Discovery of an intermolecular disulfide bond required for the thermostability of a heterodimeric protein from the thermophile *Hydrogenobacter thermophilus*

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Abstract

Factors that increase protein thermostability are of considerable interest in both scientific and industrial fields. Disulfide bonds are one of such factors that increase thermostability, but are rarely found in intracellular proteins because of the reducing environment of the cytosol. Here, we report the first example of an intermolecular disulfide bond between heteromeric subunits of a novel-type phosphoserine phosphatase from a thermophilic bacterium *Hydrogenobacter thermophilus*, which contributes to protein thermostability at the physiological temperature. Comparison of remaining soluble proteins between wild-type and cysteine-deleted mutant using SDS-PAGE revealed that the disulfide bond increases the thermostability of the whole protein by tightly connecting a subunit with low solubility to the partner with higher solubility. Furthermore, it was strongly suggested that the disulfide bond is formed

and contributes to the stability *in vivo*. This finding will open new avenues for the design of proteins with increased thermostability.

Keywords: Protein thermostability, Heterodimer, Intermolecular disulfide bond, Phosphoserine phosphatase, Protein solubility

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Abbreviations: iPSPs, metal-independent phosphoserine phosphatases; A-A, wild-type iPSP1; A-B, wild-type iPSP2; As-As, PspA C198S-PspA mutant of iPSP1; As-Bs, PspA C198S-PspB C197S mutant of iPSP2; IAA, Iodoacetamide; TCEP, Tris(2-carboxyethyl)phosphine; CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; WB, Western blotting.

Introduction

Despite considerable research efforts over the last few decades in both scientific and industrial sectors to identify factors that contribute to the thermostability of proteins [1-4], no single or universal factor responsible for protein thermostability has been identified.[5-8] However, comparisons of protein homologs between mesophilic and (hyper-) thermophilic organisms, and the mutagenic screening of thermostable proteins have revealed that electrostatic surface interactions, hydrogen bonding, compact protein packing, intrinsic secondary structure propensity and disulfide bond formation all contribute to thermostability.[2, 9-12]

The formation of intracellular disulfide bonds is considered to be extremely rare because of the reducing environment of the cytoplasm.[13-15] However, crystal structure analyses have revealed that several intracellular proteins from thermophilic organisms contain disulfide bonds within or between subunits that contribute to thermostability.[7, 13, 16] In addition, thermophilic microorganisms, particularly hyperthermophiles, are reported to have a higher ratio of intracellular disulfide bonds compared to mesophiles.[16, 17] For this reason, a number of researchers have attempted to create thermostable proteins for industrial applications by artificially introducing disulfide bonds.[18-21] However, the disulfide bonds found in crystal structures or those that have been introduced manually are limited to intrasubunit bonds or those between two identical subunits.

Novel-type serine-synthesizing enzymes, termed metal-independent phosphoserine phosphatases (iPSPs; EC 3.1.3.3), were recently identified and characterized from the thermophilic bacterium *Hydrogenobacter thermophilus*, which grows optimally at 70-75°C.[22-24] *H. thermophilus* has two types of iPSPs, iPSP1 and iPSP2. The former is a homodimer of PspA subunits, and the latter is a heterodimer of PspA and PspB subunits. Although PspA and PspB share 35% amino acid sequence identity and contain a conserved catalytic domain of the histidine phosphatase superfamily, only the PspA subunit shows substantial PSP activity.[22, 25] K_m values of iPSP1 and iPSP2 for phosphoserine are comparable while V_{max} of iPSP2 is almost the half of iPSP1 [22], suggesting that monomeric PspA is the minimum unit for the activity but dimerization stabilizes whole structure of iPSPs. Although homodimers of PspBs have not been detected in *H. thermophilus*, this type of PSP enzyme is not likely formed, because co-expression of PspA and PspB is essential for PspB accumulation in the

cytosol of *Escherichia coli*. In addition, no member of this superfamily protein appears to function as a chaperone.[22]

Crystal structure analysis of iPSP1 revealed that this protein forms an intermolecular disulfide bond between the two C198 residues at the interface of the PspA subunits.[25] As the C198 residue of PspA is conserved in PspB as C197, it is expected that iPSP2 can also form an intermolecular disulfide bond between PspA and PspB. We therefore hypothesized that these intermolecular disulfide bonds are necessary for the thermostability of iPSP1 and iPSP2. To confirm this hypothesis, here, the existence of a disulfide bond in iPSP2, both in purified soluble protein and under *in-vivo* conditions, was investigated, and the contribution of this bond to the thermostability of iPSP2 was then examined.

Materials and Methods

Construction of Plasmids for Site-directed Mutants

The genes encoding the PspA (**HTH_0103**) and PspB (**HTH_0183**) subunits of *H. thermophilus* TK-6 (**IAM 12695, DSM 6534**) were previously cloned into the expression vectors pCDFDuet-1 and pET21c (Novagen, Darmstadt, Germany), respectively.[25] The constructed plasmids were then mutated to express C198S and C197S mutants of the PspA and PspB subunits, in which the 198^{th} and 197^{th} cysteine residues, respectively, were converted to serine. The mutated plasmids were constructed using the primer pairs 5'-ATAACCAGCCATCTGGGAGAGTTT-3' and 5'-AGATGGCTGGTTATGTTAAGCTTTAG-3' (for PspA), and 5'-AAACTTTCCCACACAAGACAGCTTAC-3' and 5'-TGTGTGGGAAAGTTTGTTTAGATAAACC-3' (for PspB), and Prime STAR

Mutagenesis Basal Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions.

