Glycine-15 in the Bend between Two α-Helices Can Explain the Thermostability of DNA Binding Protein HU from *Bacillus stearothermophilus*

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ABSTRACT: On the basis of sequence comparison of thermophilic and mesophilic DNA binding protein HUs, *Bacillus stearothermophilus* DNA binding protein HU (*Bst*HU) seems to gain thermostability with a change in amino acid residues present on the molecular surface. To evaluate the contribution of exchange of each amino acid to the thermostability of *Bst*HU, we constructed three mutants, *Bst*HU-T13A (Thr^{13} to Ala), *Bst*HU-G15E (Gly^{15} to Glu), and *Bst*HU-T33L (Thr^{33} to Leu), in which the amino acids in *Bst*HU were changed to the corresponding ones in *Bacillus subtilis* DNA binding protein HU (*Bsu*HU). Stability of the mutant proteins was determined from thermal-denaturation curves. Replacement of Gly^{15} located in the turn region between α1 and α2 helices (HTH motif), with Glu (*Bst*HU-G15E), resulted in a decrease in thermostability, and the T_m value was 54.0 °C compared to the T_m value of 63.9 °C for *Bst*HU. The mutants, *Bst*HU-T13A and *Bst*HU-T33L, were, by contrast, slightly more stable ($T_m$values of 67.0 and 65.6 °C for *Bst*HU-T13A and *Bst*HU-T33L, respectively) than the wild type. We then generated the *Bsu*HU mutant protein *Bsu*HU-E15G, where Glu^{15} in *Bsu*HU was in turn replaced by Gly, and we analyzed the thermostability. This substitution clearly enhanced the melting temperature by 11.8 °C (T_m value: 60.4 °C for *Bsu*HU-E15G) compared to the value for *Bsu*HU (T_m: 48.6 °C). Thus, Gly^{15} in the HTH motif of *Bst*HU has an important role in the thermostability of *Bst*HU. Characterization of the stucture of the *Bst*HU-G15E by ^{1}H-NMR analysis showed that solvent accessibility of amide proton of Ala^{21} in the mutant was significantly increased compared with that of wild type, which means that the structure of the HTH motif in the N-terminal region in the mutant was changed to a more open conformation, thereby avoiding the interaction of Ala^{21} with either Ser^{17} by hydrogen bond or Ala^{11} by hydrophobic interaction.

When attempting to produce an enhanced stability of proteins, the molecular basis by which a protein has gained stability against stringent conditions has to be understood at the amino acid level. It is known that proteins from thermophiles are generally more stable than are corresponding ones from mesophiles; therefore, thermophiles are the traditional source of stable proteins. This is also the case for the procaryotic histone-like DNA binding protein HU.

The DNA binding protein HU (HU),[1] being ubiquitous in both eubacteria and archaebacteria, is a small, basic dimeric protein with 90−92 amino acid residues (M_r of 9500 for the monomer) and occurs as a homotypic dimer in solution (Drlica & Rouviere-Yaniv, 1987). Since HU binds nonspecifically to both single-stranded and double-stranded DNA as well as to RNA, it has been thought to play a major role in physical packing of the bacterial chromosome (Geider & Hoffmann-Berling, 1981). However, recent *in vivo* and *in vitro* studies on the *Escherichia coli* HU revealed that HU may be implicated in replication (Dixon & Kornberg, 1984; Bramhill & Kornberg, 1988; Hwang & Kornberg, 1992), inversion (Johnson et al., 1986; Wada et al., 1989), transposition (Lavoie & Choconas, 1990; Morisato & Kleckner, 1987), and repair (Castaing et al., 1995; Boubrik & Rouviere-Yaniv, 1995), as a "DNA chaperone" (Travers et al., 1994). Moreover, it has been reported that the *Bacillus subtilis* HU (*Bsu*HU) is required for DNA recombination mediated by β-recombinase (Alonso et al., 1995).

Four DNA binding protein HUs from Bacilli (two thermophiles: *Bacillus stearothermophilus* and *Bacillus caldolyticus*, and two mesophiles: *B. subtilis* and *Bacillus globigii*), have been isolated and their structures well studied as a model system for nucleic acid−protein interaction (Imber et al., 1982; Dijk et al., 1983). In particular, for *B. stearothermophilus* HU (*Bst*HU), the primary structure was determined both by protein chemical methods (Kimura & Wilson, 1983) and by nucleotide sequencing (Kawamura et al., 1995). The three-dimensional structure was first analyzed at a resolution of 2.8 Å (Tanaka et al., 1984) and has recently been refined at 2.1 Å (White et al., 1989), and the model for nucleic acid−HU interaction has been proposed.

