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Contribution of a Salt Bridge to the Thermostability of DNA Binding Protein HU from *Bacillus stearothermophilus* Determined by Site-Directed Mutagenesis

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We have employed site-directed mutagenesis to evaluate the contribution of the amino acid residues, Glu[34], Arg[37], and Lys[38], to the thermostability of the thermophilic protein, *Bacillus stearothermophilus* DNA binding protein HU (*Bst*HU), relative to the mesophilic homologue, *Bacillus subtilis* HU (*Bsu*HU). Mutants *Bst*HU-E34D and *Bst*HU-K38N, in which Glu[34] and Lys[38] were individually replaced by the corresponding residues, Asp[34] and Asn[38], in *Bsu*HU, exhibited decreased thermostabilities by 2.08 and 2.17 kJ/mol, respectively, whereas mutant *Bst*HU-R37K with Lys instead of Arg at position 37 showed no change in thermostability. These results suggested that Glu[34] and Lys[38] contribute to the thermostability of *Bst*HU by forming a possible salt bridge on the hydrophilic surface. The role of Glu[34] as a salt bridge partner was corroborated by generating *Bst*HU mutant protein *Bst*HU-E34Q, in which the Glu residue was changed to Gln; this substitution clearly decreased the stability of the protein by 2.71 kJ/mol. The contribution of this favorable salt bridge to the thermostability was further investigated as the salt and pH dependencies of the stabilities of the wild-type *Bst*HU, *Bst*HU-K38N, and *Bst*HU-E34D. As for salt dependency, the stability of the wild-type relative to those of the two mutants decreased with an increase in ionic strength, indicating that the electrostatic interaction between Glu[34] and Lys[38] indeed significantly contributes to the thermostability of *Bst*HU. As for pH dependency, the difference in thermostability between the wild-type *Bst*HU and mutant *Bst*HU-K38N showed that the mutant protein was as stable as the wild-type protein at pH 2.0, however, at neutral pH, the mutant protein was less stable than the wild-type protein. In contrast, the difference between the melting temperatures of mutant *Bst*HU-E34D and the wild-type did not change as a function of pH. This suggested that the Glu[34] residue may play an important role in the thermostability not only as a partner in the salt bridge with Lys[38], but also by stabilizing the α-helix from residue 18 to 35 in *Bst*HU. On the basis of the present results, together with the previous results [Kawamura *et al.* (1996) *Biochemistry* 35, 1195-1200], three mutations, Glu[15] to Gly, Asp[34] to Glu, and Asn[38] to Lys, were simultaneously introduced into mesophilic protein *Bsu*HU to demonstrate their contributions to thermostability. This combination of multiple thermostabilizing mutations generated a more stable mutant protein HU, showing a T_m value of 64.5°C compared to 48.6°C for the wild-type *Bsu*HU.

Key words: bacilli, DNA binding protein HU, salt bridge, site-directed mutagenesis, thermostability.

A thorough understanding of the molecular means by which a protein gains thermostability is one of the major subjects in protein biochemistry. In general, two main strategies have been used to address this subject. One has been the introduction of a series of mutations into a given protein, such as bovine trypsin inhibitor (*1*), T4 lysozyme (*2*), *Bacillus* neutral proteases (*3*), and *Escherichia coli* RNase HI (*4*), followed by analysis of the effect of the mutations on the protein thermostability. These studies have revealed various factors important for protein thermostability.

The other approach has been comparative studies on proteins which are available from organisms living under different temperature conditions. In particular, Menendez-Arias and Argos (*5*) statistically compared the amino acid sequences of mesophilic and thermophilic organisms, and suggested a general principle for the engineering of a thermostable protein. In our own study, four DNA binding protein HUs from bacilli (two thermophiles: *Bacillus stearothermophilus* and *Bacillus caldolyticus*, and two mesophiles: *Bacillus subtilis* and *Bacillus globigii*) were used as model proteins for a study on protein thermostability, and the correlation between protein thermosta-

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Abbreviations: *Bst*HU, *Bacillus stearothermophilus* DNA binding protein HU; *Bsu*HU, *Bacillus subtilis* DNA binding protein HU; HU, DNA binding protein HU.

bility and optimum temperatures was examined (*6*).

Bacterial DNA binding protein HU (HU), an abundant protein and ubiquitous in the eubacterial kingdom, is a small and basic protein, composed of 90-92 amino acid residues, and occurs as a homotypic dimer in solution (*7*). HU binds to DNA in a sequence-independent manner and has been thought to play an important role in the structure of the bacterial nucleoid, being involved in replication (*8*), inversion (*9*), transposition (*10*), and repair (*11*), as a DNA chaperon (*12*). HUs from four bacilli were isolated and their protein structures well studied as model systems for nucleic acid-protein interactions (*13, 14*). In particular, the three-dimensional structure of *B. stearothermophilus* HU (*Bst*HU) was analyzed by both X-ray crystallographic (*15, 16*) and NMR analyses (*17, 18*), and a model for the nucleic acid-HU interaction has been proposed.