Heterologous Protein Expression and Purification

iPSP1 (A-A), iPSP2 (A-B) and the corresponding dimeric proteins formed with the PspA C198S and PspB C197S mutated subunits were expressed in *E. coli* BL21-Codon Plus (DE3)-RIL and then purified using the protocol described previously, with a minor modification.[22] Here, the heat treatment of cell lysate at 80°C was omitted, as the present study was focused on protein thermostability. Instead, the cell lysate was applied to a Q-Sepharose Fast-flow column (GE Healthcare) equilibrated with buffer containing 20 mM Tris-HCl (pH 8.0) and was then eluted with a gradient of NaCl from 0 to 1 M in the same buffer. The fraction containing iPSPs were further purified using Butyl-Toyopearl and MonoQ columns, as described previously.[22] For performing the elution from the Butyl-Toyopearl column, the first ammonium sulfate concentration was decreased to 20% saturation.

Reductive and Non-reductive SDS-PAGE

Reductive and non-reductive SDS-PAGE [26] were conducted using a 5% stacking and 10% separating gel with and without DTT in the loading buffer, respectively. Samples to be analyzed by reductive SDS-PAGE were mixed with loading buffer (4 mM DTT, final concentration) and incubated at 95°C for 10 min prior to separation. After SDS-PAGE, the separated proteins were stained with CBB, and Image J software was used to quantify the band intensity of stained proteins.

Enzyme Assays

PSP activity was assayed by measuring the production of inorganic phosphate, as described previously with minor modifications.[22] Briefly, the reaction mixture contained 200 mM HEPES-NaOH (pH 8.0 at room temperature), 10 mM L-phosphoserine, 1.0 mM EDTA (pH 8.0), and enzyme solution (total volume =50 μL). The reaction mixture was incubated for 7 min at 70 °C for iPSPs proteins. One unit of PSP activity was defined as the amount of enzyme producing 1 μmol of inorganic phosphate per min.

Thermostability Analysis

One mL of 20 mM Tris-HCl (pH 8.0) with 1 mM EDTA containing 400 μg of purified proteins were incubated at 70, 75, 80, 85, and 90°C for 10 min, and were then placed into ice-water. After 30 min, the precipitants were removed by centrifugation at 20,000×g for 30 min. Ten μL of the supernatants were subjected to SDS-PAGE analysis to confirm the residual proteins in the soluble fraction. Additionally, the supernatents diluted 20 times were subjected to enzyme assays to measure the residual enzyme activity per volume of the sample.

Western Blotting

Rabbit antisera for PspA and PspB were prepared by Eurofins Operon, Japan using synthesized peptides (^{67}AEAKNLEVIKED^{78} for PspA and ^{83}MSFGEYEGKH^{92} for PspB) as antigens. For WB, proteins separated on SDS-PAGE gels were transferred to PVDF membranes, which were then blocked for at least 4 h at room temperature using TBST buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) containing 5% (w/v) skim milk. Blocked membranes were probed overnight at 4°C with PspA or PspB antiserum (1/1000 and 1/250 dilutions,

149 respectively) in TBST containing skim milk. After washing the membranes three
150 times in TBST, they were probed with goat anti-rabbit IgG (pAb, HRP conjugate;
151 Enzo) in TBST (1/1000 dilution). After washing the membranes twice in TBST, once
152 in TBST without Tween 20, and once in distilled water, the immunopositive spots
153 were visualized using a POD Immunostain Set (Wako) as directed by the
154 manufacturer.
155
156 **Protein Assay**
157 Protein concentrations were measured using the Bradford protein assay (Bio-
158 Rad) with bovine serum albumin as the standard.
159
160 **Fluorescent Labeling of Cysteines Involved in Disulfide Bonds**
161 A slightly modified method of Boutz et al.[16] was used to fluorescently label
162 the cysteines that formed disulfide bonds. Briefly, *H. thermophilus* or *E. coli* cell
163 pellets corresponding to 650 μg protein were suspended in 0.1 mL lysate buffer (20
164 mM Tris-HCl pH 8.0, 10 mM NaCl, 1 mM EDTA, and 20 mM iodoacetamide [IAA])
165 and centrifuged at 20,000×g for 5 min. The washed cell pellets were resuspended in
166 0.1 mL lysate buffer, lysed on ice by sonication, and then centrifuged at 20,000 ×*g* for
167 10 min. SDS and lysate buffer were added to the supernatant to yield 500 μL sample
168 containing 1% SDS (final concentration). The protein samples were denatured by
169 heating at 95°C (2 min for *E. coli,* 4 min for *H. thermophilus*) and then mixed with
170 26.3 μL of 400 mM IAA solution to block free cysteine thiols. After a 30-min
171 incubation in the dark at room temperature, IAA was diluted approximately 1000 fold
172 by adding excess amounts of lysate buffer containing 0.1% SDS, but without IAA,
173 and the sample was then concentrated using ultrafiltration spin columns (Vivaspin