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[1] Abbreviations: *Bst*HU, *Bacillus stearothermophilus* DNA binding protein HU; *Bsu*HU, *Bacillus subtilis* DNA binding protein HU; HU, DNA binding protein HU; *hbst*, *Bacillus stearothermophilus* DNA binding protein HU gene; *hbsu*, *Bacillus subtilis* DNA binding protein HU gene; HTH, α-helix−turn−α-helix; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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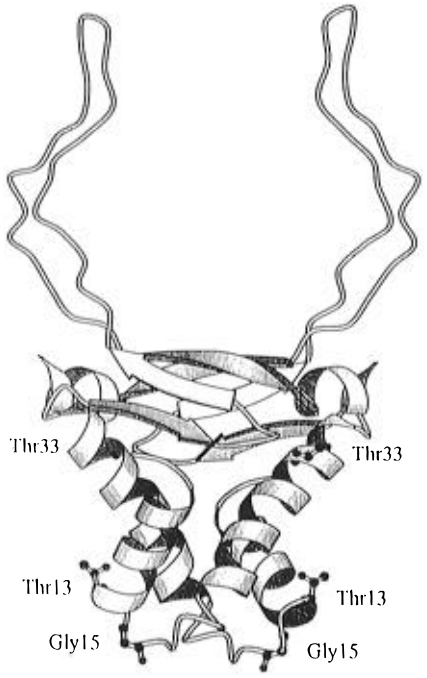

FIGURE 1: Tertiary structure of the dimer of *Bst*HU. The protein model is from Tanaka et al. (1984). It should be noted that the residues in the distal region of the arm between strands 2 and 3 (59−70) were tentatively included in this figure. Side chains are shown only for residues where the contribution to the thermostability of *Bst*HU was examined in this study.

Furthermore, almost all ^{1}H, ^{13}C, and ^{15}N resonance assignments for *Bst*HU have been defined in NMR experiments (Vis et al., 1994).

On the basis of sequence comparison of these four DNA binding protein HUs from two thermophiles and two mesophiles, the relative thermostability in terms of the amino acid differences between the four proteins and three-dimensional structure of *Bst*HU was discussed (Wilson et al., 1990). The study showed that 14 amino acid differences between the thermophilic and mesophilic proteins are restricted to the molecular surface not implicated in the mode of DNA binding. Thus, these changes might give rise to additional hydrogen bonds and salt bridges that would contribute to the thermal stabilization of the thermophilic HU. Subsequently, we constructed an expression plasmid of *Bst*HU in *E. coli*, in which the *Bst*HU gene was placed under control of the T7 phage promoter and *Bst*HU was produced in large quantities in *E. coli* (Kawamura et al., 1995). The recombinant protein thus obtained had a circular dichroism spectrum identical to that of the authentic protein and bound to DNA to the same extent as seen with the authentic protein.

To evaluate the contribution of individual amino acid changes between thermophiles and mesophiles to the thermal stabilization of *Bst*HU, we made use of site-directed mutagenesis of the *Bst*HU gene. We report here effects on the thermostability of *Bst*HU when we replaced two threonine residues, Thr^{13} and Thr^{33}, in α-helices and Gly^{15} in the bend between α-helices (HTH motif), as shown in Figure 1. This addresses the previous discussion in terms of *Bst*HU thermostability, as to whether the extra hydrogen bonds derived from Thr^{13} and Thr^{33} are critical for stability and whether or not Gly^{15} in the HTH motif has an important role in protein stability (Wilson et al., 1990).

MATERIALS AND METHODS

Materials. The DNA binding proteins HUs, *Bst*HU, and *Bsu*HU were isolated from *B. stearothermophilus* and *B. subtilis*, respectively, as described by Groch et al. (1992). Bacterial strains used as cloning hosts were *E. coli* JM109 and BL21(DE3). Plasmid pUC18 and expression plasmid pET5a were obtained from TaKaRa. Restriction enzymes and nucleic acid modifying enzymes were obtained from GIBCO BRL and used as recommended by the supplier. [α-^{32}P]dCTP was purchased from Amersham Japan. Heparin−agarose for purification of mutant proteins was purchased from Sigma. All other chemicals were of analytical grade, specified for biochemical use.

