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ORIGINAL PAPER

Evangelos Christodoulou · Constantinos E. Vorgias

The thermostability of DNA-binding protein HU from mesophilic, thermophilic, and extreme thermophilic bacteria

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Abstract Based on primary structure comparison between four highly homologous DNA-binding proteins (HUs) displaying differential thermostability, we have employed in vitro site-directed mutagenesis to decipher their thermostability mechanism at the molecular level. The contribution of the 11 amino acids that differ between the thermophilic HU*Bst* from *Bacillus stearothermophilus* (T_m = 61.6°C) and the mesophilic HU*Bsu* from *Bacillus subtilis* (T_m = 39.7°C) was evaluated by replacing these amino acids in HU*Bst* with their mesophilic counterparts. Among 11 amino acids, three residues, Gly-15, Glu-34, and Val-42, which are highly conserved in the thermophilic HUs, have been found to be responsible for the thermostability of HU*Bst*. These amino acids in combination (HU*Bst*-G15E/E34D/V42I) reduce the thermostability of the protein (T_m = 45.1°C) at the level of its mesophilic homologue HU*Bsu*. By replacing these amino acids in HU*Bsu* with their thermophilic counterparts, the HU*Bsu*-E15G/D34E/I42V mutant was generated with thermostability (T_m = 57.8°C) at the level of thermophilic HU*Bst*. Employing the same strategy, we generated several mutants in the extremely thermophilic HU*Tmar* from *Thermotoga maritima* (T_m = 80.5°C), and obtained data consistent with the previous results. The triplet mutant HU*Tmar*-G15E/E34D/V42I (T_m = 35.9°C) converted the extremely thermophilic protein HU*Tmar* to mesophilic. The various forms of HU proteins were overproduced in *Escherichia coli*, highly purified, and the thermostability of the mutants confirmed by circular dichroism spectroscopy. The results presented here were elucidated on the basis of the X-ray structure of HU*Bst* and HU*Tmar* (our unpublished results), and their mechanism was proposed at the molecular level. The results clearly show that three individual local interactions located at the helix-turn-helix part of the protein are responsible for the stability of HU proteins by acting cooperatively in a common mechanism for thermostability.

Key words Thermostability · DNA-binding protein HU · Mutations · CD spectroscopy

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E. Christodoulou · C.E. Vorgias (✉)
Faculty of Biology, Department of Biochemistry and Molecular Biology, National and Kapodistrian University of Athens, Panepistimiopoli-Zographou, 15784 Athens, Greece
Tel. +30-10-7274-514; Fax +30-10-7274-158
e-mail: cvorgias@biol.uoa.gr

Introduction

A feature of psychrophilic, thermophilic, and extreme thermophilic bacteria is their ability to survive and grow in an environment that can be considered as extreme from an anthropocentric point of view. The survival mechanisms of these organisms in part results from the appropriate adaptation (psychrophilicity, mesophilicity, or thermophilicity) of their individual components. During the past decade, considerable attention has been given to proteins because they comprise the actual machinery of the cells.

We have now reached the stage of providing some answers to the question: why are some proteins more thermostable than others? Thermostability versus protein engineering is an attractive scientific field because it can produce knowledge of protein structure–function-stability relationships at the molecular level (Argos et al. 1979; Matthews et al. 1987; Alber et al. 1987; Menendez-Arias and Argos 1989; Frömmel and Sander 1989; Karpusas et al. 1989; Nosoh and Sekiguchi 1990; Pace 1990; Bennell et al. 1991; Eijsink et al. 1992; Kimura et al. 1992; Maras et al. 1992; Sauer and Lim 1992; Ishikawa et al. 1993; Fersht and Serrano 1993; Matthews 1993; Kelly et al. 1993; Russell et al. 1994; Matthews 1995; Korolev et al. 1995; Yip et al. 1995; Russel and Taylor 1995; Pace and Scholtz 1997). Of particular biotechnological interest are the enzymes derived from organisms living in extreme conditions (Vorgias and Antranikian 2000).

HU is a small, basic, prokaryotic nonsequence-specific DNA-binding protein. A comprehensive review of homologous DNA-binding proteins (HUs) has been published (Drlica and Rouvriere-Yaniv 1987). The three-dimensional (3-D) structure of the HU protein from the thermophilic

bacterium *Bacillus stearothermophilus* (HU*Bst*) has been elucidated (Tanaka et al. 1984; White et al. 1989, 1999), and the protein is extensively used as a model system to study protein-DNA interactions of the histone-like protein family to which the integration host factor (IHF) protein also belongs (Rice et al. 1996).

We have previously described the cloning and overproduction of several HU proteins from three different thermophilic and two mesophilic bacilli (Padas et al. 1992) and more recently from the extreme thermophilic marine eubacterium *Thermotoga maritima* (HU*Tmar*) (Christodoulou and Vorgias 1998).

Several reasons support our decision to investigate the thermostability of these proteins: (i) the 3-D structure of HU*Bst* has been determined in crystals by X-ray crystallography (Tanaka et al. 1984; White et al. 1989, 1999) and in solution by nuclear magnetic resonance (NMR) (Vis et al. 1995, 1998; Boelens et al. 1996) and we attempt to interpret our results on a structural basis (Wilson et al. 1990); (ii) we previously completed analysis of the 3-D structure of HU*Tmar* at high resolution (Christodoulou et al., unpublished data); (iii) HU is a small protein, about 90 residues, and the similarity between thermophilic HU*Bst* and its mesophilic homologue from *Bacillus subtilis* (HU*Bsu*) is very high; (iv) HU*Tmar* is extremely thermophilic and shows lower homology to HU*Bst* and HU*Bsu*; (v) HU exists in solution as a homodimer with a molecular weight of 18 kDa, which allows us to investigate which interactions within the dimer interface contribute to dimer formation and the overall stability of the molecule; (vi) HU proteins do not contain cysteine and therefore their thermostability is independent of sulfur bridges; and (vii) as we have cloned the genes of these proteins, they can be easily overexpressed in *Escherichia coli* and prepared in large amounts and high purity for biochemical, structural, and biophysical studies. All these reasons together with the fact that HU on one hand is a small protein and on the other hand is a homodimer make it an attractive model to address questions concerning stability, homodimerization, and adaptation to extreme temperatures.

The main goal of this study was to elucidate the structural features of HU proteins, at the amino acid level, that are responsible for their thermostability and to pinpoint specific areas of the molecule which contribute to the stability of HU proteins. The results clearly show that only a few amino acids are responsible for the thermostability of HUs and that these amino acids are located in strategic positions, "hotspots," within the molecule.