174 5,000 MWCO; Sartorius Stedim). Samples were reduced with 10 mM tris(2-
175 carboxyethyl)phosphine (TCEP; final concentration; adjusted to pH 7.0 with NaOH)
176 during a 30-min dark incubation at room temperature. Following disulfide bond
177 cleavage, samples were reacted within 50 μM 7-diethylamino-3-(4'-
178 maleimidylphenyl)-4-methylcoumarin (CPM) in the dark at room temperature for 30
179 min for the fluorescent labelling of free thiols. Proteins were then separated by non-
180 reducing SDS-PAGE on a 12% acrylamide gel, and CPM-labeled protein bands were
181 visualized by excitation at a wavelength of 365 nm. Precision Plus Protein™ Dual
182 Color Standards (Bio-Rad) were used as protein molecular weight markers.
183
184 **Results**
185
186 **Construction of Mutant Proteins**
187 Mutated iPSP1 and iPSP2 proteins were constructed to confirm the presence of
188 intermolecular disulfide bonds between the PspA and PspB subunits in soluble form.
189 C198 of PspA and C197 of PspB were changed to serine, because serine appears to
190 effectively suppress sulfur chemistry without influencing protein structure.[27]
191 Hereafter, wild-type iPSP1 and iPSP2 are referred to as A-A and A-B, respectively,
192 and the mutant forms of each recombinant protein are called As-As and As-Bs,
193 respectively.
194 The wild-type and mutant proteins were heterologously expressed using the
195 same procedure in *E. coli.* The elution patterns of the mutants during the purification
196 by column chromatography exhibited similar profiles as the respective wild-type
197 proteins, suggesting that the overall structure was not changed by the mutations. The

198 homogeneity of the purified proteins was confirmed by SDS-PAGE and CBB staining.
199 It was also confirmed that the mutations did not affect the K_m and V_{max} values.
200
201 **Detection of Intermolecular Disulfide Bonds by Non-reducing SDS-PAGE**
202 To determine if intermolecular disulfide bonds are present not only in the
203 crystal of A-A, but also in the soluble form of A-A and A-B, SDS-PAGE analysis of
204 A-A, A-B, and the generated mutants were performed under non-reducing conditions.
205 Two distinct bands of 24.0 and 38.0 kDa were detected when A-A was subjected to
206 non-reduced SDS-PAGE (Fig. 1A), whereas only the 24.0-kDa band, which was
207 consistent with the predicted molecular weight of the PspA subunit (24.6 kDa), was
208 detected from As-As, as expected. In contrast, a single major protein band of 45.0
209 kDa was observed when A-B was subjected to non-reducing SDS-PAGE (Fig. 1A),
210 whereas 23.5- and 24.5-kDa bands, corresponding to PspB (estimated molecular mass
211 of 23.5 kDa) and PspA, respectively, were detected when reduced A-B or non-
212 reduced As-Bs were analyzed by SDS-PAGE (Fig. 1A, B). The two monomeric size
213 bands were also detected when As-B and A-Bs were subjected to non-reduced SDS-
214 PAGE (data not shown). In addition, a single major protein band was observed when
215 A-A, As-As, A-B or As-Bs was subjected to native-PAGE (Fig. 1C), and a single
216 peak corresponding to the dimeric form of each protein was observed by size
217 exclusion chromatographies (data not shown). Therefore, the 38.0- and 45.0-kDa
218 proteins detected in the non-reduced SDS-PAGE analyses were A-A and A-B dimers,
219 respectively. These results clearly indicated that heterologously expressed and
220 purified A-A and A-B have intermolecular disulfide bonds between C198 of PspA
221 and between C198 of PspA and C197 of PspB, respectively in the soluble form. From
222 the CBB-stained band intensities in the non-reduced SDS-PAGE gels, the ratio of

proteins containing an intermolecular disulfide bond was estimated to be 35% for A-A and 97% for A-B.

To determine whether the intermolecular disulfide bonds between the PspA and PspB subunits also exist in A-A and A-B obtained from *H. thermophilus* lysate, Western blotting (WB) was performed using anti-PspA or PspB antiserum. The specificity of anti-PspA and PspB antisera to each subunit was confirmed using purified A-A and A-B (Fig. 2). When anti-PspA antiserum was reacted with reduced *H. thermophilus* lysate, a distinct band was observed at 24.5 kDa, confirming the presence of monomeric PspA subunits (Fig. 2A). However, when the anti-PspA antibody was reacted with non-reduced lysate, the 24.5-kDa band had markedly reduced intensity and additional bands of 38.0 and 43.0 kDa were also observed (Fig. 2A). These two bands most likely corresponded to A-A and A-B protein dimers that contained an intermolecular disulfide bonds. Although greater cross-reactivity with proteins in the *H. thermophilus* lysate was observed with the anti-PspB antiserum, a 23.5-kDa band corresponding to monomeric PspB was detected in reduced lysate (Fig. 2B). Moreover, a 43.0-kDa band was present in the non-reduced lysate sample, also suggesting that PspB forms a heterodimer with PspA, and that the two subunits are interconnected by a disulfide bond.