Site-Directed Mutagenesis. The genes *hbst* and *hbsu* were ligated to the plasmid pUC18, as described previously (Kawamura et al., 1995), and used for the site-directed mutagenesis. All mutants used in the present study were generated by the unique site elimination method (Deng & Nickoloff, 1992) principally using U.S.E. Mutagenesis kits (Pharmacia). T4 DNA polymerase was replaced by the Klenow fragment (TaKaRa). The synthetic oligonucleotides used were a 30 mer: 5′GCGGTCGCTGAAGCAAGCGGTCTTTCCAAA3′ for *Bst*HU-T13A, 24 mer: 5′GTCGCTGAAACAAGCGAGCTTTCC3′ for *Bst*HU-G15E, 27 mer: 5′TTTGATTCGATTCTAGAAGCGCTGCGA3′ for *Bst*HU-T33L, and 24 mer: 5′GTCGCTGAAGCAAGCGAGCTTTCC3′ for *Bst*HU-T13A/G15E. The *Bsu*HU mutant *Bsu*HU-E15G was made with 28 mer: 5′CTTTTTTAGACAATCCGCTTGCTTCTGC3′. Structures of all mutants were confirmed by DNA sequence determination by the dideoxy chain termination method (Sanger et al., 1977), using *Bca*BEST sequencing kits (TaKaRa).

Expression of the Mutant Genes and Purification of Mutants. The site-specific mutants of pET-*hbst*, a plasmid for expression of *Bst*HU, were transformed into *E. coli* BL21-(DE3), and the transformants were cultivated as described elsewhere (Kawamura et al., 1995). After confirmation of the overexpression of recombinant proteins by SDS−PAGE, the recombinant proteins were purified by a heparin−agarose column. The purity of each mutant protein was checked by SDS−PAGE, and amino acid substitution of each mutant was confirmed by protein sequencing with the aid of a gas-phase sequencer (Shimadzu PSQ-1).

Circular Dichroism Spectra. CD spectra were measured using a JASCO J 720 spectropolarimeter at given temperatures. Proteins were dissolved in 5 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl, and the protein concentration was 0.15 mg/mL. The path length of the cells used for the wavelength region 200−250 nm was 1 cm.

Thermal Denaturation. Thermal denaturation curves were obtained by monitoring the CD value at 222 nm and temperature indicated. The wavelength (222 nm) used is sensitive to the presence of any secondary structure in the protein. The cuvette containing the sample was heated for 10 min at a given temperature by a thermostatically regulated circulating-water bath. The fraction of native protein was calculated from the CD values by linearly extrapolating the pre-transition and post-transition base lines, based on the assumption that the CD values of the pre-transition and post-transition reflect those of the folded and unfolded proteins, respectively. The temperature of the midpoint of the

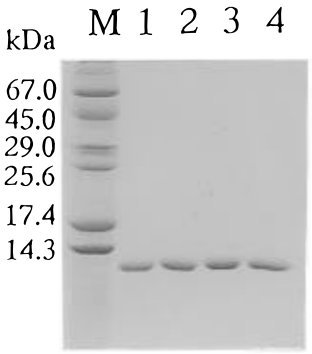

FIGURE 2: SDS−PAGE of *Bst*HU and three mutant *Bst*HUs. Lane 1, *Bst*HU; lane 2, *Bst*HU-T13A; lane 3, *Bst*HU-T33L; lane 4, *Bst*HU-G15E. M indicates the standard proteins including bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), α-chymotrypsinogen (25.6 kDa), myoglobin (17.4 kDa), and lysozyme (14.3 kDa).

transition, T_m, at which half of each protein sample unfolds, was determined from curve fitting of the fraction of native protein versus temperature plots. 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 the relationship given by Becktel and Schellman (1987), $\Delta\Delta G = \Delta T_m \Delta S_m$(wild type), where, ΔT_m is the change in T_m of the mutant protein relative to 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 *Bst*HU-G15E were dissolved in 20 mM sodium phosphate buffer (99.95% D_2O or H_2O/D_2O (90/10)) containing 200 mM NaCl at pH 6.92. The protein concentration was 1 mM. ^{1}H-NMR measurements were carried out on a JNM-A600 (600 MHz) NMR spectrometer. Chemical shifts were measured relative to the external standard, TSP (0 ppm). All ^{1}H-NMR resonances of *Bst*HU have been previously assigned (Vis et al., 1994).