Materials and methods

Materials

Enzymes used in the cloning procedures were from Boehringer Mannheim (Mannheim, Germany) and AGS (Heidelberg, Germany). The sequencing kit Sequenase was purchased from United States Biochemical (Cleveland, OH, USA) and the in vitro M13 site-directed mutagenesis kit was purchased from Amersham (Buckinghamshire, UK). The column chromatography media were from Pharmacia (Uppsala, Sweden) and all other chemicals were from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany), and of the highest analytical grade. Synthetic oligonucleotides were prepared at the EMBL DNA central synthesis laboratory and purified by high-performance liquid chromatography (HPLC) (Cynchropak 300 C_{18} 6.5 μm; MZ Analysentechnik, Mainz, Germany). Bacteria strains were supplied from DSM (German Collection for Microorganisms). The strains used for this study were *Bacillus stearothermophilus* DSM 22, growth temperature 55°C; *Bacillus subtilis* DSM 675, growth temperature 30°C; and *Thermotoga maritima* DSM 3109, growth temperature 80°C.

Cloning and mutagenesis of *hupbst, hupbsu*, and *huptmar* genes

The cloning of HU genes was carried out as described previously (Padas et al. 1992; Christodoulou and Vorgias 1998; Sambrook et al. 1989). Plasmids were purified using the Qiagen plasmid preparation kit (Qiagen, Hilden, Germany). In vitro site-directed mutagenesis was performed using the M13 system from Amersham. The resulting mutations were verified by sequencing using the primer extension method (Sanger et al. 1977).

Overproduction and purification of HU wt and mutants

Various wild-type (wt) *hup* genes and their mutants were cloned into the pET-3a or pET-11a expression vectors and introduced in to the BL21(DE3) and BL21(DE3)pLysS expression host cells (Studier et al. 1990). HU wt or mutants were overproduced and purified as described previously (Padas et al. 1992; Christodoulou and Vorgias 1998).

Briefly, in a routine protein preparation, 1 l cell culture was induced with 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 h. The induced bacteria were collected by low-speed centrifugation and washed once with ice-cold buffer W [50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF)]. All further procedures were carried out at 0–4°C unless otherwise specified. The bacterial paste was resuspended in 5 ml of buffer A [20 mM Tris-HCl, pH 8.0, 1 mM ethyldiaminetetraacetic acid (EDTA), 0.1 mM PMSF, 20 mM NaCl, 0.1% (w/v) Triton X-100] per gram of bacterial paste. The cells were disrupted by sonication for 10 min. The total extract was clarified by centrifugation in an SS-34 rotor (Sorvall, Paris, France) at 20,000 *g* for 20 min. The supernatant was adjusted to 38% saturation in ammonium sulfate. After 30-min stirring, the nonprecipitated material was similarly separated by centrifugation. The soluble supernatant was dialyzed against buffer B (10 mM sodium phosphate, pH 8.0, 1 mM EDTA, 0.1 mM PMSF) overnight. HU-enriched fractions were applied to a 10-ml heparin Sepharose CL-6B column. Bound proteins were eluted

between 0 and 1.5 M NaCl, stepwise. Collected fractions were analyzed by 0.1%–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). HU fractions were combined and diluted 1 : 3 with buffer C (10 mM sodium phosphate, pH 6.0, 1 mM EDTA, 0.1 mM PMSF). The pH was adjusted to 6.0 and the protein solution was applied to a Mono-S fast protein liquid chromatography (FPLC) column. HU was eluted between 350 and 400 mM NaCl via a short linear gradient. In the case of HU*Tmar* wt and mutants, the elution from the heparin-column was achieved at 1.2 M NaCl. The final step provides a very pure and concentrated HU protein. The overall yield varies between 10 and 25 mg of highly purified HU protein from 1 l of bacterial culture (Christodoulou and Vorgias 1998).

Thermal denaturation studies by circular dichroic spectroscopy

Circular dichroism (CD) spectra were recorded on a J715 spectropolarimeter (Jasco, Tokyo, Japan). The instrument was calibrated using a 1 mg/ml aqueous solution of (+)-10-camphosulfonic acid (CSA) (Sigma-Aldrich, Munich, Germany) in a 1-mm cell. This compound has a molar ellipticity of 2.36×10^3 at its CD maximum of 290.5 nm and a molar ellipticity of -4.9×10^3 at its CD minimum of 192.5 nm. A cuvette (165-QS; Hellma, Essex, UK) of 0.2-mm cell length was used, and its temperature was increased or decreased at a rate of 50°C/h. The protein amount is one of the most important parameters to be determined to achieve accurate and reliable CD measurements. The best quality spectra for reproducibility of molar ellipticity were obtained at 0.2 mg/ml protein concentration. HU proteins, both wt and mutants, were examined reversibly under these experimental conditions. The fraction of native protein was calculated from the CD values by linearly extrapolating the pre- and posttransition baselines, respectively. The CD spectrophotometer was interfaced to a personal computer, and the collected data were processed using the sigmoidal fitting of Boltzmann's equation.

Protein concentration

HU protein concentration was determined using various methods (Bradford 1976; Gill and von Hippel 1989; Lowry et al. 1951; Pace et al. 1995) as well as total amino acid analysis. Finally, we used the equation: 1 OD_{230nm} of HU = 0.60 mg.

Protein analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis

Analyses at 0.1% SDS – 15% PAGE were run according to the Laemmli procedure (Laemmli 1970). The gels were run at a constant current of 30–40 mA at room temperature and stained with Coomassie brilliant blue G-250.

DNA-binding assay

DNA binding of wt and mutant HUs was measured by affinity chromatography on a calf thymus dsDNA-cellulose column. The column was run in 20 mM sodium phosphate buffer at pH 8.0, and bound HUs were eluted stepwise between 50 and 1,000 mM NaCl.

Urea-Triton PAGE

The electrophoretic mobility of HU protein in 2 M urea and in the presence of 1%(v/v) Triton X-100 as described by Aitken and Rouvière-Yaniv (1979) was used to examine possible oxidation of HU wt and mutant proteins and to trace possible structural changes.

Results

Primary structure comparison of HU*Bst*, HU*Bsu*, HU*Tmar*, and HU*Tth*

The primary structures of HU*Bst*, HU*Bsu* (Padas et al. 1992), and HU*Tmar* (AC L23541) deduced from the gene sequences were aligned and compared with the primary structure of the thermostable HU from *Thermus thermophilus* (HU*Tth*) (Tanaka et al. 1984) (Fig. 1). There is a considerable degree of homology among these four HUs (Table 1). The secondary structure elements indicated in Fig. 1 were drawn based on the 3-D structure of HU*Bst* (White et al. 1999).