On the basis of sequence comparison of the four HUs from bacilli and the three-dimensional structure of *Bst*HU, the relative thermostability with respect to amino acid differences between the four proteins was discussed (*6*). This study revealed 14 amino acid replacements between the thermophilic and mesophilic proteins, which are almost all restricted to the molecular surface not implicated in the mode of DNA binding. Thus, these amino acid replacements might give rise to additional hydrogen bonds and/or salt bridges that would contribute to the thermostability of the thermophilic HU.

We decided to employ site-directed mutagenesis to assess the individual amino acid replacements which might contribute to the thermostability of the thermophilic HU. In the previous study, we showed that Gly^{15} in the bend between two α-helices in the N-terminal region is essential for the thermostability of *Bst*HU (*19*). In this study, we have extended this analysis to Glu^{34}, Arg^{37}, and Lys^{38}: in the crystal structure of *Bst*HU, these three form a cluster of oppositely charged residues on the hydrophilic surface, Glu^{34} sitting between Arg^{37} and Lys^{38}, and are expected to form a salt bridge. Since these three residues are replaced by Asp^{34}, Lys^{37}, and Asn^{38}, respectively, in mesophilic protein *Bsu*HU, this analysis would be expected to address the question as to whether or not a salt bridge on the hydrophilic surface is responsible for the thermostability of *Bst*HU.

EXPERIMENTAL PROCEDURES

Materials—The DNA binding protein HUs, *Bst*HU and *Bsu*HU, were prepared from *B. stearothermophilus* and *B. subtilis* cells, respectively, according to the method described by Groch *et al.* (*20*). The bacterial strains and plasmids used in the present study were the same as those previously reported (*19*). Restriction enzymes and DNA modifying enzymes were purchased from either GIBCO BRL or MBI Fermentas, and used as recommended by the suppliers. DNA ligation and *Bca*Best sequencing kits were purchased from TaKaRa. [α-^{32}P]dCTP was purchased from Amersham Japan. Heparin-agarose for the purification of mutant proteins was obtained from Sigma. All other chemicals were of analytical grade specified for biochemical use.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out by the unique site elimination method (*21*), using the Chameleon™ double-stranded site-directed mutagenesis kit supplied by Stratagene. The mutagenic primers used in the present study are listed in Table I. Mutations were confirmed by DNA sequencing using the dideoxy chain termination method (*22*) to ensure that no alterations other than those expected had occurred.

Production and Purification of Mutants—Restriction digests obtained with *Nde*I and *Bam*HI were employed to move a mutant gene from pUC18 into the expression vector, pET5a. An overproducing strain for each mutant protein was constructed by transforming *E. coli* BL21-(DE3) cells with one of the resultant plasmids. The overproduction and purification of the mutant proteins were performed as described previously (*19, 23*). The purity of the mutant preparations was assessed by SDS-PAGE, and each mutation was confirmed by protein sequencing with the aid of a gas-phase sequencer PSQ-1 (Shimadzu).

Circular Dichroism Spectra—CD spectra were obtained at 25°C with a Jasco J-720 spectropolarimeter. Proteins were dissolved to a final concentration of 0.15 mg/ml in 5 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. The path-length of the cells was 1 cm for far-ultraviolet CD spectra (200-250 nm).

Thermostability—Since Welfle *et al.* (*24, 25*) showed the strong dependency of the CD spectral properties and the stability of *Bsu*HU under a variety of conditions, all measurements were therefore made on samples prepared using the same procedure and the same type of spectrophotometer. The stabilities of the wild-type and mutant proteins were determined by monitoring the change in circular dichroism (CD) at 222 nm as a function of temperature. The protein concentration was 0.15 mg/ml. The buffer solutions were 5 mM phosphate and 0.2 M NaCl below pH 4, and 5 mM sodium phosphate and 0.2 M NaCl above pH 5. As for salt dependency, the buffer solutions were 5 mM sodium phosphate, pH 7.0, containing 0, 0.1, 0.2, and 0.5 M NaCl, respectively. The protein was incubated for 10 min at a given temperature in a thermostatically regulated circulating-water bath until it was completely unfolded. Then the temperature was decreased to ensure that the transition was reversible. Assuming that only the folded and unfolded states contribute to the measured CD value, the low- and high-temperature baselines were extrapolated to the transition region and the

TABLE I. **Mutagenic primers used in this study.** [a]The mismatched position in each oligonucleotide is underlined. [b]The template DNA used to produce each mutant by means of the unique site elimination method is also listed.

Mutant	Template[b]	Mutagenic primer[a]
*Bst*HU-E34D	Wild-type *Bst*HU gene	5′-GATTCGATTACAGATGCGCTGCGAAAAGG-3′
*Bst*HU-R37K	Wild-type *Bst*HU gene	5′-GAAGCGCTGAAAAAAGGCGATAAAG-3′
*Bst*HU-K38N	Wild-type *Bst*HU gene	5′-GCGCTGCGAAACGGCGATAAAGTG-3′
*Bst*HU-E34D/K38N	Mutant *Bst*HU-E34D gene	5′-GCGCTGCGAAACGGCGATAAAGTG-3′
*Bst*HU-E34Q	Wild-type *Bst*HU gene	5′-GATTCGATTACACAAGCGCTGCGAAAAGG-3′
*Bsu*HU-E15G/D34E/N38K	Mutant *Bsu*HU-E15G gene	5′-GGATTTTATCACCCTTTTTAAGTGCCTCTAAGATCG-3′