Nomenclature. 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 Thr^{13} in the wild type is replaced with Ala is referred to as *Bst*HU-T13A).

RESULTS

Production and Characterization of Mutant Proteins. The mutant proteins were expressed in *E. coli* using the expression vector pET5a and were overproduced in a similar amount to the wild type *Bst*HU recombinant protein; the yields of proteins from 1 L of culture broth were 15−20 mg for all mutant proteins. The proteins were purified as done for the wild type protein (Kawamura et al., 1995) and proved to be homogeneous, by SDS−PAGE (Figure 2) 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 wild type protein was checked by examining the CD spectrum in the 200−250 nm region. As shown in Figure 3, the CD

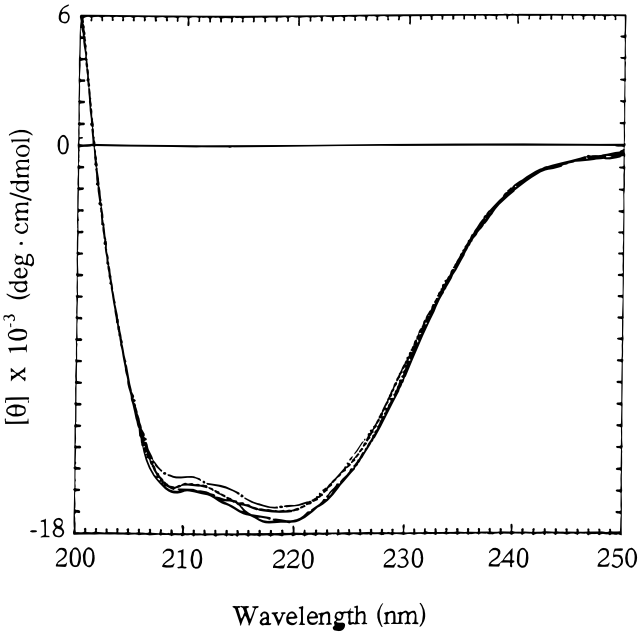

FIGURE 3: Circular dichroic spectra of *Bst*HU and three mutant *Bst*HUs in the far-ultraviolet region. The spectra were measured on a JASCO-J 720 spectropolarimeter at 25 °C. The protein concentration was 0.15 mg/ml, as described in Materials and Methods. —, − −, ···, and − · − indicate the CD spectra of *Bst*HU, *Bst*HU-T13A, *Bst*HU-G15E, and *Bst*HU-T33L, respectively.

spectra of mutant proteins in the short-wavelength region (200−250 nm), indicating the backbone polypeptide chain conformation, were essentially the same as that of the wild type *Bst*HU. Therefore, we concluded that the backbone conformation of the mutant proteins is practically the same as that of *Bst*HU.

Thermostabilities of Mutant Proteins. To compare the stabilities of the three mutant proteins, *Bst*HU-T13A, *Bst*HU-G15E, and *Bst*HU-T33L, with that of the wild type *Bst*HU, against thermal denaturation, CD spectra of the wild type *Bst*HU and three mutants were measured, and then the magnitude of the CD band at 222 nm was followed at different temperatures. Welfle et al. (1992, 1993) showed a strong dependence of CD spectral property and the stability of *Bsu*HU, under a variety of conditions. Therefore, for this analysis all measurements were made on samples prepared using the same procedure and the same type of spectrophotometer, as described in Materials and Methods. As a typical example, the CD spectra of the wild type *Bst*HU incubated at 30−80 °C are shown in Figure 4. All mutants in this analysis 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 three mutant proteins are shown in Figure 5A. By 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 the T_m of 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 1. The melting temperature T_m for *Bst*HU derived from the present study was 63.9 °C, which

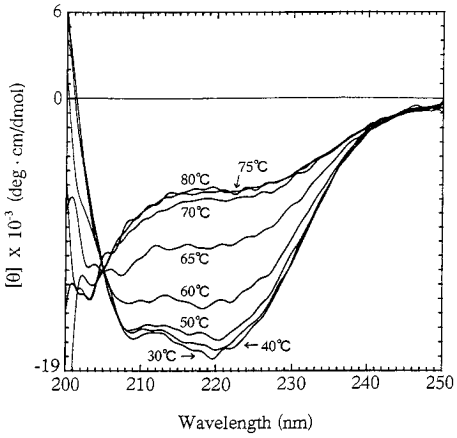

FIGURE 4: Circular dichroic spectra of *Bst*HU incubated at different temperatures. *Bst*HU was incubated at a given temperature for 10 min, and then the CD spectrum was measured in the same manner as described for Figure 3.

was close to reported values 64 °C (Wilson et al., 1990) and 68 °C (Dijk et al., 1983).