The currently known HU proteins number nearly 100, and the number is constantly increasing. HU molecules can be divided into three functionally and structurally distinct parts. The C-terminus part of the molecule is the putative DNA-binding domain (DBD), which is highly conserved and has a high content of basic amino acids. The middle part of the molecules contains the consensus region "GFGXF"

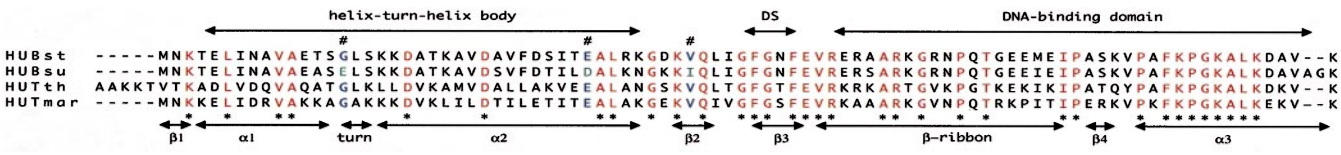

Fig. 1. Alignment of the amino acid sequences of HU*Bst*, HU*Bsu*, HU*Tth*, and HU*Tmar*. The position of the secondary structure elements derived from the three-dimensional structure of HU*Bst* is shown. α_1, α_2, and α_3 are the three α-helices; β_1, β_2, and β_3 are β-sheets; DS, dimerization signal; # indicates G15, D34, and I42 which are highly conserved in the thermophilic homologous DNA-binding proteins (HUs)

Table 1. Identity scores between the HU*Bst*, HU*Bsu*, HU*Tth*, and HU*Tmar* proteins and their domains

Protein	HU*Bst*	HU*Bsu*	HU*Tmar*	HU*Tth*
HU*Bst*	100			
HU*Bsu*	87.7% HTH: 77.8% DBD: 100%	100		
HU*Tmar*	61.1% HTH: 53.3% DBD: 67.5%	51.1% HTH: 33% DBD: 67.5%	100	
HU*Tth*	55.5% HTH: 44.4% DBD: 65%	53.3% HTH: 40% DBD: 65%	55.5% HTH: 44.4% DBD: 65%	100

Helix-turn-helix body (HTH), (residues: 1–45); DNA-binding domain (DBD), (residues: 51–90)
The dimerization signal (DS) peptide is not included because it is identical in all HUs

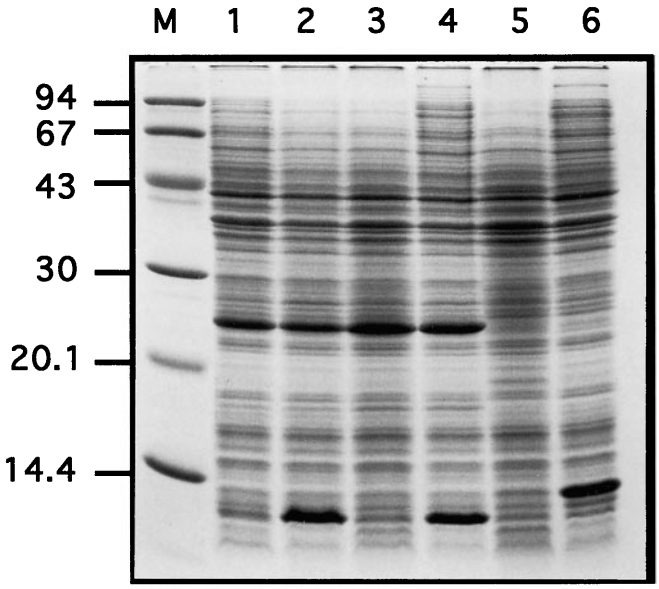

Fig. 2. 0.1% sodium dodecyl sulfate (SDS) – 15% polyacrylamide gel electrophoresis (PAGE) of total *Escherichia coli* protein extract before (*lanes 1, 3, 5*) and after (*lanes 2, 4, 6*) 3-h induction with isopropyl-beta-D-thiogalactopyranoside (IPTG) of the BL21(DE3)pLysS cells harboring the plasmids pCV*hubst* (*lanes 1, 2*) and pCV*hubsu* (*lanes 3, 4*), and BL21(DE3) cells harboring the plasmid pCV*hutmar* (*lanes 5, 6*). *Lane M* contains molecular weight markers in kDa as indicated on the left side of the figure

within the HU family. This pattern is known to create a hydrophobic core in the interface between two HU subunits, and because it facilitates the formation of a very stable dimer, it is called dimerization signal (DS). The N-terminus of the HU molecule has a helix-turn-helix (HTH) motif containing some β-sheet elements. This part comprises the main body of the protein and is less conserved in the primary structure compared to the rest of the molecule. Table 1 summarizes the degree of identity of the HTH and DBD among the four HUs used in this study. The HTH part of the molecule has the highest degree of variation, and therefore it may be concluded that this reflects the differential thermostability among the four HUs.

Strategy for designing mutants

The two highly homologous HU proteins, i.e., the thermophilic HU*Bst* and the mesophilic HU*Bsu*, are different in only 11 amino acids. There are no deletions or insertions between the two sequences, even though HU*Bsu* has 2 additional amino acids at the C-terminus.

This small number of amino acid differences between the two HUs provides a good starting point to study the thermostability of these proteins. At the first stage, we investigated all the amino acid residues that differ between HU*Bst* and HU*Bsu* and their effect on the stability of thermophilic HU*Bst*. This was carried out by stepwise replacement of the 11 amino acids in HU*Bst* with their HU*Bsu* counterparts and by adding the two extra amino acids to the C-terminus of HU*Bst*. The second step was to combine only the mutations shown to have a significant effect on the thermostability of HU*Bst* and to determine their effect on the melting temperature (T_m) of the protein. The third step was to select only those mutations with significant destabilization effect on HU*Bst* and introduce them in mesophilic HU*Bsu* to examine whether they are involved in thermostabilization of HU*Bsu* protein.

The final step in our study was to predict mutants based on the results obtained and the primary structure comparison of the four HUs presented in Fig. 1, produce them, and study their effect on the thermostability of extremely thermophilic HU*Tmar*. Primary structure comparisons of HU molecules detected only three amino acids at the N-part of the molecule; G15, D34, and I42 are highly conserved in thermophilic HUs (Fig. 1, indicated by the symbol # on the upper part of the alignments), suggesting a significant role of these amino acids in HU molecules.