fraction of material that is folded was calculated as a function of temperature. The temperature of the midpoint of the transition, T_m, at which half of the protein is unfolded, the equilibrium constant for the unfolding reaction is unity and ΔG is zero, was determined by curve fitting of the fraction of the native protein *versus* temperature. The entropy change of unfolding at T_m, ΔS_m, and the enthalpy change of unfolding at T_m, ΔH_m, were calculated by van't Hoff analysis. The difference between the free-energy change of unfolding of the mutant proteins and that of the wild-type protein at T_m of the wild-type protein, $\Delta\Delta G$, was estimated by means of the relationship given by Becktel and Schellman (*26*), $\Delta\Delta G = \Delta T_m \Delta S_m$ (wild-type), where ΔT_m is the change in T_m of the mutant protein relative to that of the wild-type protein, and ΔS_m (wild-type) is the entropy change of the wild-type protein at T_m.

NMR Measurement—*Bst*HU and the three mutant proteins, *Bst*HU-E34D, *Bst*HU-K38N, and *Bst*HU-E34D/K38N, were dissolved in 20 mM sodium phosphate buffer (99.95% D_2O) containing 0.2 M NaCl, pH 7.0. The protein concentration was 1 mM. ^{1}H-NMR measurements were carried out with a JNM = A_{600} (600 MHz) NMR spectrometer. Chemical shifts were measured relative to the external standard, TSP (0 ppm).

Nomenclature—The DNA binding proteins from *B. stearothermophilus* and *B. subtilis* are designated as *Bst*HU and *Bsu*HU, respectively. The mutants are denoted using the one-letter code with the wild type residue given first, followed by the position number, and the new residue (*e.g.*, the *Bst*HU mutant in which Glu^{34} in the wild-type is replaced with Asp is referred to as *Bst*HU-E34D).

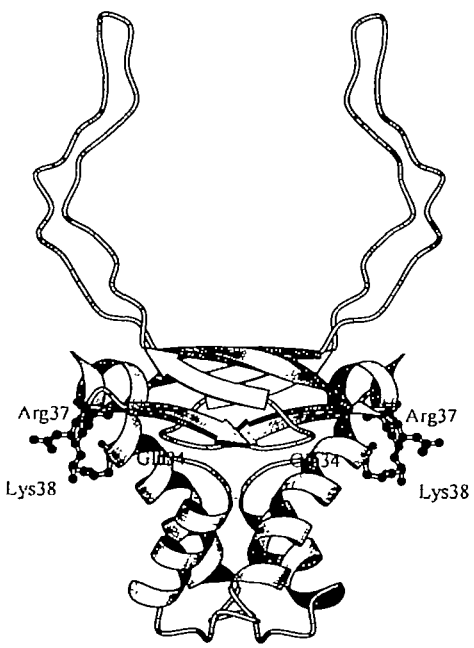

Fig. 1. **Three-dimensional structure of the dimer of *Bst*HU.** The protein model is from Tanaka *et al.* (*15*). The protein backbone is depicted by ribbons. The side chains of Glu^{34}, Arg^{37}, and Lys^{38}, whose contributions to the thermostability of *Bst*HU were examined in this study, are shown.

RESULTS AND DISCUSSION

Overproduction and Characterization of Mutant Proteins—We previously found that Gly^{15} in the α-helix-turn-α-helix motif of *Bst*HU plays an important role in the thermostability of *Bst*HU, and also showed that the enhanced thermostability of *Bst*HU, compared to that of *Bsu*HU, is not brought about by the addition of extra hydrogen bonds derived from Thr^{13} and Thr^{33} (*19*). The present study concerned mutations introduced individually or simultaneously into the protein, *Bst*HU: the replacements of Glu^{34}, Arg^{37}, and Lys^{38} in *Bst*HU with the corresponding amino acids Asp, Lys, and Asn, respectively, present in *Bsu*HU. Our objective was to determine the contribution of extra salt bridges between the three oppositely charged side chains on the molecular surface to the thermostability of *Bst*HU (Fig. 1).

Four mutant proteins, designated as *Bst*HU-E34D, *Bst*HU-R37K, *Bst*HU-K38N, and *Bst*HU-E34D/K38N, were expressed in *E. coli* BL21(DE3) cells using the expression vector, pET5a. The production level of each mutant protein was approximately the same as that of the wild-type *Bst*HU recombinant protein (*23*). The proteins were purified in the same manner as reported for the wild-type protein, and their behaviors during the purification procedures were almost identical with that of the wild-type. The amount of each mutant protein purified from a 1-liter culture was 15-20 mg. The purified proteins were homogeneous, as judged on SDS-PAGE and reverse-phase high performance liquid chromatography (data not shown). The proteins thus obtained were directly subjected to N-terminal sequence analysis, and mutations were confirmed at the amino acid level (not shown).