Thus, Gly^{15} to Glu mutation (*Bst*HU-G15E) resulted in a considerable decrease in thermostability of the protein (Figure 5A). The melting temperature, T_m, for the *Bst*HU-G15E was decreased by 9.9 °C compared to 63.9 °C for the wild type *Bst*HU (Table 1). The mutation reduced thermostability of the protein by about 8.94 kJ mol^{-1}. In contrast to Gly^{15}, replacements of Thr^{13} and Thr^{33} with Ala and Leu, respectively, have little effect on the stability: the *Bst*HU-T33L was almost as stable as the wild type ($\Delta\Delta G$: 1.54 kJ mol^{-1}) and the *Bst*HU-T13A was slightly more stable than the wild type, with a T_m value of 67.0 °C ($\Delta\Delta G$: 2.80 kJ mol^{-1}).

To confirm the contribution of Gly^{15} to the thermal stabilization of *Bst*HU, the Gly residue was introduced at position 15 in the mesophilic protein *Bsu*HU; the *Bsu*HU mutant (*Bsu*HU-E15G), where Glu^{15} in *Bsu*HU was replaced with Gly, was constructed, and thermostability was analyzed in exactly the same manner as for the *Bst*HU mutant proteins. As shown in Figure 5B and Table 1, replacement of Glu^{15} in *Bsu*HU with Gly resulted in an increase in protein stability, with a T_m value of 60.4 °C compared to 48.6 °C for the wild type *Bsu*HU. Therefore, replacement of Gly^{15} by Glu at the HTH motif in the *Bst*HU-G15E gives rise to a structural change which seems to contribute to the thermal destabilization of the mutant protein.

^{1}H-NMR Analysis of the Mutant BstHU-G15E. A crystallographic analysis of the mutant *Bst*HU-G15E, when available, will make feasible analysis of how the substitution of the Gly residue affects structure of the HTH motif. To investigate structural basis of the observed stability of the *Bst*HU-G15E, the protein *Bst*HU-G15E was subjected to ^{1}H-NMR analysis. Figure 6 shows the aromatic proton and the high-field shift methyl proton regions of ^{1}H-NMR spectra at 600 MHz of *Bst*HU (A) and *Bst*HU-G15E (B) in 99.95% D_2O. The measurements were done at 25 °C. As spectra A and B are alike, the conformation of hydrophobic residues, including Phe residues of *Bst*HU-G15E, is much the same to those of *Bst*HU.

The solvent accessibility of amide protons were then evaluated by measuring the ^{1}H-NMR spectra at various elapsed times after dissolving the protein in D_2O, and results are shown in Figure 7. The spectrum at the bottom shows the result of measurement in H_2O/D_2O (90/10), and others are in 99.95% D_2O after the elapse of time. Although the spectra of *Bst*HU and *Bst*HU-G15E are similar in H_2O/D_2O (90/10), they significantly deviate from each other in 99.95% D_2O, which means that the environment of amide protons, in terms of solvent accessibility, is altered by the mutation. In *Bst*HU, the resonances of the amide protons of Phe^{50} and Ala^{78} decreased in intensity and disappeared with increasing lapses of time, whereas the resonance of Ala^{21} remained even after 1370 min. On the other hand, for *Bst*HU-G15E, although the resonances of the amide protons of Phe^{50} and Ala^{78} disappeared in a manner similar to that seen with *Bst*HU, the resonance of Ala^{21} disappeared at an even faster rate (Figure 7).

DISCUSSION

Comparison of the amino acid sequences of the thermophilic HU proteins with those of the mesophilic HU proteins showed 14 amino acid exchanges, and the contribution of such changes to protein stability was evaluated on the basis of the crystal structure of *Bst*HU (Wilson et al., 1990). The present study concerned mutations introduced individually into the protein *Bst*HU: the replacements of two threonine residues, Thr^{13} and Thr^{33}, with the corresponding amino acids Ala and Leu, respectively, as present in *Bsu*HU. Our objective was to assess contribution of the hydrogen bond to thermal stability for *Bst*HU and the replacement of Gly^{15} in *Bst*HU with corresponding Glu in *Bsu*HU and to evaluate the effect of a steric hindrance in the bend between α1 and α2 helices on protein stability.

