Stabilization of *Escherichia coli* Ribonuclease HI by Strategic Replacement of Amino Acid Residues with Those from the Thermophilic Counterpart* 

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 Thermus thermophilus* ribonuclease H is exceptionally stable against thermal and guanidine hydrochloride denaturations as compared to *Escherichia coli* ribonuclease HI (Kanaya, S., and Itaya, M. (1992) *J. Biol. Chem.* 267, 10184–10192). The identity in the amino acid sequences of these enzymes is 52%. As an initial step to elucidate the stabilization mechanism of the thermophilic RNase H, we examined whether certain regions in its amino acid sequence confer the thermostability. A variety of mutant proteins of *E. coli* RNase HI were constructed and analyzed for protein stability. In these mutant proteins, amino acid sequences in loops or terminal regions were systematically replaced with the corresponding sequences from *T. thermophilus* RNase H. Of the nine regions examined, replacement of the amino acid sequence in each of four regions (R4–R7) resulted in an increase in protein stability. Simultaneous replacements of these amino acid sequences revealed that the effect of each replacement on protein stability is independent of each other and cumulative. Replacement of all four regions (R4–R7) gave the most stable mutant protein. The temperature of the midpoint of the transition in the thermal unfolding curve and the free energy change of unfolding in the absence of denaturant of this mutant protein were increased by 16.7 °C and 3.66 kcal/mol, respectively, as compared to those of *E. coli* RNase HI. These results suggest that individual local interactions contribute to the stability of thermophilic proteins in an independent manner, rather than in a cooperative manner.

One of the main purposes of protein engineering is to develop methods for designing protein variants with higher thermostability. Various strategies to enhance protein stability have been proposed (1–7). These strategies, as well as the findings that the effects of amino acid substitutions on protein stability are additive (8–10), encourage us to use protein engineering technology for industrial applications. However, general methods to increase protein stability have not yet been established. More information on the structure-stability relationships of proteins is required.

Elucidation of the mechanisms by which thermophilic enzymes acquire their unusual thermostability will not only yield much information on the structure-stability relationships of proteins, but also allow evaluation of strategies that have been proposed to improve protein stability. It has been shown that the thermostability of mesophilic enzymes can be dramatically enhanced by replacing a few amino acid residues or a portion of the amino acid sequence with those from the thermophilic counterpart (11–16). In some cases, thermostabilization of proteins has been rationally designed (11, 12). However, the differences in stability between mesophilic and thermophilic proteins may reflect the sum of the various forces or the interactions that stabilize the proteins. In addition, different proteins may adopt unique mechanisms for stabilization. Therefore, it is important to thoroughly investigate the differences in protein stability for a given pair of mesophilic and thermophilic proteins.

RNase H (EC 3.1.26.4) is an enzyme that specifically degrades the RNA moiety of DNA/RNA hybrids (for a review, see Ref. 17). The enzyme is distributed widely in various organisms, including *Escherichia coli* (18, 19) and *Thermus thermophilus* (20). *E. coli* RNase HI, which had been designated as RNase H until a second RNase H (RNase HI) was isolated from *E. coli* (21), and *T. thermophilus* RNase HI are composed of single polypeptide chains with 155 (22) and 166 (23) amino acid residues, respectively. Despite the relatively high amino acid sequence similarity (52% identity), *T. thermophilus* RNase H exhibits unusually high stability (23). The melting temperature of *T. thermophilus* RNase H, which was determined from thermal denaturation experiments in the presence of 1.2 M guanidine hydrochloride (GdnHCl) at pH 5.5, was 33.9 °C higher than that observed for *E. coli* RNase HI. The free energy change of unfolding in the absence of GdnHCl of *T. thermophilus* RNase H was also greatly increased by 11.8 kcal/mol at 25 °C, as compared to *E. coli* RNase HI.

*E. coli* RNase HI and *T. thermophilus* RNase H are promising materials to identify the amino acid substitutions contributing to the thermal stability of a thermophilic protein for the following reasons. 1) They are relatively small in size. 2) The structural genes of the enzymes are available, facilitating mutational analysis, and overproduction systems for *E. coli* RNase HI (24) and *T. thermophilus* RNase H (23) have been established. 3) Both *E. coli* RNase HI (25, 26) and *T. thermophilus* RNase H (23) reversibly unfold in a single cooperative fashion in thermal and GdnHCl denaturation experi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) K0652 and 60507.