Intermolecular Disulfide Bond Enhances Protein Thermostability

To confirm the function of the intermolecular disulfide bond identified between the PspA and PspB subunits, the thermostabilities of purified, electrophoretically homogeneous wild-type and mutant A-B enzymes were analyzed. A-B was targeted in this experiment as nearly all of the purified A-B heterodimers had intermolecular disulfide bonds. Because the PspB subunit does not have clear enzymatic activity but

both PspA and PspB subunits are required for the existence of iPSP2 (=A-B), thermostability was defined as the ability of both the subunits to maintain solubility after heat treatment (If only PspB precipitated, PspA may still be able to stay in the soluble fraction as A-A but it is not the thermostability as A-B but A-A). When purified A-B was incubated at 90°C, approximately 30% of the PspA and PspB subunits remained in the soluble fraction (Figs. 3A, B, E and F). In contrast, only 3% of PspA and almost no PspB retained solubility when As-Bs, which cannot form an intermolecular disulfide bond, was heat-treated at 90°C (Figs. 3C, D, E and F). The ratio of residual soluble PspA and B subunits from As-Bs was similar to that of A-B incubated with DTT (Figs. 3E and F), supporting the speculation that the observed difference in thermostability between A-B and As-Bs is attributable to the presence of an intermolecular disulfide bond. Notably, the PspB subunit from As-Bs was precipitated at lower temperature than PspA, whereas the wild-type PspA and B subunits were precipitated at almost the same conditions (Figs. 3A-D). In the case of As-Bs, 55% and 100% of PspB subunits were precipitated at 75 and 80°C, respectively, whereas only 21% and 55% of PspA subunits were precipitated at those respective temperatures (Figs. 3E and F). This indicates that the thermostability of As-Bs can be defined as the ratio of residual soluble PspB subunit. Residual PSP activity (Fig. 3G) showed the similar trend with the ratio of residual soluble PspA rather than that of PspB; In case of A-B, almost all the activity and both subunits were retained after incubation at 80°C. On the other hand, in case of As-Bs, about 50% of PspA subunit and PSP activity was retained while almost all the PspB subunit was disappeared from the supernatant after incubation at 80°C. Therefore, we concluded that the intermolecular disulfide bond of the heterodimer enhanced thermostability of

the whole protein, especially of PspB subunit, by increasing the solubility at high temperature.

Detection of Disulfide Bonds by Fluorescent Labeling

To determine whether the intermolecular disulfide bonds detected in the A-A and A-B proteins were formed *in vivo* or after cell lysis, thiols from disulfide bonds were labelled with the thiol-reactive fluorescent reagent CPM (16). For the analysis, free thiols were blocked before cell lysis by adding the alkylation reagent IAA, disulfide bonds were then reduced by treatment with TCEP, and the cleaved thiols were labeled with CPM. The proper blocking of free thiol groups by IAA and thiol labeling by CPM were confirmed by including control samples without added TCEP and IAA, respectively (Supplementary Fig. 1).

Two monomeric bands corresponding to PspA and PspB were detected from the positive control sample, purified A-B, but not from the negative control, purified As-Bs, confirming that this assay system was able to detect intermolecular disulfide bonds (Fig. 4A). In contrast, no additional bands were observed in whole cell lysates of *E. coli* cells expressing A-B compared with lysates from cells expressing As-Bs, indicating that the disulfide bond between PspA and PspB was not formed in *E. coli*. When the same amount of cell lysate from *H. thermophilus* and *E. coli* was analyzed, more bands were clearly observed in the cell lysate from *H. thermophilus* compared to *E. coli*, indicating that various proteins within *H. thermophilus* contain disulfide bonds. In addition, a relatively strong band was observed around 24.5 kDa, which is the same size as PspA, suggesting that PspA in *H. thermophilus* has a disulfide bond.

Conservation of Cysteine Residues Able to Form Intermolecular Disulfide Bonds

297 The distribution of cysteine residues with the potential to form intermolecular
298 disulfide bonds was examined among species of the order Aquificales with sequenced
299 genomes. Our previous studies suggested that the ancestor of PspA and PspB divided
300 into PspA and PspB after the family Desulfurobacteriaceae arose, but before the
301 division of Aquificaceae and Hydrogenothermaceae.[22] Multiple sequence
302 alignments of iPSP homologs from these three families using the CLUSTALW
303 program [28] showed that the cysteine residues that correspond to C198 and C197 of
304 *H. thermophilus* PspA and PspB, respectively, were conserved in all homologs from
305 Aquificaceae, except one of the two PspAs (ZP_02179977) from *Hydrogenivirga*, but
306 not in those from Hydrogenothermaceae or Desulfurobacteriaceae (Table 1). The
307 PspA of *Hydrogenivirga* without the cysteine residue was acquired by lateral gene
308 transfer from Hydrogenothermaceae [22]. In contrast, another PspA of
309 *Hydrogenivirga* (ZP_02178481), which was acquired by vertical inheritance,
310 conserved the cysteine residues. Therefore, iPSP2 (A-B) from *Hydrogenivirga* can
311 also form intermolecular disulfide bond.
312
313 **Discussion**
314 This study presents the first example of a heterodimeric protein from a
315 thermophilic bacterium with an intermolecular disulfide bond that contributes to
316 protein thermostability. The soluble forms of both heterologously expressed and
317 purified iPSP1, a homodimer of PspA (A-A), and iPSP2, a heterodimer of PspA and
318 PspB (A-B), were shown to be connected by disulfide bonds formed between the
319 198th and 197th cysteine residues of PspA and PspB, respectively (Fig. 1A). Nearly
320 100% of A-B dimers were connected by a disulfide bond. Comparison of the
321 thermostabilities between wild-type A-B, A-B under reducing conditions, and the

