Investigation of the Structural Basis for Thermostability of DNA-binding Protein HU from *Bacillus stearothermophilus*

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Site-directed mutagenesis was used to identify amino acid residues essential for the thermostability of the DNA-binding protein HU from the thermophile *Bacillus stearothermophilus* (BstHU). Two mutants, BstHU-A27S and BstHU-V42I, in which Ala27 and Val42 in BstHU were replaced by the corresponding amino acids Ser27 and Ile42, respectively, in the homologue from a mesophile *B. subtilis* (BsuHU), were less stable than the wild-type BstHU (63.9 °C), showing $T_m$ values of 58.4 °C and 60.1 °C, respectively, as estimated by circular dichroism (CD) analysis at pH 7.0. The denaturation of two mutants was further characterized using differential scanning calorimetry; the $T_m$ values obtained by calorimetric analysis were in good agreement with those estimated by CD analysis. The results suggest that Ala27 and Val42 are partly responsible for enhancing the thermostability of BstHU. When considered together with previous results, it is revealed that Gly15, Ala27, Glu34, Lys38, and Val42 are essential for the thermostability of thermophilic protein BstHU. Moreover, five thermostabilizing mutations were simultaneously introduced into BsuHU, which resulted in a quintuple mutant with a $T_m$ value of 71.3 °C, which is higher than that of BstHU, and also resulted in insusceptibility to proteinase digestion.

A thorough understanding of molecular rules governing thermostability of proteins should provide invaluable insight into basic principles for a design of novel proteins by protein engineering. Both theoretical and experimental approaches have been undertaken to examine the thermostability of proteins and many different structural principles have been postulated for increased thermostability (for reviews, see Refs. 1–3). One convincing approach is a comparative study on proteins that are available from organisms living under different temperature conditions. Recently, Vogt et al. statistically compared the amino acid sequences and tertiary structures of mesophilic and thermophilic organisms and suggested that the increased hydrogen bonds and ion pairs may provide the most general explanation for thermostability in proteins (4). We have pursued the investigation of the structural basis of thermostability of proteins using a histone-like bacterial DNA-binding protein HU (HU) as a model protein.

The HU is a small basic polypeptide chain composed of 90–92 amino acids and occurs as a homotypic dimer in solution (5). The 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 (6), inversion (7), transposition (8), and repair (9) as a DNA chaperon (10). *Bacillus stearothermophilus* HU (BstHU) is composed of 90 amino acids, and its tertiary structure has been extensively studied by both X-ray crystallographic (11, 12) and NMR spectroscopic (13, 14) methods. The BstHU has two distinct halves; the N-terminal half consists of two α-helices (α1 and α2), which are connected by a broad turn to create a V-shaped superelementary structure (HTH motif), while the C-terminal half consists mainly of a three-stranded antiparallel β-sheet sheet. In a dimeric form, a pair of HTH motifs are entangled with each other to form a tightly packed hydrophobic core domain. In addition to BstHU, we have isolated three homologous HUs from mesophilic (*Bacillus globigii* and *Bacillus subtilis*) and thermophilic (*Bacillus caldolyticus*) bacilli, and their amino acid sequences have been sequenced (15, 16). On the basis of sequence comparison of the four HUs and the known three-dimensional structure of BstHU, the relative thermostability with respect to amino acid differences between the four proteins was discussed (16). This study revealed 11 amino acid substitutions between the thermophilic and mesophilic proteins, which are almost all restricted to the molecular surface not implicated in the mode of DNA binding. Thus, it could be expected that these amino acid substitutions might give rise to additional hydrogen bonds and salt bridges that would contribute to the thermostability of the thermophilic HU. To assess the contribution of the individual amino acids to the thermostability of BstHU, we constructed BstHU mutants, in which the amino acids were individually replaced with the corresponding amino acids in *B. subtilis* HU (BsuHU) and evaluated their thermostability. Previously, it has been shown that Gly15 in the bend between two α-helices (α1 and α2) and Glu34 and Lys38 occurred at the molecular surface, significantly contributing to the thermostability of BstHU (17, 18).