The secondary structure of each mutant protein and the wild-type protein was checked by examining the CD spectrum in the 200-250 nm region. As shown in Fig. 2, few spectral changes were observed among these proteins in the short-wavelength region (200-250 nm), where the spectra

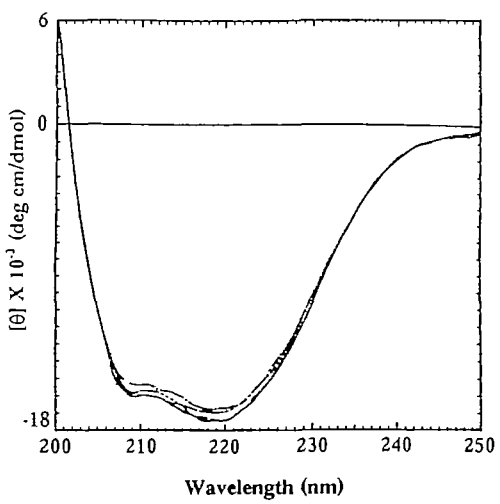

Fig. 2. **CD spectra of *Bst*HU and three mutant *Bst*HUs in the far-ultraviolet region.** Spectra were measured as described under "EXPERIMENTAL PROCEDURES." —, – –, ····, and –·– indicate the CD spectra of *Bst*HU, *Bst*HU-E34D, *Bst*HU-R37K, and *Bst*HU-K38N, respectively.

reflect the backbone polypeptide chain conformation, indicating that none of these mutations affected the secondary structure.

To further confirm that no structural changes were induced by each mutation in detail, ^{1}H-NMR spectra of three mutant proteins, *Bst*HU-E34D, *Bst*HU-K38N, and *Bst*HU-E34D/K38N, the exception being *Bst*HU-R37K, were measured and compared with that of the wild-type protein. Since the Arg^{37} to Lys mutation (*Bst*HU-R37K) did not affect the thermostability of the protein, as will be described in the following section, the structure of mutant *Bst*HU-R37K was not investigated. Figure 3 shows the aromatic proton and high-field shift methyl proton regions of the ^{1}H-NMR spectra at 600 MHz of *Bst*HU (A), *Bst*HU-E34D (B), *Bst*HU-K38N (C), and *Bst*HU-E34D/K38N (D) in 99.95% D_2O. No spectral changes in the chemical shifts caused by the mutations were detected. The lack of changes in the chemical shifts between the mutants and the wild-type was considered to be strong evidence for no large structural changes having occurred upon the mutations. Thus, the conformations of hydrophobic residues, including Phe residues which form the central core of the dimer, in the mutants are the same as those of *Bst*HU. Therefore, we concluded that the backbone conformation of the mutant proteins is practically the same as that of the wild-type *Bst*HU.

Thermostabilities of Mutant Proteins—The crystal structure of *Bst*HU includes a cluster of oppositely charged residues on its hydrophilic surface, comprising residues Glu^{34}, Arg^{37}, and Lys^{38}, that possibly weakly interact by forming exposed salt bridge(s). Comparable interactions between Glu^{34}, and Lys^{37}, and Lys^{38} are likely in another thermophilic HU from *Bacillus caldolyticus*. These residues are replaced by Asp^{34}, Lys^{37}, and Asn^{38}, respectively, in the mesophilic protein, *Bsu*HU. Wilson *et al.* (*6*) previously suggested that the shorter Asp^{34} side chain weakens possible interactions and in addition the substitution of Lys^{38} in thermophiles with Asn results in the loss of

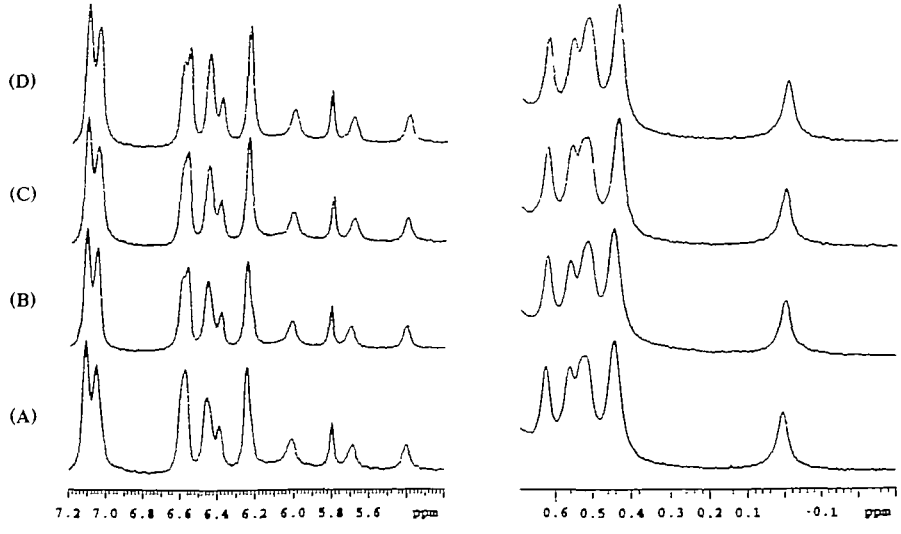

Fig. 3. **The aromatic proton and the high-field shift methyl proton regions of the 600 MHz ^{1}H-NMR spectra of *Bst*HU (A), *Bst*HU-E34D (B), *Bst*HU-K38N (C), and *Bst*HU-E34D/K38N (D) in 99.95% D_2O.** The measurements were performed at 25°C.