Protein overproduction and purification

The various HU wt and mutant proteins were overproduced in *E. coli* at the same level. For the production of wt and mutated HUs, BL21(DE3)pLysS cells were used as hosts for the expression of the plasmids pCV*hubst* and pCV*hubsu* (Padas et al. 1992; Christodoulou and Vorgias 1998). The overproduction of nearly all mutants was at the same level, about 20 mg protein/l bacterial culture. An exceptional case was the mutant HU*Bst*-A27S, which was overproduced at least threefold higher compared to the wt. Figure 2 shows SDS-PAGE protein overproduction profiles of HU*Bsu*, HU*Bst*, and HU*Tmar* before (lanes 1, 3, 5) and after (lanes 2, 4, 6) 3-h induction with 0.4 mM IPTG, respectively.

In the case of HU*Tmar,* the expression of wt and mutants of the *huptmar* gene was carried out in BL21(DE3) cells using the same procedure as for HU*Bst* and HU*Bsu*. The protein purification protocol included 4 M urea in the HU*Tmar* total bacterial extract after sonication, as also

described by Christodoulou and Vorgias (1998). The addition of urea was essential to dissociate small DNA fragments tightly bound to the protein, whereas the pure HU*Tmar* protein was fully refolded at the end of the preparation.

DNA-binding assay

To ensure that HUs wt and mutants used for the thermostability experiments retained their biological activity, we employed DNA-binding activity experiments using calf thymus double-stranded DNA-cellulose affinity column chromatography. The column was run in 20 mM sodium phosphate buffer at pH 8.0, and bound HU was eluted stepwise with 50-mM steps between 50 and 500 mM NaCl. Except for HU*Bst*-A27S, which showed reduced affinity to DNA, all other HU mutants retained their DNA-binding activity at the levels of the wt (our unpublished results). HU*Tmar* wt protein and mutants were bound very tightly to the DNA-cellulose column, and 1.0–1.2 M NaCl was required to elute them from the column (Christodoulou and Vorgias 1998).

Urea-Triton PAGE

Figure 3 shows the electrophoretic mobility of HU*Bst*, HU*Bsu*, and selected mutants in the presence of 2 M urea and 1% (v/v) Triton X-100, according to the initial protocol (Aitken and Rouvière-Yaniv 1979). The results show substantial differences between HU*Bst* and HU*Bsu* in terms of electrophoretic mobility. We have investigated the electrophoretic behavior of several HU mutants (see Fig. 3) for possible correlations between the nature of the amino acid substitution and the mobility of the protein under the experimental conditions described in Materials and methods. Comparison of wt and mutant proteins shows a clear correlation between mobility and changes in the total hydrophobicity resulting from amino acid changes.

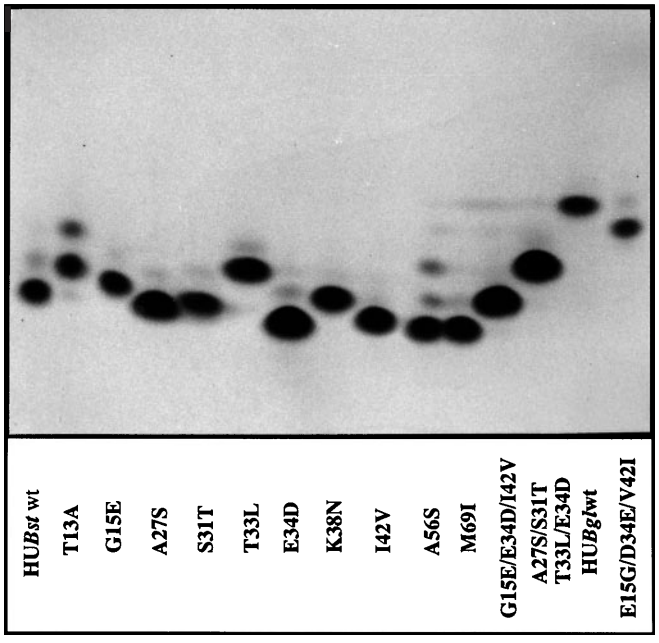

Fig. 3. PAGE with 2 M urea – 1% (v/v) Triton X-100 of HU*Bst*, HU*Bsu* wt, and several mutants as listed at the lower part of the figure

The gel electrophoretic behavior of the mutated HUs in this system can only be interpreted by assuming subtle structural changes that may significantly affect the binding of the detergent Triton X-100 to the protein. Some faint bands with lower mobility were also observed, likely caused by oxidation of methionine residues. Methionine oxidation can potentially influence the binding of the detergent to the protein or possible dimer formation (our unpublished observations). This small heterogeneity did not influence the behavior of the proteins during the thermal denaturation experiments because the amount of oxidized protein was very low. We eliminated this heterogeneity by performing protein purification under reducing conditions.

Thermal denaturation measurements by CD spectroscopy

Circular dichroism (CD) was used to monitor the secondary structure of HUs. Figure 4 shows far-UV CD full spectra of wt HU*Bsu* (a), HU*Bst* (c), and HU*Tmar* (e) at various temperatures. For these three proteins, the ellipticity signal at wavelengths longer than 220 nm was dramatically reduced as the temperature increased.

Determination of T_m of various HU wt and mutants by CD spectroscopy

The melting temperature (T_m) is conventionally defined as the temperature at which half the protein molecules are denatured and was calculated using the sigmoidal fitting of Boltzmann's equation. The thermal unfolding curves of wt and mutants of HU*Bsu*, HU*Bst*, and HU*Tmar* are shown in Fig. 4 (b, d, and f, respectively). Table 2 summarizes the results of the T_m measurements of HU*Bst* wt and mutants and describes the location of each mutation based on the 3-D structure HU*Bst*.

According to the T_m data (Table 2), there are two sets of point mutations. In the first set, HU*Bst* point mutations T13A, A27S, S31T, T33L, A56S, and M69I did not significantly affect the thermostability of the HU*Bst* protein. The second set, containing mutants G15E, E34D, K38N, and V42I, substantially reduced the T_m of the HU*Bst* protein between –10° and –1.8°C. As our goal was to identify only amino acids that can destabilize the thermostable HU*Bst* protein, we decided to combine only mutations with a significantly negative ΔT_m. Therefore, we continued our experiments by creating a set of multiple mutants containing the four amino acids in an additive manner (Table 3). The double mutant HU*Bst*-G15E/V42I has a ΔT_m of –15.6°C, which is close to the sum of the ΔT_m of the individual mutants. The triple mutant HU*Bst*-G15E/E34D/V42I reduced the thermostability of HU*Bst* by –16.5°C, nearly reaching the T_m of HU*Bsu* wt. The K38N point mutation combined in this triple mutant had only a marginal effect (ΔT_m = –17.5°C).