To this end, we extend this comparative study to the remaining six amino acid replacements: BstHU Ala27 to BsuHU Ser27, Ser31 to Thr31, Val42 to Ile42, Ala56 to Ser56, Met69 to Ile69, and Lys90 to Ala90. Gly91-Lys92. In the crystal structure of BstHU (Fig. 1), Ala27 resides on the outside of the second α-helix (α2) and is exposed to solvent. Since the Ala residue is a strong helix-forming amino acid, it could be suggested that Ala27 in BstHU would contribute to thermostability by stabilizing the second α-helix (α2). Ser31 in BstHU is located on the solvent-facing surface of the second α-helix, and its side chain forms a hydrogen bond to the main chain carboxyl of Ala27. Since an
equivalent hydrogen bond is probably formed through the side chain of the Thr27 residue in the mesophile BsuHU, the replacement from Ser27 to Thr was thought to be neutral. The Val42 in BstHU is in the β1-strand and is buried in the interior of the protein. It was therefore suggested that the larger side chain Ile in BsuHU would produce structural strain in the tightly packed core, resulting in destabilization of the mesophilic protein. Ala56 and Met69 in BstHU are substituted by Ser and Ile, respectively, in BsuHU. The former residue is on the outgoing β-ribbon closed to the disordered arms of the BstHU, and the latter is in the returning part of the disordered arm in BstHU. These residues are expected to be accessible to solvent and, therefore, were thought to have little effect on the thermostability of the protein. The C-terminal Lys90 in BstHU is replaced by the tripeptide Ala90-Gly91-Lys92 in BsuHU. It is shown that the C terminus is disposed on the molecular surface, and thus the significance of this replacement was unclear.

In the present study, we reveal that Ala27 and Val42 are essential for the thermostability of the BstHU. Furthermore, on the basis of the present and previous results, we construct a quintuple mutant HU using the BsuHU gene as a prototype and discuss its hyperthermostability and resistance to proteolytic degradations.

EXPERIMENTAL PROCEDURES

Genetics—We previously described the cloning, sequencing, and expression of the genes encoding BstHU and BsuHU (19). All genetic procedures, including site-directed mutagenesis were performed as described earlier (17, 18). The oligonucleotide primers used in this study are as follows: for BstHU-A27S, 5'-GCCGTGTACCTCGGTGTTATGTCG-3'; for BstHU-S31T, 5'-GTCTTTGATACGATTACAGAAGCGC-3'; for BstHU-V42I, 5'-GCCAAAAGGCGTATCAAATTTCACTTG-3'; for BstHU-A56S, 5'-CGCGGAGCGCTCCGCCCGGAAAGGACG-3'; for BstHU-M69I, 5'-GCCCAGAAGGAATGGATTCACTCCAAGCGC-3'; for BstHU-K90AGK, 5'-GCATTGAAGAGTGGGCGTCCGCCGGAAAAGTAAAGC-TTGG-3'; for BsuHU-S27A, 5'-CGATACAAAACGCGTGAACATC-3'; and for BsuHU-I42V, 5'-CCGACATGTTGAGCTGTTACACCGC-3'.

Purification and Characterization of Proteins—All procedures were used for production, purification, and SDS-PAGE analysis of the recombinant proteins BstHU and BsuHU and the mutants thereof have been described previously (17, 18). Thermostability of the protein was determined by monitoring the change in circular dichroism (CD) at 222 nm as a function of temperature, and thermodynamic parameters were obtained as described in Refs. 17 and 18. Since it was reported that the protein BsuHU showed the strong dependence of the CD spectral properties and stability under a variety of conditions (20, 21), we measured all proteins using the same procedure and the same type of spectrophotometer, as described in previous papers (17, 18).

Differential Scanning Calorimetry (DSC)—Calorimetric measurements were carried out with a VP-DSC (MicroCal Inc., Northampton, MA) microcalorimeter with a personal computer. The scan rate was 1.0 K/min. Sample solutions for DSC measurements were prepared by dialyzing of HU proteins dissolved in water against 0.05 M phosphate buffer at pH 7.0 exhaustively. The protein concentrations were 22–33 μM. The concentrations of the protein solutions were determined using amino acid analysis of proteins after acid hydrolysis. Data analysis was done using the Origin software (MicroCal).

NMR Spectra—1H NMR spectra were recorded at 600 MHz with a Varian Unity Plus spectrometer. All NMR measurements were carried out at pH 7 and 25 °C. Dioxane was employed as the internal standard (3.743 ppm). The pD values were the pH meter readings without adjustment for isotope effects.