cysteine mutant of A-B clearly showed that the disulfide bonds increase thermostability (Figs. 3B, D, E and F). These findings are consistent with studies reporting that tight interfacial connections between subunits mediated by hydrogen bonding [29], hydrophobic interactions [30], or disulfide bonds [31] increase protein thermostability. In addition, the importance of interactions between subunits for increasing multimeric protein solubility has already been reported [32]; however, these studies were limited to homomultimeric proteins. To our knowledge, the findings presented here are the first example of an intracellular protein that contains an intermolecular disulfide bond between heteromeric subunits that contributes to thermostability, and the contribution to thermostability was unique to heteromeric nature.

Interestingly, PspB subunits that were not connected to PspA by a disulfide bond were precipitated at lower temperature than PspA, whereas both subunits, when they were connected with an intermolecular disulfide bond, were precipitated under the same conditions; namely, both subunits start to precipitate around 85°C and about 70% of them precipitated at 90°C (Figs. 3B, D, E and F). This observation likely indicates that attachment to PspA is required for PspB to exist in the soluble fraction. Our speculation concerning this point is as follows: PspA and PspB can stably form heterodimers without a disulfide bond at 70°C or lower, and therefore the intermolecular disulfide bond is not essential below the optimal growth temperature of *H. thermophilus*. However, the intermolecular disulfide bond between PspA and PspB is necessary for the solubility of PspB at 75°C or higher because molecular motion is markedly increased at these high temperatures and the probability of detachment of the subunits is also increased. If PspB detaches from PspA, it may immediately precipitate and disappear from the soluble phase while PspA can remain in the soluble

fraction as a monomer for a very short time and then find other PspA monomer to make stable homodimer, A-A immediately. It is also supported by the result that residual PSP activity of A-B and As-Bs after heat treatment well agrees with the ratio of residual soluble PspA subunit (Fig. 3E and G). Therefore, the strong connection of PspB to PspA through the disulfide bond may prevent the precipitation of PspB. This speculation well agrees with the following observations from the present and past studies: (1) PspB does not remain in the soluble fraction when expressed without PspA in *E. coli* [22]; (2) the elution pattern of A-A and A-B from a hydrophobic column suggests that the surface of PspB has higher hydrophobicity than that of PspA; and (3) the surface charge of modeled PspB structure calculated by PyMOL was 0.0, whereas that of PspA was -4.0, suggesting that the surface electron charge of PspB is very low (data not shown). We therefore propose that intermolecular disulfide bonds between subunits with low solubility and those with higher solubility can increase the thermostability of multimeric proteins.

It is noteworthy that the intermolecular disulfide bond between PspA-PspB is essential for the PspB subunit to exist in the soluble fraction at 75°C, which is the upper limit of the optimal growth temperature of *H. thermophiles*.[24] Thus, the intermolecular disulfide bond appears to be physiologically important for this protein to maintain solubility in *H. thermophilus*. However, due to the reducing environment of the cytosol, disulfide bonds are not typically found in cytosolic proteins. In eukaryotes, disulfide bonds are formed in the lumen of the endoplasmic reticulum in reactions catalyzed by protein disulfide isomerase.[33] As the intracellular redox potential of *E. coli*, a mesophilic prokaryote, is around -200 to -300 mV, recombinant proteins with disulfide bonds may not fold properly.[17, 34] Therefore, we examined whether the disulfide bonds found in heterologously expressed and purified proteins

also exist *in vivo*. The results of a CPM assay showed that A-B does not form intermolecular S-S bonds in *E. coli* (Fig. 4), a finding that does not conflict with the above information indicating that the intracellular environment of *E. coil* is reduced. In contrast, numerous disulfide bonds were detected in total protein samples from *H. thermophilus*, in addition to the relatively strong band around 24.5 kDa that may be derived from PspA (Fig. 4). Although a band of 23.5 kDa corresponding to PspB was not clearly observed, A-B may still form an intermolecular disulfide bond in *H. thermophilus*, because PspB is estimated to have a lower molecular number than that of PspA [22] and therefore more difficult to be detected. This speculation does not conflict with the WB data that *H. thermophilus* lysate contained both A-A and A-B dimers with disulfide bonds (Figs. 2A and B).