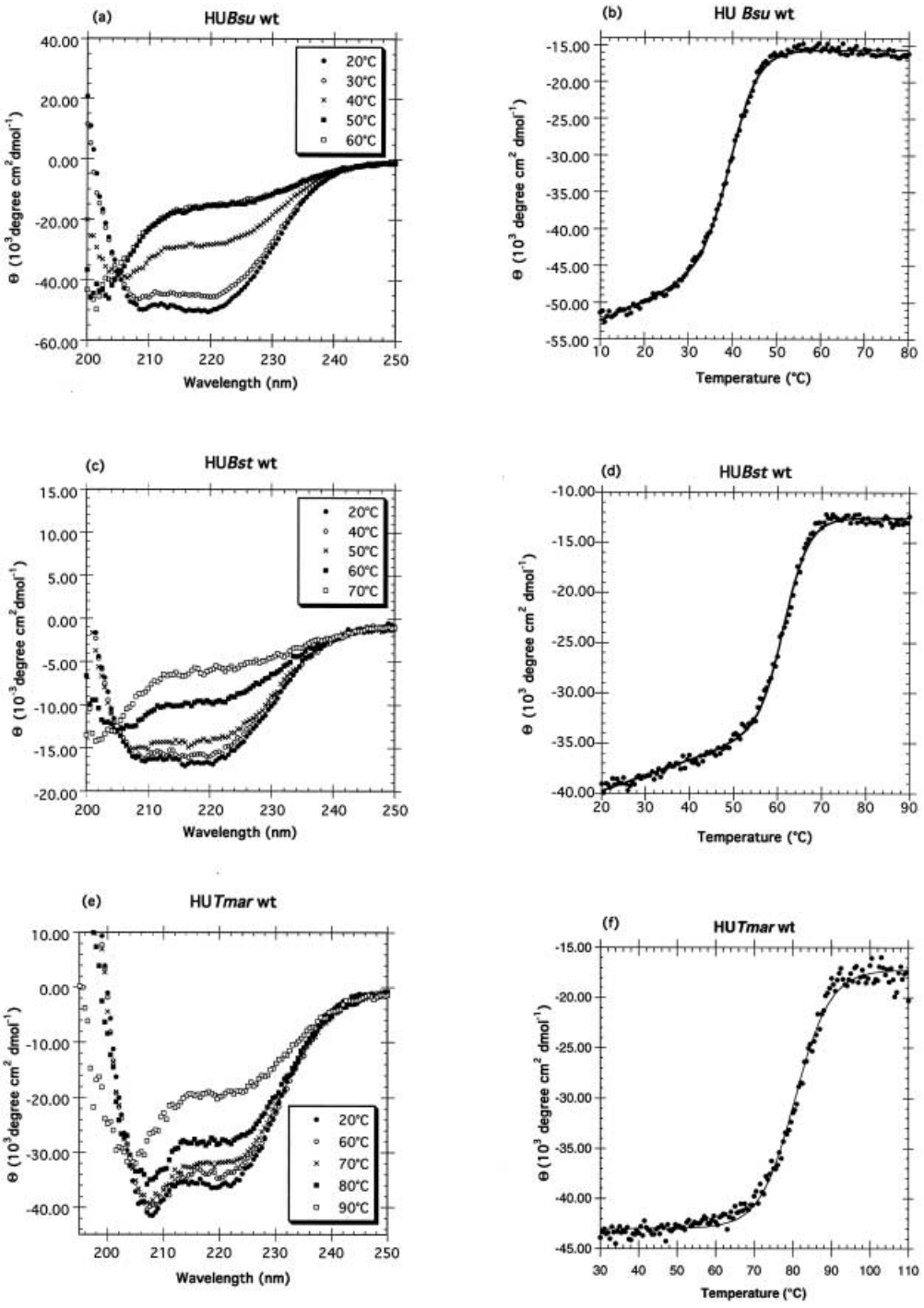

Fig. 4. Ultraviolet-circular dichroic (UV-CD) spectra of HU*Bsu* (**a**), HU*Bst* (**c**), and HU*Tmar* (**e**) at various temperatures and melting curves of HU*Bsu* (**b**), HU*Bst* (**d**), and HU*Tmar* (**f**)

In another set of mutations, we combined only the amino acids that differed between HU*Bst* and HU*Bsu* and were located on the α_2-helix (Table 3, first part). Both multimutant HU*Bst*-S31T/T33L/E34D (T_m = 66.2°C) and HU*Bst*-A27S/S31T/T33L/E34D (T_m = 64.7°C) had increased thermostability relative to HU*Bst* wt, with ΔT_m of +4.6 and +3.1°C, respectively. The second part of Table 3 summarizes the T_m determination of HU*Bsu* mutants. The HU*Bsu* mutants were designed to change the protein toward its thermostable homologue, HU*Bst*. We focused only on amino acid mutants that had a strong negative effect on the T_m of HU*Bst* to determine whether the same amino acid replacements in the reverse direction would have a positive effect on the T_m of HU*Bsu*. The point mutations of HU*Bsu*, E15G, D34E, and I42V, substantially increased the thermostability of the protein, nearly to the same extent that the reverse mutations destabilized HU*Bst*. The data demonstrate that these three amino acid residues play an important role in the thermostabilization mechanism of HU*Bst* and HU*Bsu* proteins. The combination of these three substitutions in the mutations HU*Bsu*-E15G/I42V (T_m = 55.8°C), HU*Bsu*-E15G/D34E (T_m = 56.7°C), and

Table 2. Localization of the amino acid residues that differ between HU*Bst* and HU*Bsu* and have been mutated in HU*Bst*

