Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2017

www.elsevier.com/locate/jbiosc

Function of a thermophilic archaeal chaperonin is enhanced by electrostatic interactions with its targets

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Received 12 January 2017; accepted 11 April 2017
Available online xxx

Molecular chaperonin CpkB from *Thermococcus kodakarensis* possesses a unique negatively charged carboxy-terminal region that functions in target protein recognition. In the present study, green fluorescent protein (GFP), 4-oxalocrotonate tautomerase (4OTA) and glutamine:fructose-6-phosphate amidotransferase (GFAT) were fused with a positively charged tag, selected using docking simulation *in silico*, to enhance their electrostatic interactions with CpkB. Target proteins were heated at 75°C in the presence or absence of CpkB, and the remaining enzymatic activity was measured. The half-life ($t_{1/2}$) of the positively charged tagged targets was significantly longer than that of their tagless counterparts. *Escherichia coli* cell extracts containing heterologously expressed targets (GFP, 4OTA and GFAT and their tagged variants) were incubated at 75°C in the presence or absence of CpkB, and the proportion remaining in the soluble fraction was evaluated by SDS-PAGE. Only positively charged tagged targets remained predominantly in the soluble fraction in the presence of CpkB but not in the absence of CpkB. When tagless or negatively charged tagged targets were employed, the targets were barely detected in the soluble fraction, suggesting that CpkB protected positively charged tagged proteins more efficiently than tagless targets. Attachment of a positively charged tag may be a generally applicable method for enhancing target recognition by chaperonins carrying negatively charged carboxy-terminal regions, such as the archaeal chaperonin CpkB.

[**Key words:** Group II chaperonin; Archaea; Protein trapping; Specificity; Electrostatic interaction; *Thermococcus kodakaraensis*]

Chaperones are generally considered to catalyse the folding of a wide range of target proteins due to their low specificity. Group I chaperonins such as GroEL/ES and some group II chaperonins are highly promiscuous and target a broad range of substrates through hydrophobic interactions, but they exhibit a weak bias towards several structural fold types (1,2). By contrast, the group II chaperonin ring complex/chaperonin TCP-1 (TRiC/CCT) displays higher specificity towards particular proteins through charged and polar interactions between substrates and apical domains of TRiC/CCT subunits (3,4). Group II chaperonin thermosomes assemble into a similar TRiC/CCT structure, and their apical domains are also believed to function in substrate recognition (5). However, the apical domain of thermosomes is less well studied than TRiC/CCT.

The carboxy-terminal regions of chaperonins are highly variable in amino acid sequences and are believed to function in ATP hydrolysis and substrate binding (6–9). The hyperthermophilic archaeon *Thermococcus kodakarensis* grows optimally at 85°C and possesses two chaperonins, the cold-inducible CpkA and the heat-inducible CpkB, which are involved in adaptation to low and high temperatures, respectively (10,11). These two chaperonins share high sequence identity (77%), but differ significantly in their carboxy-terminal regions. CpkA differs from CpkB by possessing a mildly hydrophobic Gly-Gly-Met (GGM) repeat sequence in its carboxy-terminal region, similar to that originally identified in *Escherichia coli* GroEL. By contrast, CpkB has a classic thermosome carboxy-terminal region that is rich in negatively charged residues. In this study, we attempted to enhance the affinity of CpkB for its target proteins by adding a positively charged tag to the target proteins that would increase electrostatic interaction between the tagged target and the negatively charged C-terminal region of CpkB.