The physiological importance of the intermolecular disulfide bond identified in iPSP1 and iPSP2 is also supported by the strict conservation of the cysteine residues corresponding to the 197^{th} or 198^{th} cysteines among homologs of these proteins in Aquificacea (Table 1). Although the cysteine residues are not conserved in PspA or PspB from Hydrogenothermaceae, it is unclear whether PspB subunits from this family are unable to remain in the soluble phase at physiological temperature. In addition, the growth temperature of many members of Hydrogenothermaceae is lower than that of several Aquificaceae species (Table 1). We speculate that the evolution of iPSP in Aquificales occurred as follows. When a single iPSP gene was duplicated to generate PspA and PspB in the ancestor of Aquificaceae and Hydrogenothermaceae, both proteins had iPSP activity and were soluble as homo- and hetero-dimers. Subsequently, PspA maintained PSP activity and solubility, whereas PspB lost PSP activity and became less soluble, but may have acquired other functions. During the evolution of PspB, the solubility of this protein might have been reduced to the point

that B-B became insoluble. However, PspB retained its ability to form heterodimers with PspA, and therefore can exist in soluble form as a heterodimer. Concurrent with the evolution of PspB in Aquificaceae, PspB inherited cysteine residues from an ancestor of Aquificaceae that allowed for the formation of a disulfide bond between PspA and PspB.

CPM assay revealed that various intracellular proteins of *H. thermophilus* contain disulfide bonds (Fig. 4A). This observation is consistent with several recent reports that several thermophilic eukaryotes have numerous intracellular proteins with disulfide bonds.[16, 17, 35] As such proteins are rare in mesophiles, it appears that the formation of intramolecular and intermolecular disulfide bonds within proteins is a common strategy for thermophiles to increase protein thermostability and allow adaptation to high temperatures. However, it remains unclear how disulfide bonds are formed in intracellular environments.[17] As *H. thermophilus* utilizes the reductive tricarboxylic acid cycle, which is used to fix CO_2 in reducing environments, it seems highly unlikely that the disulfide bond between PspA and PspB would spontaneously form in cells. Therefore, it is more likely that a specific system selectively forms disulfide bonds in thermophiles. *H. thermophilus* has several genes that are predicted to encode protein disulfide isomerases and thioredoxins, which may catalyze the formation of disulfide bonds.

In the present study, we demonstrated that an intermolecular disulfide bond contributes to the thermostability of a heterodimeric protein from a thermophilic bacterium. The disulfide bond increases the thermostability of the whole protein by specifically increases the solubility of a single subunit at high temperature connecting it to the partner. This finding provides new insight into the evolution of proteins with

high thermostability and is expected to contribute to the development of new strategies for increasing the thermostability of target proteins.

Author Contribution

Y.C. and M.I. designed this study. K.T.K and Y.C. performed the experiment. K.T.K and H.A. analyzed the data. K.T.K, Y.C., and M.I. wrote the manuscript. All the authors reviewed the results and approved the final version of the manuscript.

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Figure captions

Figure 1 Detection of monomeric and dimeric iPSPs by SDS-PAGE. Four μg of heterologously expressed and purified A-A, A-B, and their mutants were subjected to 10% SDS-PAGE (A: without reduction, B: reduced with DTT) or Native-PAGE (C). M: molecular marker.

Figure 2 Detection of iPSPs from *H. thermophilus* cell lysate by WB using anti-PspA (A) and anti-PspB (B) antisera. For anti-PspA, 0.06 μg of purified A-A, 0.1 μg of purified A-B, and 17.4 μg of *H. thermophilus* lysate were used. For anti-PspB, 10-fold higher amounts of A-A and A-B, and two-fold more lysate were used. Arrows, white arrowheads, and black arrowheads indicate monomeric PspA or PspB, dimeric A-A, and A-B, respectively. M: molecular marker.

Figure 3 Thermostability of A-B and a mutated form (As-Bs) that cannot form an intermolecular disulfide bond. SDS-PAGE analysis of A-B under non-reducing (A) or reducing (B) conditions. SDS-PAGE analysis of As-Bs under non-reducing (C) or reducing (D) conditions. The same volume of samples corresponding to 4 μg of protein before heat treatment were applied to 10% SDS-PAGE gels after heat treatment at the designated temperatures for 10 min and removal of the precipitant. NH: non-heat treated. The ratio of PspA (E) and PspB (F) remaining in the soluble phase was quantified from the band intensities using Image J software and non-heat treated samples as 100%. A-B+DTT indicate that the sample was heat treated at the designated temperatures with DTT. (G) Residual activity per volume of samples after heat treatment at designed temperatures was measured at 70°C. Band intensity or activity from the non-heat treated sample was defined as 100%.

Figure 4 Detection of intracellular proteins containing disulfide bonds. Thiols forming disulfide bonds were labeled with CPM, followed by the separation by 12% SDS-PAGE gels, and the label was then visualized by UV excitation at 365 nm (A). A black and white-converted picture is shown. The gel of (A) was stained with CBB (B). Arrowhead points the position of monomeric PspA.