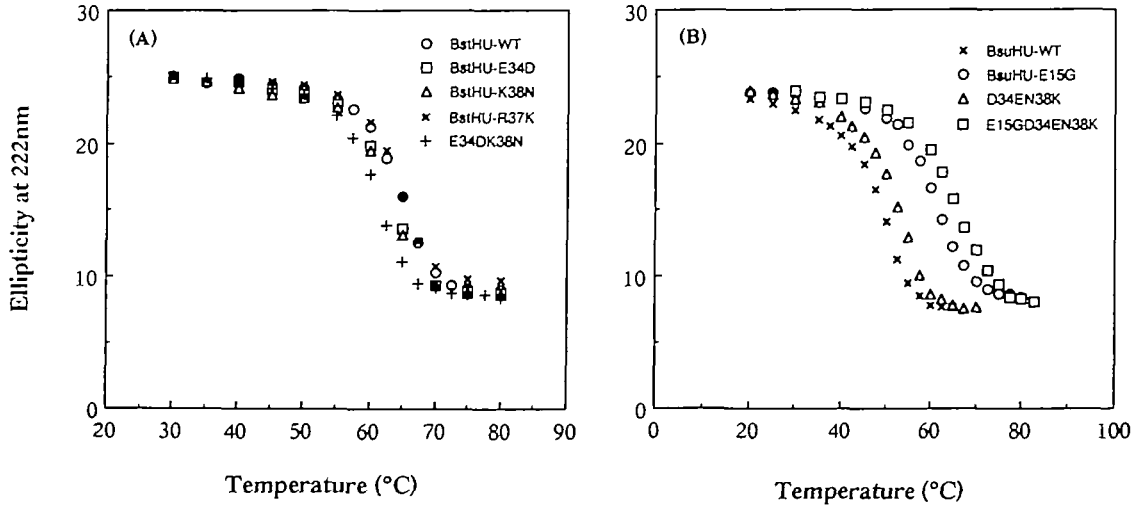

Fig. 4. **Thermal denaturation curves for *Bst*HU and *Bsu*HU, and their mutants.** (A) Temperature dependencies of the $[\theta]_{222nm}$ values of *Bst*HU (*Bst*HU-WT) and its mutants, *Bst*HU-E34D, *Bst*HU-R37K, *Bst*HU-K38N, and *Bst*HU-E34D/K38N (E34DK38N); (B) those of *Bsu*HU (*Bsu*HU-WT) and its mutants, *Bsu*HU-E15G, *Bsu*HU-D34E/N38K (D34EN38K), and *Bsu*HU-E15G/D34E/N38K (E15GD34EN38K).

the positive charge, leading to destabilization of the mesophilic protein.

In order to evaluate the contribution of the putative salt bridge(s) to the thermostability of *Bst*HU, we compared the stabilities of the three mutant proteins, *Bst*HU-E34D, *Bst*HU-R37K, and *Bst*HU-K38N, with that of the wild-type *Bst*HU, as to thermal denaturation. CD spectra of the wild-type *Bst*HU and the three mutants were measured, and then the magnitude of the CD-band at 222 nm was monitored at different temperatures. All mutants analyzed exhibited reversible denaturation: two successive thermal denaturations of the same sample gave a T_m value which differed by less than 0.5°C. The denaturation curves of the *Bst*HU wild-type and the four mutant proteins obtained at pH 7.0 are shown in Fig. 4A. Assuming a two-state transition for unfolding, the equilibrium constant between the folded and unfolded states, $K_D = D/N$, and the free energy change of unfolding, $\Delta G_D = -RT\ln K_D$, at a given temperature were calculated from each unfolding curve. The denaturation curves were also used to determine the melting temperature (T_m), the entropy change at T_m (ΔS_m), and the enthalpy change at T_m (ΔH_m). The stability of each mutant protein at T_m of the wild-type *Bst*HU was estimated using the relation, $\Delta\Delta G = \Delta T_m \Delta S_m$ (wild-type at T_m). The values thus obtained are summarized in Table II.

At pH 7.0, the Glu[34] to Asp mutation (*Bst*HU-E34D) resulted in a decrease in the thermostability of the protein (Fig. 4A). The melting temperature, T_m, of *Bst*HU-E34D was decreased by 2.3°C compared to 63.9°C for the wild-type *Bst*HU (Table II). The mutation reduced the thermostability of the protein by about 2.08 kJ/mol. Replacement of Lys[38] with Asn (*Bst*HU-K38N) also decreased the thermostability of the protein by 2.4°C ($\Delta\Delta G$: −2.17 kJ/mol), which was almost identical to the value shown by mutant *Bst*HU-E34D. These results suggested a favorable electrostatic interaction between Glu[34] and Lys[38] might contribute to the thermostability of *Bst*HU about 2.17 kJ/mol. We then attempted to replace Glu[34], a presumed salt bridge partner of Lys[38], with Gln, to investigate the importance of the carboxylate group of Glu[34]. Mutant *Bst*HU-E34Q, in which the putative salt bridge is absent, showed a decrease in stability relative to the wild-type by 3.0°C ($\Delta\Delta G$: −2.71 kJ/mol) (Table II). This strongly suggested that the carboxylate of Glu[34] does indeed undergo a stabilizing interaction with the positive charge of Lys[38], as expected from the crystal structure of *Bst*HU. To further confirm the contribution of Glu[34] and Lys[38] to the thermostability of *Bst*HU, a double mutant, *Bst*HU-E34D/K38N, in which Glu[34] and Lys[38] were simultaneously mutated to the corresponding residues, Asp and Asn, respectively, present in *Bsu*HU, was constructed and analyzed with respect to its thermostability. The results showed that the double mutant, *Bst*HU-E34D/K38N, was even less stable than the individual mutants, *Bst*HU-E34D and *Bst*HU-K38N, showing a T_m value of 60.5°C ($\Delta\Delta G$: −3.07 kJ/mol). This demonstrated the contribution of Glu[34] and Lys[38] to the thermostability of *Bst*HU, and the further decrease in thermostability of the double mutant suggested that it might be due to some residual interactions involving either the Glu[34] or Lys[38] residue.