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1 The abbreviations used are: GdnHCl, guanidine hydrochloride; PCR, polymerase chain reaction.

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ments. 4) The three-dimensional structure of E. coli RNase HI has been determined by x-ray analysis (27-29). 5) The catalytic (30) and substrate-binding (31, 32) sites of E. coli RNase HI have been identified by site-directed mutagenesis. Although the three-dimensional structure of T. thermophilus RNase H has not been determined, the sequence similarity between the E. coli and T. thermophilus enzymes suggests that they have similar three-dimensional structures. As an initial step to identify the amino acid substitutions responsible for the unusual thermal stability of T. thermophilus RNase H, we searched for the amino acid sequences of E. coli RNase HI that have poor sequence similarity to those of T. thermophilus RNase H and are associated with relatively independent secondary structures. Then, we systematically replaced these sequences with those of T. thermophilus RNase H and analyzed the effects of each replacement on the stability of E. coli RNase HI.

Here, we report that the stability of mutants of E. coli RNase HI in which the amino acid sequence in each of the four regions was replaced with the corresponding T. thermophilus RNase H sequence increased as compared to the parent protein. We also report that the effect of each replacement on protein stability was cumulative.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—The expression vector pJLA503 (33) was obtained from Medac Gentechology. Plasmid pAK600 was previously constructed (31). Competent cells of E. coli HB101 (F-, hsdS20 (rK-, mK+), recA13, ara-13, proA2, lacY1, galK2, rpsL20 (S80), xyl-5, mtl-1, supE44, rK+) were from Takara Shuzo Co., Ltd. Cells were grown in Luria broth medium (34) containing 100 mg/liter ampicillin.

Restriction enzymes and modifying enzymes for recombinant DNA technology were obtained from Takara Shuzo Co., Ltd., Toyobo Co., Ltd., or Bethesda Research Laboratories. Ultrapure-grade GdnHCl was from Schwarz/Mann. E. coli RNase HI was prepared previously (24). Other chemicals were of reagent grade.

Plasmid Construction—Plasmid pJAL600N for the overproduction of E. coli RNase HI was constructed as follows. The rnhA gene in plasmid pAK600 was amplified using PCR. 5'- and 3'-primers were 5'-GGGATCCTAGTTAACAGGTAAG-3' and 5'-GGGGTGCGACCATCGCGAGGTTG-3', where dots represent the initiation codon of the rnhA gene, and the underlined bases show the positions of the NdeI and Sall sites, respectively. After digestion of the PCR product with NdeI and SalI, the result 500-base pair DNA fragment was ligated with the large NdeI-Sall fragment of pJLA503 to construct plasmid pJAL600N. In this plasmid, transcription of the rnhA gene is initiated by the bacteriophage λ promoter P0 and P1 in tandem and terminated by the bacteriophage fd terminator (Fig. 1). The initiation of the rnhA gene is also present on the plasmid. PCR was performed in 25 cycles with a Perkin-Elmer Cetus DNA Thermal Cycler (Model P12000) using a Gene Amp kit (Takara Shuzo Co., Ltd.) according to the procedure recommended by the supplier. All oligodeoxyribonucleotides were synthesized with an Applied Biosystems Model 380A automatic DNA synthesizer by the phosphoramidite method (35).

Mutagenesis—Alteration of the rnhA gene was carried out by site-directed mutagenesis using PCR or by cassette mutagenesis. All the codons for amino acid residues that were changed in the rnhA gene in this experiment are shown in Table I. Relevant restriction enzyme sites of pJAL600N are shown in Fig. 1. Alteration of the codons for the amino acid residues located at the NH2 or COOH terminus was carried out by cassette mutagenesis. The 30-base pair NdeI-BglII fragment or the 100-base pair SstII-SalI fragment from pJAL600N was replaced by the chemically synthesized mutant. Alteration of the amino acid codons located between the BglII and SalI sites was carried out with PCR as described by Higuchi (36). Briefly, two primary PCR products, which overlap in sequence, were first obtained from an appropriate DNA template. One was generated with the 5'- or the 3'-mutagenic primer or both were usually designed to create a new restriction enzyme site within the gene, the mutants were initially screened by restriction enzyme mapping of plasmid DNA prepared from Amp'-transformants of E. coli HB101. The production and purification of the mutant proteins from HB101 transformants were carried out as described for the wild-type protein mutant N4830-1 bacteriophage fd phage pJAL600N (37). The synthetic levels and the purities of the mutant proteins were analyzed by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (37). Each mutation was confirmed by amino acid sequence analysis as previously described (38).