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Figure 1

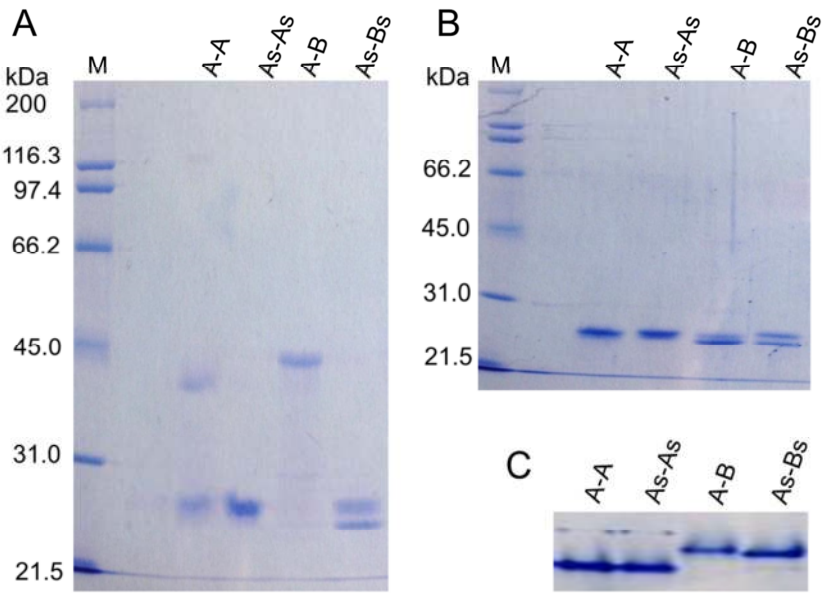

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Figure 2

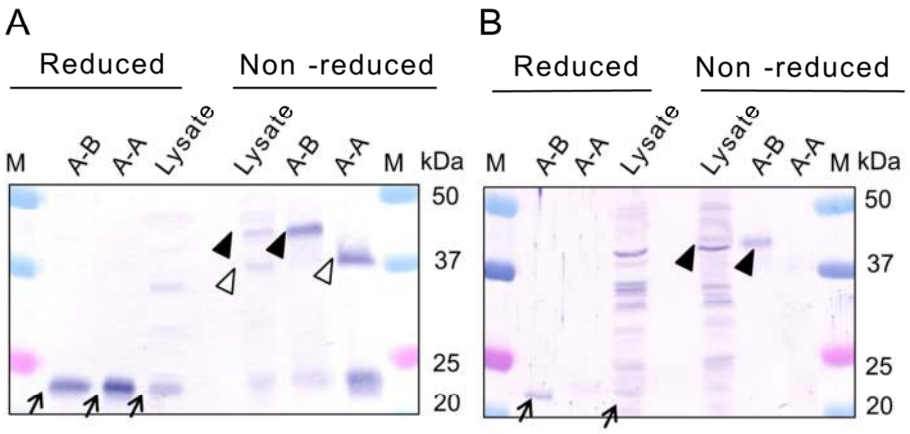

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Figure 3

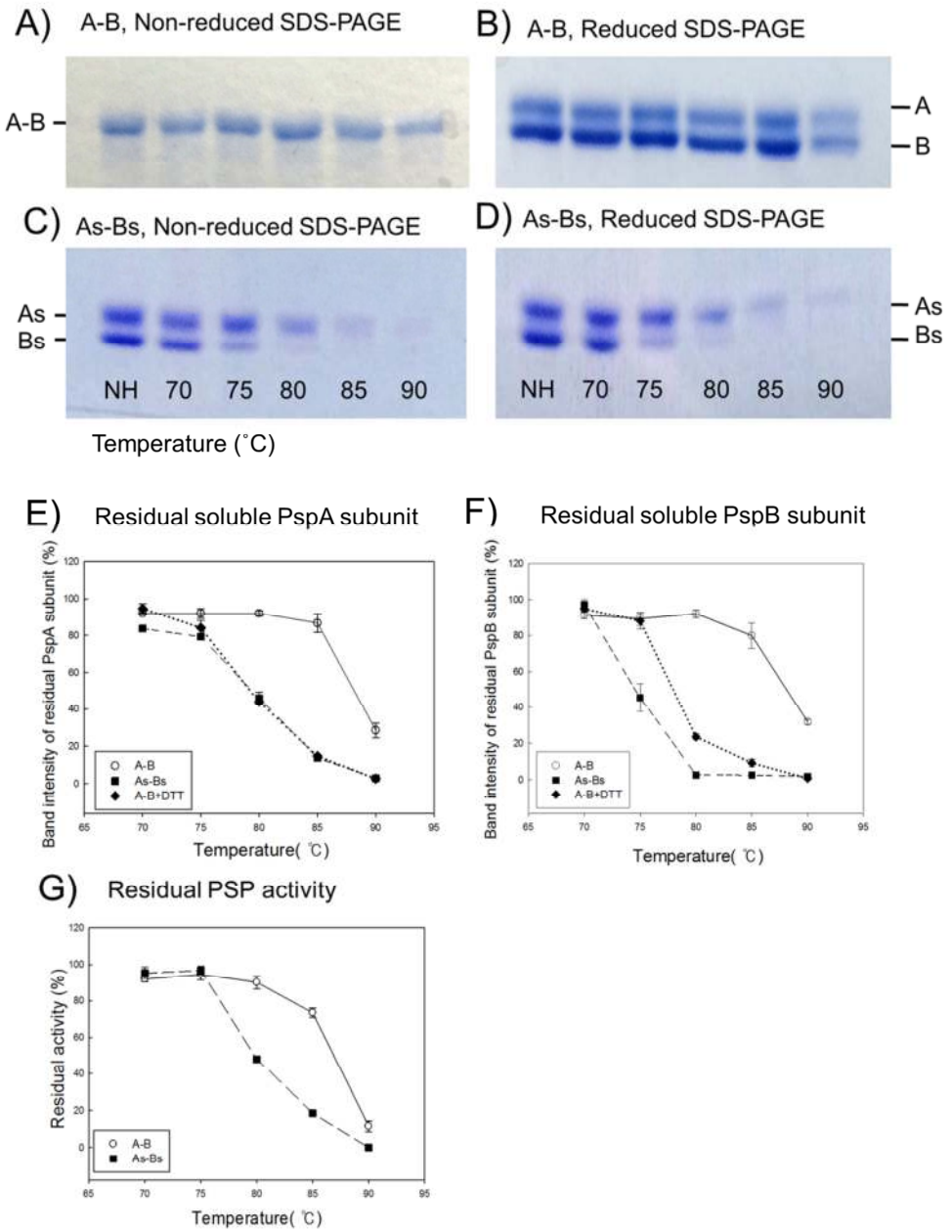

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Figure 4

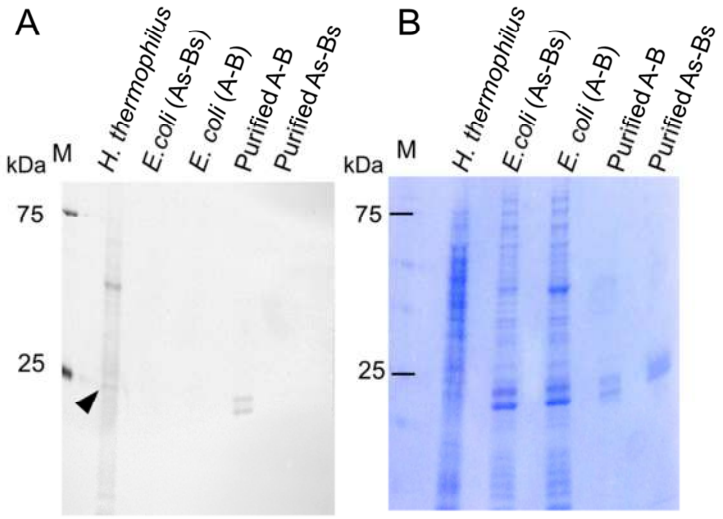

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Fig. S1.