From HU*Bst* to HU*Bsu*	T_m (°C)	ΔT_m (°C)	Localization in the structure	Comments
HU*Bst* wt	61.6			
T 13 A	62.5	+0.9	Surface of α_1-helix, solvent exposed	Possible stabilization due to increased alanine content
G 15 E	51.6	–10.0	Center of turn between α_1 and α_2 -helix	Destabilization due to disruption of the tight packed in V-shape α_1- and α_2-helix
A 27 S	60.4	–0.8	Center of α_2-helix	Serine unfavorable in helix, possibly due to shared H-bonds to the helical backbone
S 31 T	63.2	+1.6	Surface α_2-helix, solvent exposed	Loss of existing H-bond between S31 and A35, increased hydrophobicity
T 33 L	64.0	+2.4	Surface α_2-helix, solvent exposed	Loss of existing H-bond between S31 and A35, increased hydrophobicity
E 34 D	59.8	–1.8	Charge cluster on the surface of α_2-helix	Possible influence on charge strength
R 37 K	61.9	+0.3	Charge cluster on the surface of α_2-helix	Possible influence on charge strength
K 38 N	58.8	–2.8	Charge cluster on the surface of α_2-helix	Possible influence on charge strength
V 42 I	59.2	–2.4	β-Ribbon	Possible packing effect in the hydrophobic pocket formed between V42, F50, I32, A35, L44, and M1
A 56 S	62.1	+0.6	β-Ribbon	Point to the solvent
M 69 I	64.1	+2.5	β-Ribbon	Point to the solvent
HU*Bst* + AG	63.3	+1.6	C-terminus α_3-helix	Point to the solvent

The primary and tertiary structure is based on the high-resolution 3-D structure of HU*Bst*
The comments are based on comparison of the location and types of amino acid substitutions with those previously identified as mutations involved in protein stability
These data allow us to propose stabilization mechanisms for some of the mutant proteins

Table 3. Summary of the wild type (wt) and mutated HU proteins and their effects on the melting temperature as determined by circular dichroism (CD)

Mutation	T_m (°C)	ΔT_m (°C)	Localization in the structure
From HU*Bst* to HU*Bsu*[a]			
Combined mutations			
G 15 E / V 42 I	46.0	–15.6	Turn $\alpha_{1,2}$-helix / β_2-strand
G 15 E / E 34 D / V 42 I	45.1	–16.5	Turn $\alpha_{1,2}$-helix/α_2-helix/β_2-strand
G 15 E / E 34 D / V 42 I / K 38 N	44.1	–17.5	Turn $\alpha_{1,2}$-helix/α_2-helix/β_2-strand
α_2-Helix mutations			
S 31 T / T 33 L / E 34 D	66.2	+4.6	α_2-Helix
A 27 S / S 31 T / T 33 L / E 34 D	64.7	+3.1	α_2-Helix
From HU*Bsu* to HU*Bst*[b]			
HU*Bsu* wt	39.7		
Single mutations			
E 15 G	53.9	+14.2	Turn $\alpha_{1,2}$-helix
D 34 E	45.1	+5.4	α_2-Helix
I 42 V	43.4	+0.3	β_1-Strand
Combined mutations			
E 15 G / D 34 E	55.8	+16.1	Turn $\alpha_{1,2}$-helix/β_2-strand
E 15 G / I 42 V	56.7	+17.0	Turn $\alpha_{1,2}$-helix/α_2-helix
E 15 G / D 34 E / I 42 V	57.8	+18.1	Turn $\alpha_{1,2}$-helix/α_2-helix/β_2-strand
E 34 G / D 34 E / N 38 K / I 42 V	56.7	+17.0	Turn $\alpha_{1,2}$-helix/α_2-helix/β_2-strand
From HU*Tmar* to HU*Bsu*[c]			
HU*Tmar* wt	80.5		
Single mutations			
G 15 E	55.8	–24.7	Turn $\alpha_{1,2}$-helix
E 34 D	72.7	–7.8	α_2-Helix
V 42 I	70.9	–9.6	β_1-Strand
Combined mutations			
G 15 E / E 34 D	52.1	–28.4	Turn $\alpha_{1,2}$-helix/β_2-strand
E 34 D / V 42 I	63.4	–17.1	Turn $\alpha_{1,2}$-helix/α_2-helix
G 15 E / E 34 D / V 42 I	35.9	–44.6	Turn $\alpha_{1,2}$-helix/α_2-helix/β_2-strand

T_m, melting temperature
[a]Point and combined amino acid replacements from HU*Bst* to HU*Bsu*
[b]Point and combined amino acid replacements from HU*Bsu* to HU*Bst*
[c]Point and combined amino acid replacements from HU*Tmar* to HU*Bsu*

HU*Bsu*-E15G/D34E/I42V (T_m = 57.8°C) increased the T_m of HU*Bsu* close to that of HU*Bst* wt.

Finally, we extrapolated the results on the extremely thermophilic HU*Tmar* and design several mutants as described earlier. The alignments shown in Fig. 1 indicate that the amino acids at positions 15, 34, and 42 are conserved only in the thermostable partners HU*Bst*, HU*Tth*, and HU*Tmar*. Because these three amino acids have been shown to be very important for the thermostability of HU*Bst*, they were replaced with their mesophilic counterparts on HU*Tmar* to determine whether they are also involved in its thermostability. The three point mutations of HU*Tmar*, G15E, E34D, and V42I, were constructed, overproduced, and purified, and their T_m was determined (see Table 3 , third part). It is noteworthy that the single mutant HU*Tmar*-G15E (T_m = 55.8°C) caused a drastic destabilization of HU*Tmar*. However, the HU*Tmar*-E34D point mutation (T_m = 72.7°C) and HU*Tmar*-V42I (T_m = 70.9°C) had little effect.

The data from the set of combined mutants of these three amino acid residues (Table 3 , third part) show that the combination of HU*Tmar*-G15E/E34D decreased the thermostability of HU*Tmar* by nearly 28.4°C. When we combined the mutant HU*Tmar*-V42I (ΔT_m = –9.6°C) with HU*Tmar*-E34D (ΔT_m = –7.8°C), a clear additive effect occurred. Finally, we included the point mutation V42I on HU*Tmar*-G15E/E34D and formed the triplet mutant HU*Tmar*-G15E/E34D/V42I. The addition of V42I had a further additive effect on the T_m of HU*Tmar*-G15E/E34D mutant (ΔT_m = –44.6°C), reaching T_m = 35.9°C, which is very close to the T_m of mesophilic HU*Bsu*.

The chemical denaturation of both HU*Bst* and HU*Bsu* was examined using various concentrations of urea as described in Methods. The unfolding of both HUs by urea was examined by both CD spectroscopy and urea-gradient PAGE. The CD experiments showed that HU*Bst* and HU*Bsu* were 50% unfolded at 3.0 and 2.5 M urea, respectively (data not shown).