Protein Concentration—The protein concentration was determined from UV absorption with an absorption coefficient of A280 = 2.02, which assumed that all mutant proteins, except for those in which both Trp235 and Trp250 were replaced by Phe, had the same absorption coefficient as that of wild-type E. coli RNase HI (38). The absorption coefficient of the mutant proteins, in which Trp235 and Trp250 were replaced by Phe, was modified to A280 = 1.42 by determining the protein concentration by the method of Bradford (29) using wild-type E. coli RNase HI as the standard protein. This value was in good agreement with the A280 value of 1.48 calculated by using ε = 1576 M⁻¹ cm⁻¹ for Tyr (x 5) and 5225 M⁻¹ cm⁻¹ for Trp (x 4) at 280 nm (40).

Enzymatic Activity—The RNase H activity was determined by measuring the radioactivity of the acid-soluble digestion product from the substrate, a 14H-labeled M13 DNA/RNA hybrid, as previously described (25). One unit of enzymatic activity is defined as the amount of enzyme producing 1 pmol of acid-soluble material/min at 37 °C. The specific activity is defined as units of enzymatic activity/milligram of protein.

Circular Dichroism Spectra—The CD spectra were measured on a J-600 spectropolarimeter (Japan Spectroscopic Co., Ltd.). Spectra were obtained at 25 °C in 10 mM sodium acetate buffer (pH 5.5) containing 0.1 M NaCl. The protein concentration was 0.15 mg/ml, and the optical path length was 2 mm.

Thermal Denaturation—Thermal denaturation curves were determined by monitoring the CD value at 220 nm as the temperature was increased. Proteins were dissolved in 10 mM glycine HCl buffer (pH 3.0) containing 1 mM dithiothreitol or in 20 mM sodium acetate buffer (pH 5.5) containing 1 mM GdnHCl and 1 mM dithiothreitol. The protein concentration was 0.15 mg/ml, and the optical path length was 2 mm.

The temperature of the cuvette containing the sample solution was raised at a rate of ~0.7 °C/min. The temperature of the sample solution was directly measured by a Takara D641 thermostat. All mutant proteins examined, reproduced unfolded in a single cooperative fashion under these experimental conditions. On the assumption that
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**Table 1**

Correspondence between amino acid substitutions and codon substitutions

| Amino acid change | Codon       |
|-------------------|-------------|
| Addition of Met-1 | ATG         |
| Addition of Asn-3 | AAC         |
| Addition of Pro-2 | CCC         |
| Addition of Ser-1 | TCC         |
| Met-1 → Pro       | ATG → CCT   |
| Leu-2 → Arg       | CTG → AAG   |
| Tyr-3 → Phe       | TAT → TAC   |
| Arg-4 → His       | GCG → CAC   |
| Gly-5 → Ala       | GGA → GCT   |
| Arg-6 → His       | GCG → CAT   |
| Tyr-7 → Glu       | GCC → GAA   |
| Thr-8 → Ala       | ACC → GCA   |
| Arg-9 → Cys       | GGC → TGG   |
| His-10 → Pro      | CAT → CCA   |
| Val-11 → Leu      | GTC → CTC   |
| Arg-12 → Lys      | GCG → AAA   |
| Gly-13 → Lys      | CAA → AAA   |
| Gly-14 → Ala      | GGT → GCT   |
| Gly-15 → Ala      | GCT → GCT   |
| Gly-16 → Ala      | GCA → GCA   |
| Asp-17 → Glu      | GAC → GAA   |
| Lys-18 → Arg      | AAA → GGC   |
| Lys-19 → Gly      | GAA → GGC   |
| Leu-20 → Met      | TTC → ATG   |
| Gly-21 → Ala      | GGG → GGC   |
| Gly-22 → Pro      | CAG → CCG   |
| Gly-23 → Arg      | CCA → GCG   |
| Gly-24 → Ala      | AAG → AAA   |
| Thr-25 → Arg      | GAA → GTC   |
| Thr-26 → Pro      | GAC → GCA   |
| Val-27 → Leu      | AAA → AGA   |
| Thr-28 → Phe      | GTC → TAC   |
| Glu-29 → His      | GGA → CAT   |
| Thr-30 → Phe      | GAG → GCA   |
| Ala-31 → Thr      | GCC → ACC   |
| Thr-32 → Cys      | ACA → TGT   |
| Gly-33 → Pro      | GGC → CCA   |
| Asp-34 → Glu      | GAC → GAA   |
| Lys-35 → Arg      | AAA → GGC   |
| Tyr-36 → Phe      | TAT → TAC   |
| Gly-37 → Arg      | GCG → CAT   |
| Gly-38 → Ala      | GCA → GCA   |
| Thr-39 → Pro      | GAC → GCA   |
| Val-40 → Leu      | AAA → AGA   |
| Thr-41 → Phe      | GTC → TAC   |
| Glu-42 → His      | GGA → CAT   |
| Thr-43 → Glu      | GAC → GCA   |
| Ala-44 → Thr      | GCC → ACC   |
| Thr-45 → Cys      | ACA → TGT   |
| Gly-46 → Pro      | GCC → CCA   |
| Glu-47 → Arg      | GAC → GCA   |
| Thr-48 → Phe      | TAT → TAC   |
| Gly-49 → Ala      | GGC → CAT   |
| Val-50 → Thr      | GTC → TAC   |
| Val-51 → Ala      | GCA → GCA   |
| Addition of Tyr-1 | CCG         |
| Addition of Thr-2 | CTG         |
| Addition of His-3 | CAT         |
| Addition of Gly-4 | GAA         |
| Addition of Ala-5 | GCA         |

