**). The reaction rates at each peptide concentration are shown as the relative value to the rate in the absence of peptides. Statistical analysis was conducted using the two-tailed unpaired Student's *t* test: (∗) $p < 0.05$, (∗∗) $p < 0.01$. Reproduced from Iwai et al. [10] with permission from American Chemical Society

1. Prepare BGP-C10 ($NH_2$-EAYRRFYGPV-COOH) and BGP-C10dV ($NH_2$-EAYRRFYGP-COOH) as antigen and non-antigen peptide, respectively.
2. Mix 25 μL per well of 140 nM protein samples in PBS and 25 μL per well of antigen peptides in PBS. Incubate for 30 min at room temperature in a black microplate. Use approximately 1 μM protein stock and dilute it in PBS immediately before the assay.
3. Apply 50 μL per well of 2 μM fluorocillin in PBS containing urea, TritonX-100, Tween-20 or BSA, at varying concentrations. Use 1 mM fluorocillin stock solution in DMSO and dilute it in PBS immediately before the assay.
4. Immediately read for their fluorescence intensity at 535 nm with 485 nm at 30 °C for 2 h using a fluorescence plate reader GENios Pro.
5. Fit the initial rates of reactions to the Michaelis-Menten equation as follows:

$$v = V_{max}[S]/(K_m+[S])$$

by the nonlinear least squares algorithm of Kaleidagraph 4.1 (Synergy Software, Reading, PA, USA).

### 3.12 Clampbody Activity Assay with Nitrocefin

Evaluate the catalytic activity of Cbody-cpBLA using a colorimetric substrate nitrocefin, as shown in Fig. 4.

1. Mix 25 μL per well of 140 nM proteins in PBS and 25 μL per well of antigen samples in PBS. Incubate for 30 min at room temperature in transparent microplate. Use approximately 1 μM stock protein and dilute this in PBS immediately before the assay.
2. Apply 50 μL per well of 0–500 μM nitrocefin in PBS containing 2.4 M urea. Use 20 mM nitrocefin stock solution in DMSO and dilute it in PBS immediately before the assay.
3. Immediately read for their absorbance at 486 nm at 30 °C for 1 h using a microplate reader SH-1000.
4. Calculate the catalytic parameters following the same procedures in Subheading 3.11.

## 4 Notes

1. SHuffle T7 Express lysY is an engineered *E. coli* B strain to express proteins containing disulfide bonds in the cytoplasm. Constitutively expressed disulfide bond isomerase DsbC promotes the correction of mis-oxidized proteins into their correct form, and also works as a chaperone that can assist in the folding of proteins that do not require disulfide bonds.