Discussion

The improvement of protein stability by rational design is one of the most important issues of protein engineering because, among other reasons, it is of biotechnological relevance. Although a number of model proteins have been used to elucidate the stabilization mechanism, we are not yet at the stage of fully predicting and applying concrete rules for protein stabilization. The major difficulty in solving the problem of protein thermostability is intrinsic, occurring because the free energy difference between the folded and unfolded states of a protein is marginal. It is therefore necessary to identify proteins that can be used as model systems which fulfill the requirements that were outlined in the Introduction. However, the literature contains only a few examples of highly homologous proteins with substantial differences in thermostability that have been thoroughly studied. Another interesting approach, which has not yet been used, could be to identify and study proteins that are at the beginning of evolutionary divergence. The HU proteins employed in this study fulfill the criteria of an attractive experimental model system for thermostability studies. The homodimer nature of HU proteins and the fact that they exist as multimers in solution make them even more complicated.

The initial goal of this study was to explore the differences in the primary structure between three closely related HU proteins from mesophilic, thermophilic, and extremely thermophilic bacteria. By applying site-directed mutagenesis, we tried to assess the contribution of selected amino acid residues to the thermostability of these proteins. The aligned sequences of HU*Bst*, HU*Bsu*, and HU*Tmar* proteins with the secondary structure elements based on the 3-D structure of HU*Bst* are shown in Fig. 1, and a short description of the domains is given in the Results. The selection of the mutants was performed using a primary structure comparison (see Fig. 1) and the strategy for choosing the mutants is also described in the Results.

Because the 3-D structures of HU*Bst* and HU*Tmar* are available in high resolution, the localization of the mutated amino acids and their possible effect on thermostability can be postulated. Figure 5 is a model based on the X-ray structure HU*Bst* (PDB entry, 1 HUU) and the location of the created mutants. The mutants are predominantly localized on the main body of the molecule and are exposed to solvent; this is not consistent with the general directions of thermostabilization mechanisms summarized by Querol

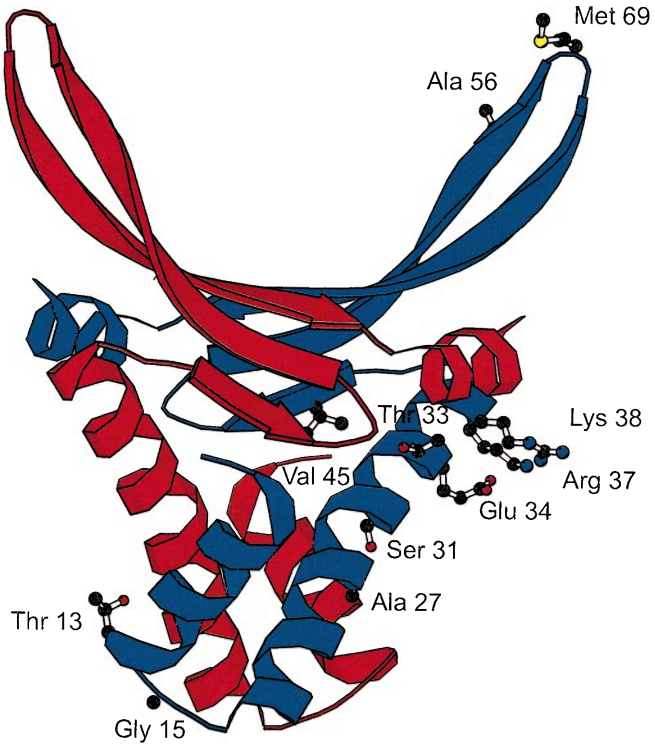

Fig. 5. Model of the X-ray structure of HU*Bst* (PDB entry, 1 HUU) and the location of the mutated amino acid residues on the structure. Only the amino acid residues that have been mutated are shown

et al. (1996). Table 2 presents the experimental results of the effect of single mutations on the T_m of HU*Bst* and comments on each mutation by proposing an explanation of the effect of each amino acid replacement. The proposed interpretation is based on the current general concept of the molecular mechanisms involved in protein stability.

As shown in Tables 2 and 3, 17 mutations were created on HU*Bst.* Of these, 11 were single, 5 combined, and 1 was an addition of two amino acids at the C-end of the molecule. The mutants G15E, D34E, and I42V, either singly or combined, substantially reduce the thermostability of HU*Bst* and are conserved in the three thermophilic HU proteins (see Fig. 1). This clearly shows that these amino acid residues are essential for the thermostability of thermophilic HUs.

Crystallographic analysis of HUB*st* localizes Gly-15 in the bend between the α_1-helix and α_2-helix, in the HTH motif (Fig. 5). Although Gly-15 is conserved among thermophilic HUs, it is replaced by Glu in mesophilic HU*Bsu*. The dihedral angle for Gly-15 in HU*Bst* is close to the region of the Ramachandran plot for a left-handed α-helix, which is energetically favorable only for Gly residues. Hence, replacement of Gly-15 by Glu in the turn structure gives rise to certain structural changes that may contribute to substantial thermal destabilization of mutant HU*Bst*-G15E. The HTH motif 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 involved in DNA binding. This result indicates that the existence of this amino acid residue in this position is important in maintaining this motif compact. The presence of Glu-15 in mesophiles must require at least some rearrangement of the local conformation, perhaps leading to some destabilization of the molecule. The inverse point mutation applied in mesophilic HU*Bsu* arises the T_m of the protein (see Table 3), again indicating the significance of this residue for the thermostability of the HTH motif of HU proteins. The HTH motif as revealed in HU*Bst* is unique and plays an important role because it is the helical core domain of the structure. No similar structure is found in the literature, except the recently elucidated structure of *Bacillus stearothermophilus* S7 ribosomal protein (Hosaka et al. 1997), which revealed a similar motif in its N-terminal half. This strongly supports the idea that the HTH motif may have been selected throughout evolution as a scaffold for stabilizing some nucleic acid-binding proteins functioning at elevated temperatures.

Mutant proteins HU*Bst*-T13A and HU*Bst*-T33L gave an unexpected result. As the hydrogen bonds to Thr-13 and the Thr-33 hydroxyl groups are lost in mutant proteins, it was expected that the mutations might decrease protein stability. However, the stability of mutant HU*Bst*-T33L was almost identical to that of the wt, and HU*Bst*-T13A was slightly more stable compared with the wt (see Table 2). The increased thermostability of mutant proteins may result from increased hydrophobicity due to Ala replacement, which not only compensated for the loss of the hydrogen bond but also improved thermostability. The Ala residue is a strong α-helix-forming amino acid, and it seems likely that the first α-helix in the HU*Bst*-T13A is stabilized by the Ala residue at position 13. The enhanced stability provided by the Ala residue at position 13 was also confirmed by mutant HU*Bst*-T13A/G15E, which was more stable than HU*Bst*-G15E (ΔT_m = +3.2°C), as also described by Kawamura et al. (1996, 1998).