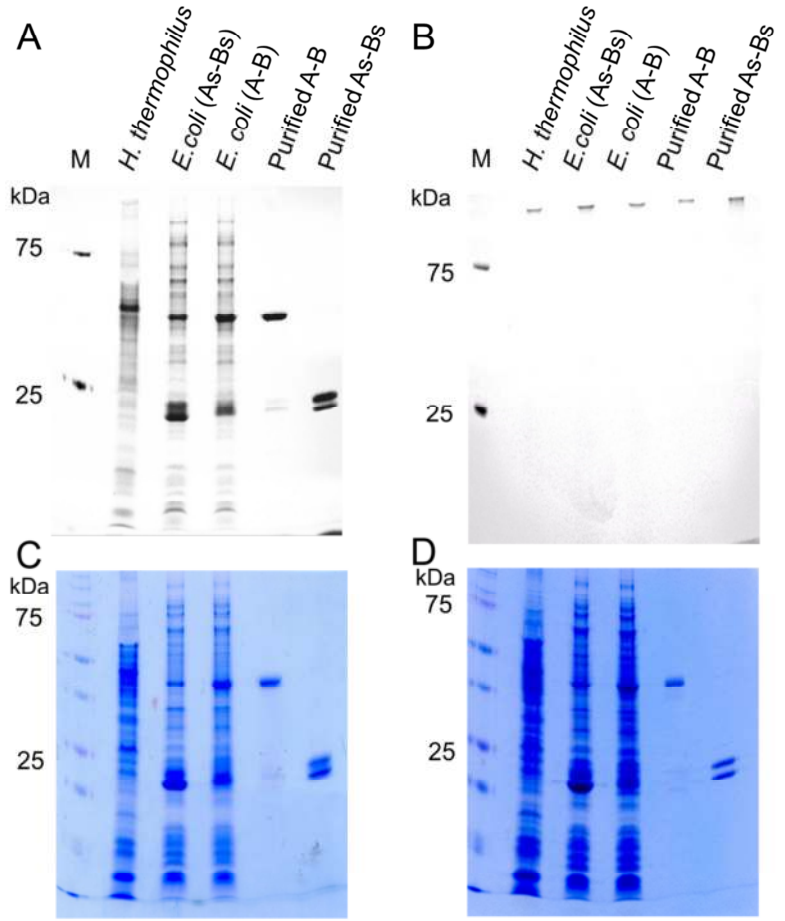

Fig. S1. Controls of CPM assay.
(A) To confirm that the CPM labeling procedure was effective, the free thiols of cysteine residues were labeled with CPM by reacting intact cells and protein samples with CPM, without blocking by IAA and reduction by TCEP. (B) To confirm that IAA blocked free thiols completely, CPM was reacted with the alkylated but not reduced samples. (C) and (D) are CBB-stained gels of (A) and (B), respectively.