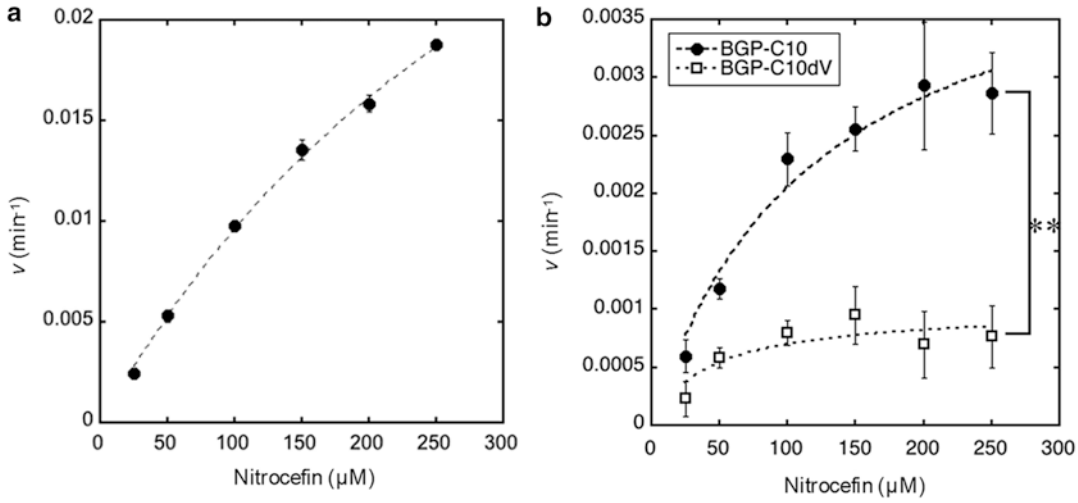


**Fig. 4** S-V plots for BLA and Cbody-cpBLA. (**a**) 2 nM BLA or (**b**) 70 nM Cbody-cpBLA was added with nitrocefin substrate in the presence or absence of antigen 1 μM BGP-C10, in PBS containing 1.2 M urea, incubated at 30 °C, and read for absorbance. Background absorbance of the samples without BLA or Cbody-cpBLA was subtracted for each condition. Initial velocities are plotted against the substrate concentration. Averages of three samples with an error bar for 1 SD are shown. Fitted curves based on the Michaelis-Menten equation are shown for each condition. Statistical analysis was conducted using the two-tailed unpaired Student's *t*-test. (**) $p < 0.01$. Reproduced from Iwai et al. [10] with permission from American Chemical Society

2. Culture supernatant (2 mL) can be precipitated with 10% trichloroacetic acid, and washed twice with cold acetone, and resuspended in 20 μL of PBS and used for SDS-PAGE.
3. The supernatant including soluble protein should be kept for SDS-PAGE.
4. Before adding to the solubilized protein, TALON metal affinity resin should be washed twice with TALON extraction buffer containing 6 M Gdn-HCl.
5. Keep stirring the buffer using a magnetic stirrer.
6. The purified enzyme in the buffer containing 15% glycerol can be stored for several months at −80 °C. The concentration of the proteins in stock solution was typically 1 μM in our hands.

## Acknowledgment

We thank Tatsuya Shinoda of Kyowa Medex Co. for generously allowing the use of the genes derived from KTM-219 IgG. This study was supported by Grant-in-Aid for Scientific Research (Grants 24360336 and 15H04191 to H.U. and Grant 46420793 to J.D.) from JSPS, Japan, and partly by Strategic International Collaborative Research Program, Japan Science and Technology Agency (JST).