In contrast to the contributions of Glu[34] and Lys[38], the replacement of Arg[37] with Lys did not affect the protein stability: mutant *Bst*HU-R37K exhibited the same stability as the wild-type *Bst*HU (Fig. 4A). This result suggested that the interaction of Arg[37] with Glu[34] in *Bst*HU might be compensated for by the positive charge on the replaced Lys residue, and the equivalent salt bridge might be formed in the mesophilic protein, *Bsu*HU. Thus, we concluded that Arg[37] does not contribute to the relative thermostability of *Bst*HU as compared to *Bsu*HU.

To confirm the contributions of Glu[34] and Lys[38] to the thermal stabilization of *Bst*HU, Glu, and Lys residues were simultaneously introduced at positions 34 and 38, respectively, in the mesophilic protein, *Bsu*HU: the *Bsu*HU mutant (*Bsu*HU-D34E/N38K), in which Asp[34] and Asn[38] in *Bsu*HU were replaced with Glu and Lys, respectively, was constructed, and its thermostability was analyzed in exactly the same manner as for the *Bst*HU mutant proteins. As shown in Fig. 4B and Table III, the melting temperature, T_m, of mutant *Bsu*HU-D34E/N38K was 52.1°C, *i.e.* 3.5°C higher than that of the wild-type *Bsu*HU (T_m value: 48.6°C). Thus, it was demonstrated that these two amino acids, Glu[34] and Lys[38], are responsible for the thermo-

TABLE II. **Parameters characterizing the thermal denaturation of *Bst*HU and its mutants.** [a]The melting temperature, T_m, is the midpoint temperature of the thermal denaturation transition shown in Fig. 4A. [b]The difference in melting temperature between the wild-type protein and the mutant proteins, ΔT_m, is calculated as $T_{m(mutant)} - T_{m(wild\text{-}type)}$. [c]The difference between the free-energy change of unfolding of the wild-type and mutants at T_m of the wild type protein, $\Delta\Delta G$, was calculated using the relationship reported by Becktel and Schellman (*26*), $\Delta\Delta G = \Delta T_m \Delta S_m$ (wild-type), as described under "EXPERIMENTAL PROCEDURES"; negative values indicate mutants less stable than the wild-type.

Protein	ΔH_m (kJ/mol)	ΔS_m (kJ/mol·K)	T_m[a] (°C)	ΔT_m[b] (°C)	$\Delta\Delta G$[c] (kJ/mol)
*Bst*HU, wild-type	304.2	0.903	63.9	–	–
*Bst*HU-E34D	317.4	0.949	61.6	−2.3	−2.08
*Bst*HU-R37K	336.8	0.999	63.9	0	0
*Bst*HU-K38N	309.7	0.926	61.5	−2.4	−2.17
*Bst*HU-E34D/K38N	324.6	0.973	60.5	−3.4	−3.07
*Bst*HU-E34Q	351.7	1.053	60.9	−3.0	−2.71

TABLE III. **Stabilities of the mesophilic protein, *Bsu*HU, and three mutants of it.** Thermal denaturation curves were obtained in exactly the same manner as for the thermophilic *Bst*HU mutants. The melting temperature, T_m, is the midpoint temperature of the thermal denaturation transition shown in Fig. 4B. ΔT_m(sum) and $\Delta\Delta G$(sum) represent the sums of the ΔT_m and $\Delta\Delta G$ values for the single and double mutant proteins with constituent substitutions, respectively. The data for the wild-type *Bsu*HU and mutant *Bsu*HU-E15G were obtained from the previous report (*19*).

Protein	ΔH_m (kJ/mol)	ΔS_m (kJ/mol·K)	T_m (°C)	ΔT_m (°C)	$\Delta\Delta G$ (kJ/mol)	ΔT_m(sum) (°C)	$\Delta\Delta G$(sum) (kJ/mol)
*Bsu*HU, wild-type	176.2	0.548	48.6	–	–		
*Bsu*HU-D34E/N38K	265.4	0.816	52.1	+3.5	+1.92		
*Bsu*HU-E15G	220.0	0.660	60.4	+11.8	+6.47		
*Bsu*HU-E15G/D34E/N38K	203.6	0.603	64.5	+15.9	+8.71	+15.3	+8.39

stability of *Bst*HU, a salt bridge possibly being formed between them.