Proteolysis—Tryptic and chymotryptic digestions of the proteins (1 mg/ml) were carried out at 37 °C in 0.1 M Tris-HCl, pH 8.0, with an enzyme-substrate ratio of 1:1000 and 1:5000 (w/w), respectively. Proteolytic digestion was assayed by measuring the change in the amount of the uncleaved protein, which was separated by reverse-phase HPLC on a YMC-gel C4 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. The protein was eluted with a linear gradient of 0–56% acetonitrile in 0.1% trifluoroacetic acid for 30 min. The effluents were monitored by absorption at 220 nm.

RESULTS AND DISCUSSION

Overproduction and Characterization of Mutant Proteins—Six BstHU mutant proteins, designated as BstHU-A27S, BstHU-S31T, BstHU-V42I, BstHU-A56S, BstHU-M69I, and BstHU-K90AGK, were engineered, in which Ala27, Ser31, Val42, Ala56, Met69, and Lys90 were changed to the corresponding amino acid residues in BsuHU. Expression of the mutated cDNAs was performed in Escherichia coli BL21 (DE3) cells, using the T7 system as described previously (17, 18). All mutant proteins were purified from the soluble fractions of cells so as to give a single band on SDS-PAGE. Their behaviors during the purification steps were almost identical with that of the wild type protein. The yields of protein from a 1-liter culture were 15–20 mg. The integrity of the mutant protein was confirmed by measurements of far-ultraviolet CD as described previously. The CD spectrum of each mutant was almost indistinguishable from that of the wild type, indicating that none of these mutations affected the backbone conformation (data not shown).

Thermostabilities of Mutant Proteins—To define the amino acid replacements responsible for the thermostability of BstHU, the thermostabilities of the mutant proteins were analyzed by monitoring the change in the CD value at 220 nm as a function of temperature. Fig. 2A shows the thermal denaturation curves of the wild type and the mutant proteins. In all cases, the unfolding transitions appeared to be monophasic, suggesting the absence of a folding intermediate in thermal denaturing process of global structure. Thus, on the basis of the assumption that the wild type BstHU and its mutants are denatured with a two-state model, thermodynamic parameters were calculated from the thermal denaturation curves, as summarized in Table I. Of five mutant proteins, the mutants BstHU-A27S and BstHU-V42I were less stable by −5.5 °C (ΔG ΔG = −4.97 kJ/mol) and −3.8 °C (ΔG ΔG = −3.48 kJ/mol), respectively, in Tm than the wild type BstHU. This result suggested that Ala27 and Val42 are the key residues to provide the extra thermostability to BstHU.

The contributions of Ala27 and Val42 to the thermal stabilization of BstHU were corroborated by constructing the mesophilic mutants BstHU-S27A and BstHU-I42V, in which Ser27 and Ile42 in BstHU were conversely replaced by Ala and Val, respectively, and the resulting mutants were characterized in terms of their thermostabilities. As shown in Fig. 2B and Table I, the Tm values of BstHU-S27A and BstHU-I42V were in-
Val42, are both responsible for enhancing the thermostability of conformational stability. The flexible nature. It was therefore concluded that these substitu-

creased by 5.6 °C (ΔΔG = 3.07 kJ/mol) and 4.0 °C (ΔΔG = 2.19 kJ/mol), respectively, compared with that of the wild-type BstHU, demonstrating that these two amino acids, Ala27 and Val42, are both responsible for enhancing the thermostability of the BstHU.

In contrast, the stabilities of BstHU-A56S and BstHU-M69I were almost identical to that of the wild type, and the mutants BstHU-S31T and BstHU-K90AGK were slightly more stable than the wild type. Two residues Ala36 and Met69 are located in the solvent-exposed arm region. In the crystal structure of BstHU, the top part of the arm is not visible because of its flexible nature. It was therefore concluded that these substitutions in a mobile, solvent-exposed environment do not affect the conformational stability. The Tm values of the mutants BstHU-

TABLE I
Parameters characterizing the thermal denaturations of BstHU, BstHU-K90AGK, and their mutants.