the unfolding equilibrium of these proteins follows a two-state mechanism, the pre- and post-transition base lines were linearly extrapolated, and the difference in free energy change between the folded and unfolded states ($ΔG$) was calculated as described by Pace (43). The free energy change in H2O ($ΔG^{(H2O)}$) and the measurement of the dependence of $ΔG$ on the GdnHCl concentration ($m$) were determined by a least-squares fit of the data from the transition region to the following equation:

$$ΔG = ΔG^{(H2O)} - m[\text{GdnHCl}] (42).$$

The midpoint of the GdnHCl denaturation curve ($[\text{GdnHCl}]_M$) was the concentration of GdnHCl at which the $ΔG$ value became 0.

**RESULTS**

**Mutagenesis**—Comparison of the three-dimensional structure of E. coli RNase HI with the hypothetical structure of T. thermophilus RNase H indicates that the amino acid substitutions between these two enzymes are mostly localized in the COOH-terminal region; the region consisting of the NH$_2$ terminus and the loop between βB and βC; and the region consisting of αII, αIII, αIV, and βE (23). In this study, the amino acid sequences of E. coli RNase HI in these regions were replaced with the corresponding sequences from T. thermophilus RNase H. In addition, the amino acid sequences forming the loop structure were also chosen as a target sequence to be replaced. The amino acid residues involved in the loop regions are generally accessible to the solvent and are expected to contribute to protein stability in an independent manner, rather than in a cooperative manner. Finally, as shown in Fig. 2, the amino acid sequences in the nine regions (R1–R9) of E. coli RNase HI were replaced with the corresponding T. thermophilus RNase H sequences to analyze their effects on the stability of E. coli RNase HI. R1 and R2 are the NH$_2$- and COOH-terminal regions of the protein molecule. All other regions involve loops or loop-like structures. The

![Fig. 2. Alignment of amino acid sequences of E. coli RNase HI and T. thermophilus RNase H.](image-url)

The amino acid sequences of E. coli RNase HI (22) and T. thermophilus RNase H (23) have previously been determined. The ranges of the five α-helices and the five β-strands of E. coli RNase HI are shown along the sequences according to Katayanagi et al. (27). Numbers above the sequences indicate the positions of the residues relative to the initiator methionine of E. coli RNase HI. The nine regions (R1–R9) in which the amino acid sequences of E. coli RNase HI were replaced by the corresponding T. thermophilus RNase H sequences are boxed.
localizations of these regions in the crystal structure of *E. coli* RNase HI are shown in Fig. 3.

The mutant proteins of *E. coli* RNase HI in which the amino acid sequence in each region from R₁ to R₈ was replaced with the corresponding *T. thermophilus* RNase HI sequence are designated as R₁-R₈-RNase HI, respectively. For the mutant proteins with multiple replacements, the replaced regions are separated by a slash, as in R₁-R₂-R₈-RNase HI. The mutant proteins in which Gly₆₀⁰ was inserted between Gln₈₆ and Trpᵢ¹ in which Gln¹¹³ was replaced by Pro are designated as G₆₀⁰-RNase H and Pᵢ¹¹₃-RNase H, respectively. The protein in which all the amino acid residues, except for Ghu¹⁰⁴, in region R₇ were replaced with those of *T. thermophilus* RNase H is designated as R₇E₁⁰⁴-RNase H. The mutant protein in which His₉⁰ was replaced by Pro is identified as R₉-RNase H, and not as P₉⁰-RNase H.