Salt Dependency of Stabilities—An electrostatic interaction is expected to be weakened at high salt concentrations. Therefore, if a salt bridge between Glu^{34} and Lys^{38} indeed makes an important contribution to the thermostability of *Bst*HU, the difference in stability between the wild-type protein and the mutant proteins is expected to decrease with increasing salt concentration.

Thus, in order to better assess the contribution of the possible salt bridge to the thermostability of *Bst*HU, the salt dependency of the stabilities of the mutant proteins, *Bst*HU-E34D and *Bst*HU-K38N, was examined by circular dichroism-monitored reversible thermal denaturation as a function of ionic strength. The melting temperatures, enthalpies and entropies of unfolding, and the free energies of unfolding of the wild-type and the two single mutants at pH 7.0 are listed in Table IV.

In fact, the relative stability decreased progressively with increasing salt concentration: the two mutant proteins were less stable than the wild-type below 0.2 M NaCl, whereas they were almost the same as the wild-type in the presence of 0.5 M NaCl (ΔT_m: −0.4°C), indicating that a high ionic strength (0.5 M NaCl) almost completely abolishes the electrostatic interaction between Glu^{34} and Lys^{38}. Thus, it was concluded that Glu^{34} and Lys^{38} in *Bst*HU surely contribute to the thermostability by forming a favorable salt bridge.

pH Dependency of Stabilities—It is known that the signature of an electrostatic interaction is its pH dependence. The pK_a values for the side chain carboxylates of Asp and Glu in solution are approximately 4. Although this value may, of course, be affected in the folded protein, at pH 2.0 most aspartates and glutamates in the protein would be expected to be largely noncharged and thus not to undergo significant electrostatic interactions. In contrast, at neutral pH, however, most aspartates and glutamates are negatively charged, and any electrostatic interaction in which they participate will be manifest. In the absence of any other effects, a favorable interaction of Glu^{34} and Lys^{38} in *Bst*HU is therefore expected to have little effect on the thermostability at pH 2.0 and to affect the protein stability at neutral pH.

Thus, the pH dependency of the stabilities of the mutant proteins, *Bst*HU-E34D, *Bst*HU-K38N, *Bst*HU-E34D/K38N, and *Bst*HU-E34Q, was investigated in the range of pH 2.0 to 7.0. This was determined by circular dichroism-monitored reversible thermal denaturation as a function of pH. The results obtained are summarized in Table V. The mutant, *Bst*HU-E34D, in which Glu^{34} is replaced by Asp^{34} with a shorter side chain, exhibited about 2°C lower stability than that of the wild-type at neutral pH. However, the T_m of this variant relative to the wild-type was 1.7°C lower even at pH 2.0, where both the aspartate in the mutant and the glutamate in the wild-type are expected to lose most of their charge, indicating that the observed destabilization is largely pH independent. These results suggested that the destabilization arising from the Glu to Asp mutation at neutral pH is not simply due to the salt bridges weakened by the shorter Asp^{34} side chain. The decreased stability observed for mutant *Bst*HU-E34D at pH 2.0 also suggested that there is a distinct thermodynamic preference for Glu at position 34. This view was also supported by the pH dependency of the stabilities of the mutant proteins, *Bst*HU-E34D/K38N and *Bst*HU-E34Q: the stabilities of the two mutants at pH 2.0 were almost identical with that of mutant *Bst*HU-E34D (Table V). The most likely explanation for the lower stability of mutant protein *Bst*HU-E34D relative to that of the wild-type at pH 2.0 is the lower "helix forming tendency" of an Asp residue than a Glu residue. In other words, since a Glu residue is a stronger α-helix forming amino acid than an Asp residue (*27*), the second α-helix (pos. 18-35) in *Bst*HU might be stabilized by the Glu residue at position 34.

The mutant protein, *Bst*HU-K38N, in which the positive charge of the side chain of Lys^{38} is lost, exhibited essentially the same stability as that of the wild-type *Bst*HU at pH 2.0. As the pH increased, however, this mutant melted at a lower temperature than the wild-type, which is indicative of a favorable salt bridge between the Glu^{34}-Lys^{38} charged pair in *Bst*HU. Thus, it was concluded that the positive charge of Lys^{38} is critical for the thermostability of *Bst*HU.

One possibility that needs to be considered is stabiliza-

TABLE IV. **Salt dependency of the stabilities of *Bst*HU and two mutants of it at pH 7.0.** $\Delta\Delta G$ values were calculated for each ionic strength as the difference between the values for the wild-type and each mutant. All values are the averages of three determinations.

Protein	NaCl (M)	T_m (°C)	ΔH_m (kJ/mol)	ΔS_m (kJ/mol·K)	ΔT_m (°C)	$\Delta\Delta G$ (kJ/mol)
*Bst*HU, wild-type	0	52.9	216.5	0.664		
	0.1	57.8	268.7	0.812		
	0.2	63.9	304.2	0.903		
	0.5	65.4	265.9	0.785		
*Bst*HU-E34D	0	50.7	255.5	0.789	−2.2	−1.46
	0.1	56.3	333.9	1.014	−1.5	−1.22
	0.2	61.6	317.4	0.949	−2.3	−2.08
	0.5	65.0	332.7	0.984	−0.4	−0.31
*Bst*HU-K38N	0	49.6	226.1	0.700	−3.3	−2.19
	0.1	54.9	323.9	0.987	−2.9	−2.35
	0.2	61.5	309.7	0.926	−2.4	−2.17
	0.5	65.0	310.0	0.917	−0.4	−0.31

TABLE V. **pH dependency of the stabilities of *Bst*HU and three mutants of it.** All measurements were performed using the same buffers, the same cells, and identical experimental conditions. All values are the averages of at least three determinations.