| Protein            | ΔHm (kJ/mol) | ΔSm (kJ/mol·K) | Tm (°C) | ∆G (kJ/mol) |
|--------------------|--------------|----------------|---------|-------------|
| BstHU              | 304.2        | 0.903          | 63.9    | -4.97       |
| BstHU-A27S         | 319.6        | 0.964          | 58.4    | -5.5        |
| BstHU-S31T         | 338.8        | 0.949          | 65.8    | +1.9        |
| BstHU-V42I         | 228.8        | 0.687          | 60.1    | -3.8        |
| BstHU-M69I         | 351.2        | 0.983          | 63.9    | 0           |
| BstHU-K90AGK       | 296.6        | 0.876          | 65.7    | +1.8        |
| BstHU              | 304.2        | 0.903          | 63.9    | -4.97       |
| BstHU-S27A         | 186.9        | 0.571          | 54.2    | +5.6        |
| BstHU-I42V         | 256.1        | 0.787          | 52.6    | +4.0        |

Fig. 2. Thermal unfolding curves for BstHU, BsuHU, and their mutant proteins. A, temperature dependences of [θ]222 nm values of BstHU (○) and its mutants, BstHUA27S (△), BstHU-S31T (□), BstHU-V42I (●), BstHUA56S (×), BstHUM69I (○), and BstHU-K90AGK (△). B, temperature dependences of [θ]222 nm values of BstHU (○) and its mutants, BsuHU-S27A (□) and BsuHU-I42V (△).

Fig. 3. Typical excess heat capacity curve of the wild type BstHU. The increments of excess heat capacity were 6 kJ/mol.

S31T and BstHU-K90AGK were higher by 1.9 °C and 1.8 °C than that of the wild type BstHU. The crystal structure of the BstHU shows that Ser27 resides on the solvent-facing surface, clustering together with Thr13 and Ala27, and forms a hydrogen bond to the main chain carbonyl of Ala27. Therefore, the increased stability of BstHU-S31T might be due to the rearrangement of a hydrogen bond formed between the side chain oxygen of the substituted Thr27 and the main chain carbonyl of Ala27. Another plausible interpretation is that the introduction of the larger side chain might give some preferential van der Waals contacts with Thr13 and/or Ala27. The mutant BstHU-K90AGK was also stable as compared with the wild type. At present, no explanation for thermostable property of this mutant has been obtained.

In order to understand the molecular basis for destabilization of the mutant proteins, BstHU-A27S and BstHU-V42I, their structural features were examined by 1H NMR spectroscopic analysis. As a result, the chemical shift change of some resonances were observed around aromatic regions at 6.2, 6.26, and 7.0 ppm in BstHU-A27S and 6.2, 6.28, and 6.49, and 7.0 ppm in BstHU-V42I, as compared with that of the wild-type HU (data not shown). Although the assignment of these resonances in the BstHU has still not been reported, Kakuta classified these to be derived from Phe residues (Phe29, Phe47, Phe50, and Phe79) in BstHU by DQF-COSY. It is therefore likely that the replacement of Ala27 or Val42 by Ser or Ile, respectively, may cause rearrangement of some Phe residues occupying the interior of the molecule.

DSC Measurements of BstHU and Its Mutant Proteins—The present study, together with previous studies (17, 18), suggests that five amino acid residues, Gly15, Ala27, Glu34, Lys38, and Thr13, are essential for the thermostability of BstHU. To corroborate their involvement in thermostability, the thermal denaturations of five mutants (BstHUG15E, BstHUA27S, BstHE34D, BstHUK38N, and BstHU-V42I) as well as the wild type were further characterized by DSC measurements. A typical excess heat capacity curve of the wild type BstHU is shown in Fig. 3. The examined proteins, as is the case for the BstHU, gave single peaks in calorimetric measurements; the Tm values of the wild type and its five mutants could be calculated from these curves, as given in Table II. This measurement clearly showed the thermal destabilization of five mutant proteins as compared with the wild type and demonstrated the involve-