Overproduction and Purification—The mutant proteins were overproduced in *E. coli* HB101 cells containing the appropriate variant plasmid of pJAL600N. Only R₃-R₈-RNase H accumulated in cells in an insoluble form after sonication lysis and could not be purified. All other mutant proteins were recovered in a soluble form after sonication lysis and were purified to homogeneity with yields of >80%. As a typical example, the amounts of R₁-, R₂-, R₅/R₆-, R₇/R₈-, and R₉-R₁-R₈-RNases H, purified from 1-liter cultures, varied from 1.4 to 4.0 mg. The yields of the other mutant proteins purified from 1-liter cultures varied from 20 to 80 mg.

**Enzymatic Activity**—The specific activities of the mutant proteins are summarized in Table II. It is notable that the mutant enzymes G₆₀⁰-RNase H and R₉-RNase H exhibited extremely low specific activities as compared to that of the wild-type protein. In addition, the specific activities of the mutant proteins in which the amino acid sequences in region R₉ or R₈ or both were replaced with those of *T. thermophilus* RNase HI were considerably decreased as compared to that of the wild-type protein. All other mutant proteins exhibited specific activities similar to that of the wild-type protein. The specific activities of the mutant proteins with multiple region replacements were similar to or slightly less than the hypothetical values, which were calculated assuming that the effect of each region replacement on the enzymatic activity is cumulative.

| Protein | Specific activity | Type of protein |
|---------|------------------|-----------------|
| Wild-type | 20 100 Units/mg | WT |
| R₁-R₈-RNase H | 18 90 | WT |
| R₂-RNase H | 21 110 | WT |
| R₃-RNase H | 21 110 | WT |
| R₄-RNase H | 8.0 49 | A |
| C₆₀⁰-RNase H | 1.1 5.5 | WT |
| R₅-RNase H | 18 90 | WT |
| R₆-RNase H | 4.1 21 | B |
| Pᵢ¹¹₃-RNase H | 20 100 | WT |
| R₇E₁⁰⁴-RNase H | 5.7 29 | B |
| R₈-RNase H | 21 110 | WT |
| R₉-R₈-RNase H | 9.6 4.8 | WT |
| R₁/R₂-RNase H | 20 100 90 | WT |
| R₁/R₈-RNase H | 19 95 99 | WT |
| R₂/R₃-R₈-RNase H | 17 85 110 | WT |
| R₃/R₈-RNase H | 3.1 16 23 | B |
| R₅/R₈-RNase H | 2.8 14 8.4 | C |
| R₆/R₈-RNase H | 3.4 17 36 | A |
| R₇/R₈-RNase H | 15 75 99 | WT |
| R₈/R₉-R₈-RNase H | 2.9 15 9.2 | C |
| R₆/R₇-R₈-RNase H | 0.69 3.5 7.6 | C |
| R₆/R₇/R₈-RNase H | 1.1 5.5 8.3 | C |

![Fig. 3. Main chain folding of *E. coli* RNase HI.](image)

The localizations of these regions in the crystal structure of *E. coli* RNase HI are indicated in the crystal structure of *E. coli* RNase HI by solid circles and sticks, where circles represent the positions of α-carbons. Numbers indicate the positions of the amino acid residues relative to the initiator methionine of the protein. The C₆₀ model structure of *E. coli* RNase HI was kindly provided by K. Katayanagi.

**TABLE II**

**Enzymatic activities and types of CD spectra of mutant *E. coli* RNase HI proteins**

The hydrolysis of the M13 DNA/RNA hybrid with the wild-type and mutant *E. coli* RNase HI proteins was carried out at 30°C for 15 min in 10 mM Tris-HCl (pH 8.0) containing 10 mM MgCl₂, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 10 μg/ml bovine serum albumin. Errors, which represent the 67% confidence limits, are all at or below ±30% of the values reported. Hypothetical enzymatic activities of the mutant enzymes with multiple region replacements, relative to that of the wild-type protein (% of wild type), were calculated by multiplying the relative enzymatic activities (percent of wild type) of the mutant proteins with single region replacements. The CD spectra of types A-C are shown in Fig. 4. WT indicates the spectrum that is identical to that of the wild-type protein.