Three mutant proteins thus designed, *Bst*HU-T13A, *Bst*HU-G15E, and *Bst*HU-T33L, were purified to apparent homogeneity from the soluble fractions of cells, and behaviors in the purification procedures were almost identical with that of the wild type. None of these mutations affected the secondary structure, as indicated by the far-ultraviolet CD spectra at 25 °C. Thus, one amino acid substitution does not affect the backbone structure of the mutant protein.

A comparison of the denaturation curves of the mutant proteins and wild type showed that only the mutation Gly^{15} to Glu reduces the stability of the protein by about 8.94 kJ mol^{-1} at 63.9 °C. The important role of the Gly residue in protein stability was further demonstrated by the *Bsu*HU mutant protein *Bsu*HU-E15G, where the mutation Glu^{15} to Gly enhanced thermostability of the protein by 6.47 kJ mol^{-1} (Table 1). Crystallographic analysis of *Bst*HU localizes Gly^{15} in the bend between the two α-helices and shows that this region of conformational space is expected to be energetically favorable only for the glycine residue. Hence, replacement of Gly^{15} by Glu in the turn structure gives rise to certain structural changes which may contribute to thermal destabilization of the mutant protein *Bst*HU-G15E.

This was examined by ^{1}H-NMR analysis of the *Bst*HU-G15E. Although ^{1}H-NMR spectra of the mutant protein and wild type were almost identical, measurements made at different times suggested that the solvent accessibility of amide proton of Ala^{21} differs between the proteins *Bst*HU and *Bst*HU-G15E; Ala ^{21} in the mutant protein becomes more accessible to the solvent than does Ala^{21} in the wild type.

In the *Bst*HU, Ala^{21} locates in the second α-helix with its amide proton hydrogen-bonded to the carbonyl oxygen of Ser^{17} which is the N-terminal residue of the second α-helix.

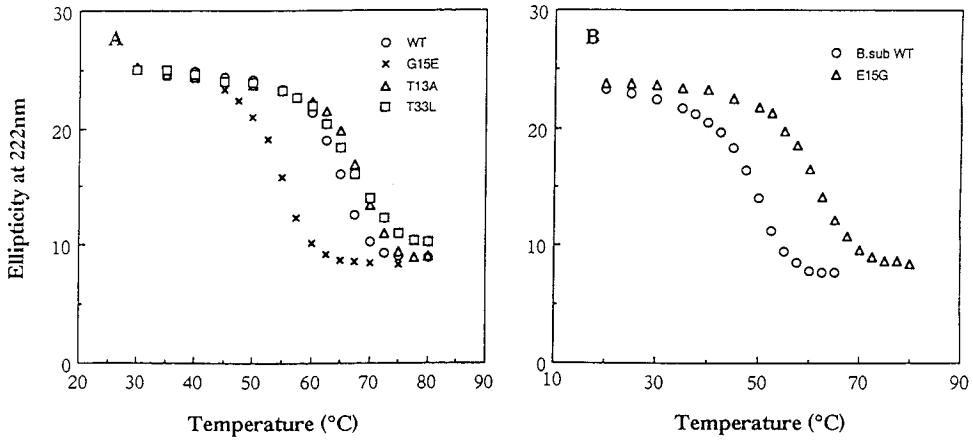

FIGURE 5: Thermal unfolding curves for *Bst*HU, *Bsu*HU, and their mutant proteins. (A) Temperature dependencies of $[\theta]_{222nm}$ values of *Bst*HU (WT) and its mutants, *Bst*HU-T13A (T13A), *Bst*HU-G15E (G15E), and *Bst*HU-T33L (T33L); (B) those of *Bsu*HU (B.sub WT) and its mutant *Bsu*HU-E15G (E15G).