2 Y. Kakuta, unpublished results.
Taking the present result, the five thermostabilizing mutations strongly suggests that characterizing the thermal denaturation are summarized in those of the five single mutant proteins with the constituent greater thermostability of the thermophilic protein residues that reside far from each other contribute to the individual local reinforcements derived from the amino acid each other and nearly additive. This result suggests that the calculated value. This result indicated that the effects of the mutations seems to be somewhat greater than the sum of the Bsu value of 71.3 °C compared with 48.6 °C for the wild-type protein BstHU, and the thermostability of the resultant mutant (quintuple mutant) was examined, exactly in the same manner as described above. The thermal denaturation curves of the wild type BstHU and the quintuple mutant as well as those of the five single mutant proteins with the constituent amino acid substitutions are shown in Fig. 4. The parameters characterizing the thermal denaturation are summarized in Table III. The simultaneous introduction of the five mutations greatly increased the thermostability of the protein with the Tm value of 71.3 °C compared with 48.6 °C for the wild-type BstHU. The stabilization energy arising from the quintuple mutations seems to be somewhat greater than the sum of the constituent single substitutions but only by about 8.9% of the calculated value. This result indicated that the effects of the mutations on the thermostability are roughly independent of each other and nearly additive. This result suggests that the individual local reinforcements derived from the amino acid residues that reside far from each other contribute to the greater thermostability of the thermophilic protein BstHU. Interestingly, the Tm value of the quintuple mutant of BstHU was higher than that of BstHU. As shown in Table I, the mutation of amino acids in BstHU to those of BstHU such as BstHU-S111T was not always less stable, indicating that the amino acid sequence of BstHU was not optimized for thermal stability. Considering this idea, the higher stability of the quintuple mutant of BstHU may depend on the stabilization of the folded state rather than the destabilization of the unfolded state, because mutations were involved in the formation of salt bridge or the introduction of favorable intermolecular interaction. Therefore, it is likely that the thermostabilizing mutations contribute to the decrease in the rate constant of unfolding.

**Conclusion**—This series of studies on the thermostability of BstHU revealed that its extra thermostability relative to the mesophilic protein BstHU seems to be achieved mainly by stabilization of two α-helices (α1 and α2) with Gly15, Ala27, and Glu34 and by improvement of the intermolecularly close packing in the hydrophobic core with Ala27 and Val42 (Table IV). Moreover, the additional salt bridge between Glu34 and Lys38 on the hydrophobic surface is found to be also responsible for enhancing the thermostability of BstHU (Table IV). The amino acid residues found to be responsible for thermostability of BstHU are mapped in the crystal structure of BstHU (Fig. 6). The Gly15 residue locates on the bend between α1- and α2-helices and is found to stabilize the conformation of the HTH motif. The replacement of Gly15 by the corresponding residue Glu in BstHU caused a remarkable decrease in the thermostability of the BstHU-G15E: ΔTm = −9.9 °C by CD analysis (17) and ΔTm = −10.1 °C by DSC measurement (Table II). Further, Ala27 and Glu34 locate at the second α-helix (α2) and may be involved in stabilization of its conformation because of their enhanced α-helix structural propensity. Two α-helices (α1 and

| Protein           | Tm value | ΔTm | ΔG  | ΔTm (sum) | ΔG (sum) |
|-------------------|---------|-----|-----|-----------|----------|
| Wild type BstHU   | 65.8    | 11.8| +4.0| +24.9     | +13.65   |
| BstHU-G15E        | 55.7    | 2.19| +4.0| +24.9     | +13.65   |
| BstHU-S27A        | 54.2    | 5.6 | +4.0| +24.9     | +13.65   |
| BstHU-I42V        | 52.6    | 2.19| +4.0| +24.9     | +13.65   |
| BstHU-E15G        | 52.1    | 3.07| +4.0| +24.9     | +13.65   |
| BstHU-N38K        | 50.1    | 3.07| +4.0| +24.9     | +13.65   |
| BstHU-D34E/       | 48.6    | 11.8| +4.0| +24.9     | +13.65   |
| BstHU-S27A/E34D/  | 48.6    | 11.8| +4.0| +24.9     | +13.65   |
| BstHU-I42V/K38N   | 52.6    | 2.19| +4.0| +24.9     | +13.65   |
| BstHU-V42I        | 59.6    | 6.47| +4.0| +24.9     | +13.65   |
| Quintuple mutant  | 71.3    | 24.9| +4.0| +24.9     | +13.65   |

**Table II**

*The Tm values of the wild type BstHU and its mutants obtained by DSC measurement and CD analysis.*

DSC measurements of proteins were carried out as described under “Experimental Procedures” and the same values estimated by CD analysis were reported in Refs. 16 and 17.

**Table III**

*Stability of the mesophilic protein BstHU and its five mutants*

Thermodynamic parameters were calculated from the thermal denaturation curves described in Fig. 2. The table summarizes the results of four independent experiments.