MATERIALS AND METHODS

Microorganisms, media and growth conditions The microorganisms (strains) used in this study were *E. coli* DH5α [genotype: F$^-$ Φ80d*lac*ZΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ$^-$ *thi*-1 *gyrA96 relA1*] (Agilent Technology, Santa Clara, CA, USA) and *E. coli* BL21(DE3) codon-plus RIL [genotype: *E. coli* B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ$^-$ (DE3) *endA* Hte (*argU ileY leuW*; Cam^r)] (Agilent Technology). Lysogeny broth (LB) medium containing (per l) 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl was used for plasmid construction, while 2YT medium containing (per l) 10 g of yeast extract, 16 g of tryptone and 5 g of NaCl was used for protein expression. *E. coli* DH5α was routinely cultivated at 37°C in LB medium. Ampicillin (50 μg ml^{-1}) was added to the medium for selection of transformants. All reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Structural modelling All gene and protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) sequence database (12). The 3D structure of the full length CpkB octamer was built using Modeller 9.12

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.04.008

(13) using the chaperonin α-subunit of *Thermococcus* strain KS-1 (PDB ID: 1Q2V) as a template. Because of the high sequence identity between CpkB and 1Q2V (~83%), the structural model was built using the default setting of Modeller. Each construction generated three end models, and the one with the lowest DOPE score was selected.

Docking of the fusion tag to the CpkB target protein A protein docking model of CpkB octamer was simulated by AutoDock 4.2.6 (The Scripps Research Institute, San Diego, CA, USA). Ligands were initially drawn using ChemBioDraw Ultra 13.0 (PerkinElmer, Waltham, MA, USA), and energy minimization was performed with Chem3D Ultra 13.0 using the MMFF94 force field (PerkinElmer, CA, USA). Optimized ligand candidates were then saved in PDBQT format. According to the atom number limit of AutoDock, a dimer (α- and β-chains) from the modelled CpkB octamer was chosen as the receptor and saved in PDBQT format. The dimensions of the grid box were set at 110, 110, 85 (x, y, z), and the centre of the box was placed on Gly542 in the β-chain. Various fusion tags were designed and docked onto the CpkB dimer model, and their binding energy was estimated (Table S1).

DNA manipulation DNA manipulation was performed according to standard techniques described by Sambrook and Russell (14). Plasmid DNA was prepared from *E. coli* cells using a GenElute plasmid Miniprep Kit (Sigma–Aldrich, St. Louis, MO, USA). DNA sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) and a model 3130 capillary DNA sequencer (Applied Biosystems, Waltham, MA, USA). All primers used in this study are listed in Table 1.

Construction of expression plasmids The green fluorescent protein (GFP) gene fused with a His-tag was amplified from plasmid AcGFP1 (Clontech Laboratories, Inc., Kusatsu, Japan) with primers histag-GFP-Fw and histag-GFP-Rv. The PCR product was digested with restriction enzymes BamHI and SalI and ligated with plasmid pQE81L (Qiagen, Hilden, Germany) that was also previously digested with BamHI and SalI. The resultant construct was confirmed by nucleotide sequencing with primer pQE81L-Fw and designated pQE81L-histag-GFP. Using the QuikChange site-directed mutagenesis method (Agilent Technology), signal tags S1 (GGRRGR), S2 (GKGKK) and S3 (GGDDGD) were introduced into the pQE81L-histag-GFP construct immediately before the His-tag of GFP with primer pairs HGFP-S1-Fw and HGFP-S1-Rv, HGFP-S2-Fw and HGFP-S2-Rv, and HGFP-S3-Fw and HGFP-S3-Rv, respectively. The resulting constructs harbouring S1, S2 and S3 signal tags were confirmed by nucleotide sequencing with primer pQE81L-Fw and designated pET21a-S1-histag-GFP, pET21a-S2-histag-GFP and pET21a-S3-GFP, respectively.

Genes for 4-oxalocrotonate tautomerase (4OTA) and glutamine:fructose-6-phosphate amidotransferase (GFAT) were obtained from *E. coli* strain BL21 genomic DNA. Gene *4ota* was initially amplified by PCR with primer pair 4OTA-flank-Fw and 4OTA-flank-Rv, and the His-tag region was then introduced at the carboxy-terminus of *4ota* using primers 4OTA-histag-Fw and 4OTA-histag-Rv by nested PCR. The amplified DNA was introduced into the NdeI and EcoRI sites of the pET21a plasmid (Merck Millipore, Billerica, MA, USA). The resulting construct was confirmed by nucleotide sequencing with the T7 terminator primer (Merck Millipore) and designated pET21a-4OTA-histag. The signal tag S1 was fused to the amino-terminus of *4ota* and then introduced into plasmid pET21a-4OTA-histag using the QuikChange site-directed mutagenesis method with oligonucleotides 4OTA-his-S1-Fw and 4OTA-his-S1-Rv. The resulting plasmid was confirmed by nucleotide sequencing and designated pET21a-4OTA-histag-S1.