The residue Glu-34 together with Arg-37 and Lys-38 in HU*Bst* creates a cluster of oppositely charged residues and appears to be very disordered in the crystal structure, as revealed by their high B values. In addition to involvement in the stabilization of the second α_2-helix, because of its intrinsic α-helix-forming property, Glu-34 is also found to be responsible for enhancing the thermostability of HU*Bst* by forming an extra salt bridge with Lys38. Substitution of Glu-34 by Asp, with a shorter side chain, reduces the strength of the salt bridge and decreases the thermostability of the protein (ΔT_m, –1.8°C). Substitution of Lys-38 with Asn in the thermophilic protein means a loss of the positive charge and might be implicated in destabilization of the protein (see Table 2). 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 surface of proteins contributes little to protein stability (Serrano et al. 1990; Horovitz et al. 1990; Sali et al. 1991). In contrast, Vogt et al. (1997) reported that the salt bridge, together with the hydrogen bond, is the main explanation for the thermostability of proteins.

Mutation of Val-42 to Ile is another significant mutant regarding the thermostability of the HU protein. Val-42 occurs at the beginning of the β_1-strand in the vicinity of the α_2-helix. Val-42 is surrounded by the side chains of Ala-35, Ile-32, Phe-50, and Leu-44, and by Met-1. All these residues form a very hydrophobic pocket. The Val residue has a shorter side chain than Ile and serves as a more tightly packed core for the HU molecule. Introduction of an Ile at this position will push residue Ile-32, and repulsion between position Leu-42 (β_1-strand) and Ile-32 (α_2-helix) might occur, causing a negative effect on thermostability (ΔT_m = –2.4°C); this is an interior apolar-to-apolar substitution that alters the packing without accompanying hydrophobicity changes and substantially destabilizes the protein.

The terminal Lys-90 in thermophiles is replaced by the tripeptide Ala-90–Gly-91–Lys-92 in the mesophiles. There are no obvious interactions suggesting a contribution of these three residues to the stability of the structure, although the T_m was slightly elevated.

Our studies have shown that some replacements of the amino acid residues in HU*Bst* with the corresponding amino acids in HU*Bsu* resulted in thermostabilized proteins. It is thus suggested that the thermophilic protein HU*Bst* has not evolved to optimize the protein structure in terms of thermostability. It is generally known that many proteins have been selected during evolution to be marginally stable. The generated side-chain clusters at positions 27, 31, 33, and 34 in the α_2-helix of the HU*Bst* protein resulted from increased thermostability (see Table 3), suggesting that this part of the molecule is not locally optimized with respect to stability.

The mutations that change the stability of HU*Bsu* to HU*Bst* (see Table 3), that is, the E15G, D34E, and I42V

HU*Bsu* mutants, increased thermostability in a manner that converted the mesophilic HU*Bsu* to its thermophilic homologue HU*Bst.* Regarding extremely thermophilic HU*Tmar*, only three amino acids were selected for replacement based on the results described for HU*Bst* and HU*Bsu*, which are suggested by the alignment in Fig. 1. As expected, the point HU*Tmar*-G15E mutants have shown a strong destabilization effect ($\Delta T_m = -24.7°C$), likely for the same reason as for the other HU proteins. Furthermore, both E34D and V42I have a dramatic effect on the stability of the protein. Our unpublished crystallographic data have clearly shown that Glu-34 from one subunit makes a salt bridge with the Lys-13 residue of the other subunit in the homodimer. By replacing Glu-34 with Asp, this salt bridge is destroyed and the molecule is considerably destabilized. The point mutant V42I also has a significant destabilization, likely for the same reason as for HU*Bst*. Finally, the triplet G15E/E34D/V42I converts the extremely thermophilic HU*Tmar* to a mesophilic protein with T_m very close to that of HU*Bsu* (see Table 3).

Vis et al. (1998) have reported the heterogeneity of conformational states of HU*Bsu* on folding. Current calorimetric studies to interpret the mechanisms of unfolding of HU wt show that the three HUs used in this study have different folding/unfolding pathways. HU proteins have fairly low enthalpy of unfolding because of the flexible arms, which create an unusual surface/volume ratio (unpublished results). An extensive thermodynamic study is under way and is the subject of another report (in preparation). The results presented in this article are similar to those presented by Kawamura et al. (1996, 1997, 1998) using the same system.

None of the mutants prepared for this study influenced the DNA-binding capacity of HU. It was expected that little effect, if any, on the DNA-binding activity would occur on changing amino acid residues that are mainly located in the compact main body of the homodimer. Several reports suggest that the flexible arms of the HU*Bst* are predominantly responsible for the interaction of the HU*Bst* with DNA. Our current unpublished results suggest that a few amino acids, located in the main body of the molecule, are also involved in the interaction of HU with DNA. These findings are in accordance with data obtained from the HU*Bst*–DNA complex investigated by nuclear magnetic resonance (NMR) (Boelens et al. 1996; White et al. 1999), showing that DNA-binding activity and thermostabilization mechanisms are independent.

In conclusion, the targeted construction of single or combined mutants of HU proteins and determination of the stability of these mutants were employed as a general strategy to determine the amino acids responsible for the stability of thermophilic HU*Bst* and HU*Tmar*, as well as to create mutants that could stabilize mesophilic HU*Bsu*. The results presented here identified three individual amino acid residues that are responsible for the thermostability of HU*Bst*. When the same amino acids were replaced in the highly homologous mesophilic HU*Bsu* by in vitro site-directed mutagenesis, thermostability increased to the levels of thermostable HU*Bst*. We have also shown that the stabilization resulting from multiple mutations is additive. Furthermore, the same amino acid residues are involved in the stability of the extremely thermophilic HU*Tmar* protein, which can be converted to the mesophilic HU*Bsu* by replacement of three amino acids with their mesophilic counterparts. Our model molecule with molecular weight of 18 kDa is not an average protein; nevertheless, the number of mutants that were able to give rise to such changes are only a small percentage of the differences between the extremely thermophilic, thermophilic, and mesophilic HUs.

Our results support the hypothesis that the thermostability mechanisms of these three HU proteins share a common basis. Further crystallographic studies on various HU mutants are required to clarify their thermostability mechanism at the molecular level. We could not elucidate the thermostability mechanism of the HU proteins in this study, but the experimental data will aid our efforts to decipher the thermostabilization mechanism of HU proteins.

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