Table 1: Parameters Characterizing the Thermal Denaturation of *Bst*HU, *Bsu*HU, and Their Mutants

protein	ΔH_m (kJ mol^{-1})	ΔS_m (kJ mol^{-1} K^{-1})	T_m[a] (°C)	ΔT_m[b] (°C)	$\Delta\Delta G$[c] (kJ mol^{-1})
*Bst*HU, wild type	304.2	0.903	63.9		
*Bst*HU-T13A	276.8	0.814	67.0	+3.1	+2.80
*Bst*HU-G15E	271.2	0.829	54.0	−9.9	−8.94
*Bst*HU-T33L	245.0	0.724	65.6	+1.7	+1.54
*Bst*HU-T13A/G15E	262.1	0.794	57.2	−7.2	−6.50
*Bsu*HU, wild type	176.2	0.548	48.6		
*Bsu*HU-E15G	220.0	0.660	60.4	+11.8	+6.47

[a] The melting temperature, T_m, is the temperature of the midpoint of the thermal denaturation transition shown in Figure 5A and B. [b] The difference in the melting temperature between the wild type and mutant proteins, ΔT_m, is calculated as T_m(mutant) − T_m(wild type). [c] The difference between the free-energy change of unfolding of the wild type and mutant proteins at T_m of the wild type protein, $\Delta\Delta G$, was calculated by the relationship given by Becktel and Schellman (1987), $\Delta\Delta G = \Delta T_m \Delta S_m$ (wild type) as described in Materials and Methods; negative values indicate less stable mutants than the wild type.

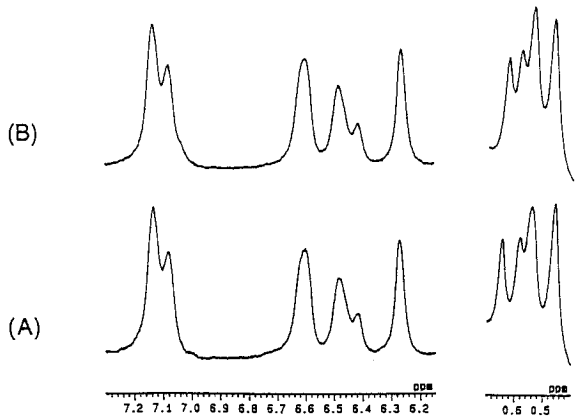

FIGURE 6: The aromatic proton and the high-field shift methyl proton region of ^{1}H-NMR spectra at 600 MHz of *Bst*HU (A) and the mutant *Bst*HU-G15E (B) in 99.95% D_2O. The measurements were done at 25 °C.

The side chain Ala^{21} makes contact with Ala^{11} in the first α-helix. The contact of these two residues is important for formation of the HTH motif. Increase in solvent accessibility of the amide proton of Ala^{21} by the mutation suggests that the HTH motif became a somewhat more open structure. The change should include the local conformational change at position 15 due to replacement of Gly^{15} with Glu. It was therefore demonstrated that the local conformational change