Gene *gfat* was initially amplified with primers GFAT-flank-Fw and GFAT-flank-Rv from *E. coli* BL21 genomic DNA. A His-tag was introduced at the carboxy-terminal region of the *gfat* gene by nested PCR using primers GFAT-histag-Fw and GFAT-histag-Rv. The resulting product was digested with HindIII and XhoI and introduced into plasmid pET21a. The resulting construct was confirmed by nucleotide sequencing and designated pET21a-GFAT-histag. The S1 signal tag was introduced into the pET21a-GFAT-histag construct immediately in front of the initiation ATG codon of the *gfat* gene using the QuikChange method with primers S1-GFAT-his-Fw and S1-GFAT-his-Rv. The obtained plasmid was confirmed by nucleotide sequencing using the T7 promoter primer (Merck Millipore) and designated pET21a-S1-GFAT-histag.

Protein expression and purification Recombinant CpkB was expressed in *E. coli* BL21(DE3) RIL bearing plasmid pECPK (15) at 25°C. Cells were disrupted by sonication, and crude cell extracts were treated at 85°C for 30 min. The soluble fraction containing CpkB was obtained by centrifugation (15,000 ×*g*, 10 min, 25°C). CpkB was purified by repeated anion-exchange chromatography steps on HiTrap Q HP 5/5 and MonoQ HR 5/5 columns (GE Healthcare, Chicago, IL, USA) as reported previously (15).

For GFP, 4OTA, and GFAT expression, competent *E. coli* BL21(DE3) codon-plus RIL cells were transformed with the appropriate construct harbouring *gfp*, *4ota* or *gfat* (pQE81L-histag-GFP, pET21a-4OTA-histag and pET21a-GFAT-histag, respectively) or their signal-tagged variants (pET21a-S1-histag-GFP, pET21a-S2-histag-GFP, pET21a-S3-GFP, pET21a-4OTA-histag-S1 and pET21a-S1-GFAT-histag). Protein expression was induced at 25°C when the OD_{660} reached 0.4 by the addition of IPTG (1 mM). After a 10 h induction at 25°C, cells were harvested by centrifugation and resuspended in 100 mM Tris–HCl buffer (pH 9.0, buffer A). Cells were disrupted by sonication, and soluble fractions (supernatants) were obtained by centrifugation (15,000 ×*g*, 15 min, 25°C). Supernatants were applied to a Ni-NTA column (Qiagen) and eluted with 200 mM imidazole in buffer A. Eluted fractions were further purified by anion-exchange chromatography on a MonoQ HR 5/5 column with a linear gradient of NaCl (0–0.5 M) in buffer A.

TABLE 1. Primers used in this study.