Protein	pH	T_m (°C)	ΔH_m (kJ/mol)	ΔS_m (kJ/mol·K)	ΔT_m (°C)	$\Delta\Delta G$ (kJ/mol)
*Bst*HU, wild-type	2.0	48.4	229.3	0.714		
	3.0	61.3	301.1	0.901		
	4.0	64.1	265.2	0.788		
	5.0	64.3	354.8	1.052		
	7.0	63.9	304.2	0.903		
*Bst*HU-E34D	2.0	46.7	239.7	0.750	−1.7	−1.21
	3.0	59.0	328.1	0.988	−2.3	−2.07
	4.0	62.2	321.4	0.959	−1.9	−1.50
	5.0	62.3	282.3	0.842	−2.0	−3.00
	7.0	61.6	317.4	0.949	−2.3	−2.08
*Bst*HU-K38N	2.0	48.1	234.0	0.729	−0.3	−0.21
	3.0	58.6	222.2	0.670	−2.7	−2.43
	4.0	61.9	267.7	0.799	−2.2	−1.73
	5.0	61.7	291.6	0.871	−2.6	−2.73
	7.0	61.5	309.7	0.926	−2.4	−2.17
*Bst*HU-E34D/K38N	2.0	46.5	233.4	0.731	−1.9	−1.36
	7.0	60.5	324.6	0.973	−3.4	−3.07
*Bst*HU-E34Q	2.0	47.1	222.6	0.695	−1.3	−0.93
	7.0	60.9	351.7	1.053	−3.0	−2.71

tion of the wild-type *Bst*HU by salt bridges with other charged groups in the vicinity. This seems virtually impossible, because the Glu and Lys residues at positions 34 and 38 are located on the fully solvent-exposed surface and make no contact with other charged residues in the crystal structure of *Bst*HU.

It has been reported that the engineered electrostatic interaction between pairs of mobile, solvent-exposed charged residues on the surfaces of proteins contributes little to protein stability (*28-31*). In the crystal structure of *Bst*HU, the side chains of these two residues are also exposed to the solvent and quite mobile, the average B values for atoms within the side chains being about 100 Å^2 (*15*). The present study, however, demonstrated that Glu^{34} and Lys^{38} in *Bst*HU contribute to the thermostability by undergoing a favorable interaction on the hydrophilic surface and, in addition, the Glu^{34} residue plays an important role in the thermostability by stabilizing the α-helix from 18 to 35 in *Bst*HU.

Combination of Thermostabilizing Mutations—The previous study revealed that *Bsu*HU mutant protein *Bsu*HU-E15G, in which the essential Gly^{15} for the thermostability of *Bst*HU was introduced at position 15 instead of Glu in the mesophilic protein, *Bsu*HU, was about 12°C more stable than the wild-type *Bsu*HU (*19*). There have been several reports, on proteins such as subtilisin BPN′ (*32*), λ repressor (*33*), and RNase HI (*34*), that significant increases in protein stability can be generated by means of combinations of multiple thermostabilizing mutations. To test this approach in HU, we simultaneously introduced three thermostabilizing mutations, Glu^{15} to Gly, Asp^{34} to Glu, and Asn^{38} to Lys, into the mesophilic protein, *Bsu*HU, and then analyzed the thermostability of the resultant mutant (*Bsu*HU-E15G/D34E/N38K) in exactly the same manner as described above.

As shown in Fig. 4B and Table III, the combination of these stabilizing mutations clearly yielded a even more stable protein, with a T_m value of 64.5°C compared to 48.6°C for the wild-type *Bsu*HU. The stabilization energy arising from the triple substitution, E15G/D34E/N38K, seems to be somewhat greater than the sum of the constituent single plus double substitutions, E15G and D34E/N38K, but only by about 3.5% of the calculated value. This result indicated that the effects of the mutations on protein stability are roughly independent of each other and additive. Since the locations of Gly^{15}, and Glu^{34} and Lys^{38} are far from each other in the crystal structure of *Bst*HU, individual local reinforcements probably contribute to the greater thermostability of the thermophilic protein, *Bst*HU. In addition, the mesophilic triple mutant, *Bsu*HU-E15G/D34E/N38K, was more stable than the thermophilic protein, *Bst*HU, by 0.6°C in the T_m value. Recently, Ala^{27} and Val^{42} in *Bst*HU were found to also be responsible for the thermostability of *Bst*HU (Kawamura, S., unpublished results). If the stabilizing effects of the mutations are roughly additive, it will be possible to generate a more hyperstable protein than the thermophilic protein, *Bst*HU. This possibility is now being investigated in our laboratory. The present study, overall, indicates that the thermophilic protein, *Bst*HU, gained thermostability, through stabilization of two α-helices (α1: pos. 3-14, α2: pos.18-35) in the amino terminal region, and an additional salt bridge on the hydrophilic surface.

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