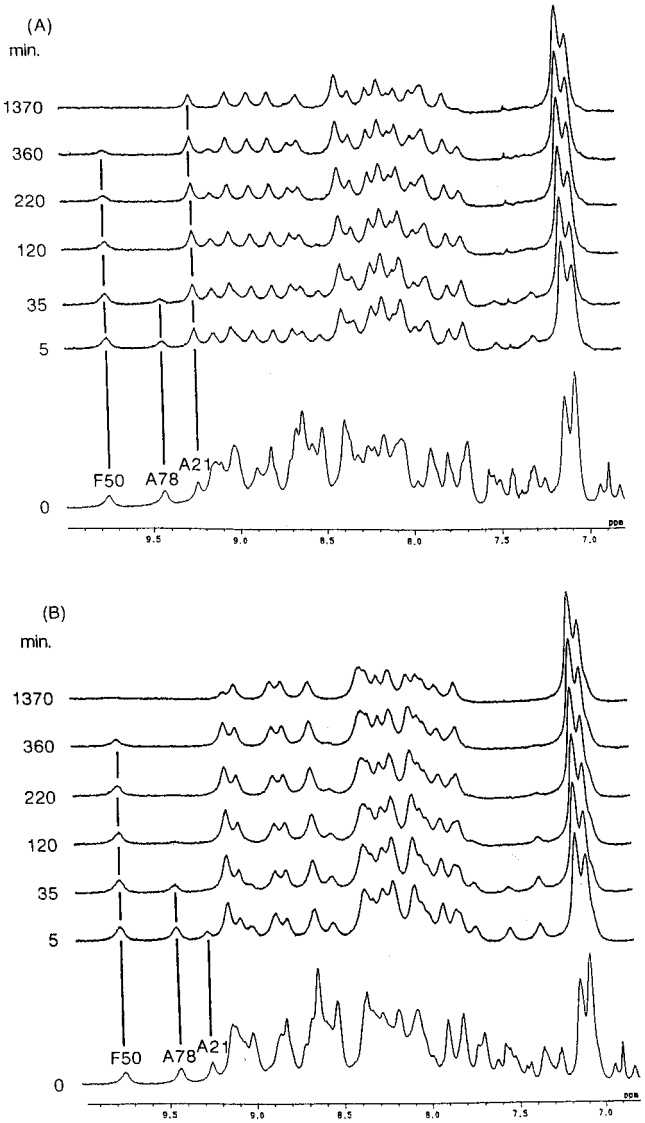

FIGURE 7: The amide proton region of the 600 MHz ^{1}H-NMR spectra of *Bst*HU (A) and the mutant *Bst*HU-G15E (B) at 25 °C at the indicated elapsed time after dissolution in D_2O. The spectrum at the bottom was recorded in H_2O/D_2O (90/10). The other spectra were recorded in 99.95% D_2O.

inevitably affects the relative orientation of the two α-helices and results in the N-terminal "melting" of the second α-helix. In other words, the protein *Bst*HU might have gained

thermostability by stabilizing two α-helices in the HTH motif in the N-terminal region.

The HTH motif present in the N-terminal region of *Bst*HU is structurally similar to that found in the operator/repressor family of DNA binding proteins, such as CAP and λ-cro, where the motif is directly involved in DNA binding. The glycine residue in the bend is known to be conserved in all of the known operator/repressor families of DNA binding proteins, but not in HUs. Our findings show the significance of glycine residues in maintaining the HTH motif, as a compact structure. When glycine was substituted for other residues, the HTH motif in DNA binding proteins other than HU, where HTH protrudes from the molecular surface, may not be maintained. In HU, on the other hand, since the same motif is in fact with the other half of the dimer, the substitution does not destroy the structure, although the compactness of the structure is to some extent lost and the thermal stability is decreased. The present result not only explains the thermal stability of *Bst*HU but also demonstrates the unique role of the HTH motif in the DNA binding protein HU.

The mutant proteins *Bst*HU-T13A and *Bst*HU-T33L gave an unexpected result. Since the hydrogen bonds to Thr^{13} and Thr^{33} hydroxy groups are lost in mutant proteins, it was expected that the mutations might decrease protein stability. However, the stability of the mutant *Bst*HU-T33L was almost identical with that of the wild type, and the *Bst*HU-T13A was stable compared to the wild type. The enhanced stability provided by the Ala residue at position 13 was further confirmed by constructing the mutant protein *Bst*HU-T13A/G15E with two substitutions; the *Bst*HU-T13A/G15E was more stable than *Bst*HU-G15E and T_m was increased by 3.2 °C, compared to the mutant *Bst*HU-G15E (Table 1). The increased thermostabilities of the mutant proteins may be due to loss of the hydrogen bond and might be compensated for by an increase in hydrophobicity of the replaced amino acid residue (Ala or Leu). In particular, since the Ala residue is a strong α-helix forming amino acid, it seems likely that the first α-helix in the *Bst*HU-T13A is stabilized by the Ala residue at position 13. Nevertheless, the increased thermostability of *Bst*HU, compared to *Bsu*HU, is not brought about by adding extra hydrogen bonds linking Thr^{13} to Ala^{9} and Thr^{33} to Phe^{29}.

The mesophilic mutant protein *Bsu*HU-E15G showed a considerable increase in the thermostability: T_m value of 60.4 °C, but is still lower by about 3.5 °C than that of *Bst*HU. Hence, other substitutions might contribute to the thermostability of *Bst*HU. The first candidate is the extra salt bridge on the molecular surface given by the residues at Glu^{34}, Arg^{37}, and Lys^{38}, where the corresponding amino acids Asp^{34}, Lys^{37}, and Asn^{38} locate in *Bsu*HU, and in particular, the substitution of Lys^{38} with Asn in the mesophilic protein means a loss of the positive charge and might be implicated in destabilization of the mesophilic protein. Another candidate is the Ala residue at position 27, where the Ser residue is located in *Bsu*HU. As with the case of the mutant *Bst*HU-T13A in this study, Ala^{27} might stabilize the second α-helix and thereby provide thermostability to *Bst*HU. All these possibilities are now being investigated in our laboratory. This study provides a molecular basis of the thermostability of *Bst*HU and gives a detailed structural basis for the DNA binding protein HU.

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