Primers	Sequence (5′ to 3′)[a, b]
pQE81L-Fw	CTCGAGAAATCATAAAAAATTTATTTGCTT
Histag-GFP-Fw	CCCCCC***GGATCC***ATGAGAGGATCTCACC-ATCACCATCACCATACGGATCCA
Histag-GFP-Rv	AAA***GTCGAC***TTACTTGTACAGCTCGTCCA-TGCCGAGAGTGATCCCGGCGGCGGT
HGFP-S1-Fw	GGTGGTCGTCGTGGTCGTTAAAGCGGCC-GCGACTCT
HGFP-S1-Rv	TTAACGACCACGGCGACCACCCTTGTAC-AGCTCGTCCATGC
HGFP-S2-Fw	GGTAAAGGTAAAAAATAAAGCGGCC-GCGACTCT
HGFP-S2-Rv	TTATTTTTTACCTTTACCCTTGTACAGC-TCGTCCATGC
HGFP-S3-Fw	GGCGGTGATGACGGCGATCACCATCAC-CATCACCATACGGATCCA
HGFP-S3-Rv	GTGATGGTGATGGTGATCGCCGTCATCA-CCGCCAGATCCTCTCATA
GFAT-flank-Fw	TTCTGGCCGGGTAACCCGGTCACATGGG-ATGAGGAGATAACATAAT
GFAT-flank-Rv	TGTCTCCTGGGAGGATTCATAAAGCAT-TGTTTGTTGGCTACGAGAAGC
GFAT-histag-Fw	CCC***AAGCTT***ATGTGTGGAATTGTTG-GCGCGATCGCGCAACGTG
GFAT-histag-Rv	ACA***CTCGAG***TTATTAGTGGTGGTG-GTGGTGGTGAGCTTATTACTCAAC-CGTAACCGATTTTGCCAG
S1-GFAT-his-Fw	ATACATATGGGTGGTCGTCGTGGTCGT-AAGCTTATGTGTGGAATTGTTG
S1-GFAT-his-Rv	CATAAGCTTACGACCACGACGACCACC-CATATGTATATCTCCTTCTTAAAG
4OTA-flank-Fw	TTTTACTGACATCTTTCAAAAGGAGC-ACAATCATGCCGCACATCGACATT
4OTA-flank-Rv	GCATTACATGCTGTAACCGGGTTTC-TTTATCAAAGCCTCCATTTGGG
4OTA-histag-Fw	CCCCCCCCCC***CATATG***CCGCACATCG-ACATTAAATGTTTTCC
4OTA-histag-Rv	AAA***GAATTC***TTATTAGTGGTGGTGGTGG-TGGTGCATGCTGTAACCGGGTTTCTT-TATCAAAGC
4OTA-his-S1-Fw	TACAGCATGCACCACCACCACCACCAC-GGTGGTCGTCGTGGTCGTTAAGAATT-CGAG
4OTA-his-S1-Rv	ACGACCACGACGACCACCGTGGTGGTGG-TGGTGGTGCATGCTGTAACCGGGTTTC-TTTATC

[a] Mutated nucleotides in each gene are underlined.
[b] Introduced restriction sites are indicated by italicized and bold type.

Protection of tagged proteins from heat denaturation in the presence of CpkB For GFP, 20 nmol of His-tagged GFP and its signal tagged variants were incubated at 75°C for 0–16 min in the presence or absence of CpkB (20 nmol as monomer) in HKM buffer (25 mM HEPES-NaOH, 100 mM KCl, 5 mM $MgCl_2$, pH 9.0) with 2 mM ATP in a total volume of 20 μl. GFP fluorescence was measured by monitoring the fluorescence (excitation = 485 nm, emission = 535 nm) using an EnVision 2014 Multilabel Reader (PerkinElmer). The intensity of GFP fluorescence before heat treatment was set to 100%, and the residual fluorescence intensity after heat treatment was measured and designated as the relative fluorescence.

For 4OTA, purified His-tagged 4OTA (60 nmol) and His- and signal S1-tagged 4OTA-S1 (60 nmol) were incubated at 75°C for 0–12 min in the presence or absence of CpkB (60 nmol as monomer) with 2 mM ATP in HKM buffer in a total volume of 20 μl. The activity of 4OTA was measured according to the reported procedure (16) using the substrate 2-hydroxymuconate synthesized as previously described (17). The rate of substrate disappearance was monitored at 295 nm using a V-550 spectrophotometer (Jasco, Tokyo, Japan).

For GFAT, GFAT (His-tagged GFAT) (30 nmol) and S1-GFAT (His-and signal S1-tagged GFAT) (30 nmol) were incubated at 75°C for 0–12 min in the presence or absence of CpkB (30 nmol as monomer) in HKM buffer with 2 mM ATP in a total volume of 20 μl, respectively. GFAT activity was measured as previously reported (18) using substrate prepared by mixing 2 μl of 500 mM fructose-6-phosphate, 1.5 μl of 1 M L-glutamine, 1 μl of 50 mM EDTA, 1 μl of 20 mM PMSF, 1 μl of 50 mM DTT and