## References


1. Stains CI, Furman JL, Porter JR, Rajagopal S, Li Y, Wyatt RT, Ghosh I (2010) A general approach for receptor and antibody-targeted detection of native proteins utilizing split-luciferase reassembly. ACS Chem Biol 5:943–952
2. Alderson RF, Toki BE, Roberge M, Geng W, Basler J, Chin R, Liu A, Ueda R, Hodges D, Escandon E, Chen T, Kanavarioti T, Babé L, Senter PD, Fox JA, Schellenberger V (2006) Characterization of a CC49-based single-chain fragment-β-lactamase fusion protein for antibody-directed enzyme prodrug therapy (ADEPT). Bioconjug Chem 17:410–418
3. Schumacher FF, Sanchania VA, Tolner B, Wright ZVF, Ryan CP, Smith MEB, Ward JM, Caddick S, Kay CWM, Aeppli G, Chester KA, Baker JR (2013) Homogeneous antibody fragment conjugation by disulfide bridging introduces 'spinostics'. Sci Rep 3:1525
4. Yokozeki T, Ueda H, Arai R, Mahoney W, Nagamune T (2002) A homogeneous noncompetitive immunoassay for the detection of small haptens. Anal Chem 74:2500–2504
5. Ueda H, Yokozeki T, Arai R, Tsumoto K, Kumagai I, Nagamune T (2003) An optimized homogeneous noncompetitive immunoassay based on the antigen-driven enzymatic complementation. J Immunol Methods 279(1–2):209–218
6. Kojima M, Iwai H, Dong J, Lim S-L, Ito S, Okumura K, Ihara M, Ueda H (2011) Activation of circularly permutated β-lactamase tethered to antibody domains by specific small molecules. Bioconjug Chem 22:633–641
7. Guntas G, Mitchell SF, Ostermeier M (2004) A Molecular switch created by in vitro recombination of nonhomologous genes. Chem Biol 11:1483–1487
8. Guntas G, Mansell TJ, Kim JR, Ostermeier M (2005) Directed evolution of protein switches and their application to the creation of ligand-binding proteins. Proc Natl Acad Sci U S A 102:11224–11229
9. Nicholes N, Date A, Beaujean P, Hauk P, Kanwar M, Ostermeier M (2016) Modular protein switches derived from antibody mimetic proteins. Protein Eng Des Sel 29:77–85
10. Iwai H, Kojima-Misaizu M, Dong J, Ueda H (2016) Creation of a ligand-dependent enzyme by fusing circularly permuted antibody variable region domains. Bioconjug Chem 27:868–873
11. Brinkmann U, di Carlo A, Vasmatzis G, Kurochkina N, Beers R, Lee B, Pastan I (1997) Stabilization of a recombinant Fv fragment by base-loop interconnection and V(H)-V(L) permutation. J Mol Biol 268:107–117
12. Motlagh HN, Wrabl JO, Li J, Hilser VJ (2014) The ensemble nature of allostery. Nature 508:331–339
13. Choi JH, San A, Ostermeier M (2013) Non-allosteric enzyme switches possess larger effector-induced changes in thermodynamic stability than their non-switch analogs. Protein Sci 22:475–485
14. Choi JH, Ostermeier M (2015) Rational design of a fusion protein to exhibit disulfide-mediated logic gate behavior. ACS Synth Biol 4:400–406
15. Choi JH, Laurent AH, Hilser VJ, Ostermeier M (2015) Design of protein switches based on an ensemble model of allostery. Nat Commun 6:6968
16. Ohmuro-Matsuyama Y, Chung CI, Ueda H (2013) Demonstration of protein-fragment complementation assay using purified firefly luciferase fragments. BMC Biotechnol 13:31
17. Ohiro Y, Ueda H, Shibata N, Nagamune T (2007) Enhanced fluorescence resonance energy transfer immunoassay with improved sensitivity based on the Fab'-based immunoconjugates. Anal Biochem 360:266–272
18. Chung CI, Makino R, Dong J, Ueda H (2015) Open flower fluoroimmunoassay: a general method to make fluorescent protein-based immunosensor probes. Anal Chem 87: 3513–3519
19. Ohmuro-Matsuyama Y, Hara Y, Ueda H (2014) Improved protein-protein interaction assay FlimPIA by the entrapment of luciferase conformation. Anal Chem 86:2013–2018
20. Kurihara M, Ohmuro-Matsuyama Y, Ayabe K, Yamashita T, Yamaji H, Ueda H (2016) Ultra sensitive firefly luciferase-based protein-protein interaction assay (FlimPIA) attained by hinge region engineering and optimized reaction conditions. Biotechnol J 11:91–99
21. de Wildt RM, Mundy CR, Gorick BD, Tomlinson IM (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat Biotechnol 18: 989–994
22. Lim S-L, Ichinose H, Shinoda T, Ueda H (2007) Noncompetitive detection of low molecular weight peptides by open sandwich immunoassay. Anal Chem 79:6193–6200
23. Ke W, Laurent AH, Armstrong MD, Chen Y, Smith WE, Liang J, Wright CM, Ostermeier M, van den Akker F (2012) Structure of an engineered β-lactamase maltose binding protein

fusion protein: insights into heterotropic allosteric regulation. PLoS One 7:e39168

24. Wright CM, Majumdar A, Tolman JR, Ostermeier M (2010) NMR characterization of an engineered domain fusion between maltose binding protein and TEM1 beta-lactamase provides insight into its structure and allosteric mechanism. Proteins 78:1423–1430

25. Tsumoto K, Shinoki K, Kondo H, Uchikawa M, Juji T, Kumagai I (1998) Highly efficient recovery of functional single-chain Fv fragments from inclusion bodies overexpressed in *Escherichia coli* by controlled introduction of oxidizing reagent—application to a human single-chain Fv fragment. J Immunol Methods 219:119–129