![Fig. 4. Thermal unfolding curves for BstHU and its mutant proteins. Temperature dependences of [θ]222 nm values of BstHU (X) and its mutants, BstHU-E15G (O), BstHU/S27A (•), BstHU-D34E/N38K (□), BstHU-I42V (+), and the quintuple mutant (○).](image)
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FIG. 5. Susceptibility of the quintuple mutant and the wild type to proteolysis by chymotrypsin and trypsin. The fraction of uncleaved protein is shown as a function of incubation time. □, BsuHU; ♦, quintuple. The amount of uncleaved protein was determined by subjecting the digests to reverse phase HPLC, by which the uncleaved protein could be separated from the chymotryptic and tryptic peptides. Rate constants were determined from the slope of semilog plots of the fraction uncleaved versus the incubation time, and then relative $k$ values (0.005 and 0.091) were calculated for chymotrypsin and trypsin, respectively, by dividing the $k$ values of the quintuple mutant by that of the wild type BsuHU.

![Graph showing fraction of uncleaved protein over time for chymotrypsin and trypsin.]

TABLE IV

| Residue | Explanation | Reference |
|---------|-------------|-----------|
| Gly^{35} | Enhanced stability of helix-turn-helix | 17 |
| Ala^{27} | Better hydrophobic intermolecular packing and enhanced $\alpha$-helix propensity | This paper |
| Glu^{34} | Formation of salt bridge with Lys^{38} and enhanced $\alpha$-helix propensity | 18 |
| Lys^{38} | Formation of salt bridge with Glu^{34} | 18 |
| Val^{42} | Better hydrophobic intermolecular packing | This paper |

a2) in BstHU adopt the HTH motif, which is structurally similar to that found in the operator/repressor family of DNA-binding proteins, such as CAP and $\lambda$-cro, where the motif is directly involved in DNA binding. Thus, the study on the thermostability of BstHU indicates the unique role of the HTH motif in the BstHU. The topology found in BstHU, where the helical core domain consists of a tightly entangled pair of the HTH motif from two protomers, is unique, and no similar structure has been found. However, the recent tertiary structural study of the B. stearothermophilus ribosomal protein S7 (a primary 16S rRNA-binding protein) revealed a similar motif in its N-terminal half region (26). Although a function of the HTH motif found in S7 is still not known, it is likely that the HTH motif may have been selected for stabilization of some nucleic acid-binding proteins as a scaffold.

Glu^{34}, in addition to involvement in the stabilization of the second $\alpha$-helix, is also found to be responsible for enhancing the thermostability of BstHU by forming an extra salt bridge with Lys^{38}. The replacement of Glu^{34} by the corresponding residue Asp^{34} in BsuHU caused a decrease in the thermostability of the BstHU-E34D: $\Delta T_m = -2.3 \, ^\circ C$ by both CD analysis (18) and DSC measurement. The contribution of a salt bridge to the thermostability of proteins is still controversial. It has been reported that the engineered electrostatic interaction between pairs of mobile, solvent-exposed charged residues on the molecular surfaces of proteins contributes little to protein stability (27–30). In contrast, Vogt et al. (4) reported that the salt bridge, together with the hydrogen bond, is the main explanation for the thermostability of proteins. In the crystal structure of the BstHU (Fig. 1), the side chains of these two residues are also exposed to the solvent and are mobile, the average B values for atoms within the side chains being about 100 $\AA^2$ (11, 12).

However, as described above, Glu^{34} may stabilize the second $\alpha$-helix by its intrinsic $\alpha$-helix forming property. Since Glu^{34} is the salt bridge partner of the Lys^{38} residue, the salt bridge formed between these two residues may somewhat stabilize the conformation of the second $\alpha$-helix (a2), thereby contributing to the thermostability of the BstHU.

This series of studies has shown that some replacements of the amino acid residues in the BstHU by the corresponding amino acids in the BsuHU, such as Thr^{13} to Ala, Ser^{31} to Ala, and Thr^{33} to Leu, resulted in thermostabilized proteins; the resulting mutants were more stable than the thermophilic parent protein BstHU (17). In this regard, it is suggested that the thermophilic protein BstHU has not evolved to optimize the protein structure in terms of thermostability. As a result, the quintuple mesophilic mutant containing five thermostable mutations has a $T_m$ that is 7.4 $^\circ C$ higher than the thermophilic protein BstHU. It is generally known that many proteins have been selected in evolution to be marginally stable. It is thus assumed that B. stearothermophilus, as is the case for other organisms, has selected the present form of the BstHU to coordinate the normal living temperature. This finding strongly supports an idea that a novel protein with hyperthermostability can be constructed by protein engineering using existing proteins in nature as prototypes.
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