**CD Spectra**—The far-ultraviolet (200–250 nm) CD spectra of the mutant proteins were classified into four groups (WT, A, B, and C) based on the shape of the troughs (Fig. 4). The type of CD spectrum for each mutant protein is summarized in Table II. A group designated as WT represents the spectrum identical to that of the wild-type protein. Type A represents the spectrum with three peaks. Type B represents the spectrum with two peaks. Type C represents the spectrum with a single peak. For the mutant enzymes with multiple region replacements, the replaced regions are separated by a slash, as in R₁-R₂-R₈-RNase H. The mutant enzyme R₉E₁⁰⁴-RNase H is designated as R₇E₁⁰⁴-RNase H.

![Fig. 4. CD spectra of *E. coli* RNase HI mutants.](image)

All mutant proteins were analyzed, and the CD spectra (---) of R₉-R₈-RNase H, R₉/R₈-RNase H, and R₉/R₉-R₈-RNase H as representatives of types A-C, respectively, are shown in comparison with that of the wild-type protein (-----). All spectra were measured as described under "Experimental Procedures."

**Notes:**

- CD spectra: The far-ultraviolet (200–250 nm) CD spectra of the mutant proteins were classified into four groups (WT, A, B, and C) based on the shape of the troughs (Fig. 4).
- Types of CD spectra: The CD spectra of types A-C are shown in Fig. 4. WT indicates the spectrum that is identical to that of the wild-type protein.
- Enzymatic activities: The specific activities of the mutant proteins are summarized in Table II. It is notable that the mutant enzymes G₆₀⁰-RNase H and R₉-RNase H exhibited extremely low specific activities as compared to that of the wild-type protein. In addition, the specific activities of the mutant proteins in which the amino acid sequences in region R₈ or R₉ or both were replaced with those of *T. thermophilus* RNase HI were considerably decreased as compared to that of the wild-type protein.
- Errors: Errors, which represent the 67% confidence limits, are all at or below ±30% of the values reported.

**Table II**

| Protein | Specific activity | Type of CD spectrum |
|---------|------------------|---------------------|
| Wild-type | 20 100 Units/mg | WT |
| R₁-R₈-RNase H | 18 90 | WT |
| R₂-RNase H | 21 110 | WT |
| R₃-RNase H | 21 110 | WT |
| R₄-RNase H | 8.0 49 | A |
| C₆₀⁰-RNase H | 1.1 5.5 | WT |
| R₅-RNase H | 18 90 | WT |
| R₆-RNase H | 4.1 21 | B |
| Pᵢ¹¹₃-RNase H | 20 100 | WT |
| R₇E₁⁰⁴-RNase H | 5.7 29 | B |
| R₈-RNase H | 21 110 | WT |
| R₉-R₈-RNase H | 9.6 4.8 | WT |
| R₁/R₂-RNase H | 20 100 90 | WT |
| R₁/R₈-RNase H | 19 95 99 | WT |
| R₂/R₃-R₈-RNase H | 17 85 110 | WT |
| R₃/R₈-RNase H | 3.1 16 23 | B |
| R₅/R₈-RNase H | 2.8 14 8.4 | C |
| R₆/R₈-RNase H | 3.4 17 36 | A |
| R₇/R₈-RNase H | 15 75 99 | WT |
| R₈/R₉-R₈-RNase H | 2.9 15 9.2 | C |
| R₆/R₇-R₈-RNase H | 0.69 3.5 7.6 | C |
| R₆/R₇/R₈-RNase H | 1.1 5.5 8.3 | C |
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Whereas the former has a minimum at 214 nm, the latter has a minimum at 217 nm. Type A and B spectra were only observed for the mutant proteins that involve the replacement of the amino acid sequences in regions R₄ and R₇, respectively. Type C spectra were only observed for the mutant proteins that involve the replacement of the amino acid sequences in both of these regions. These results suggest that the effect of each region replacement on the CD spectrum is cumulative. However, the differences in the CD spectra were faint, and the spectra of all the mutant proteins basically resembled one another. This suggests that none of the mutant proteins is markedly changed in its tertiary structure.

**Thermal Stability**—To examine whether replacement of the amino acid sequence in each region with the corresponding thermophilic RNase H sequence enhances the stability of E. coli RNase HI, the thermal stabilities of all the mutant proteins were determined at both pH 3.0 and 5.5. At pH 3.0, E. coli RNase HI is destabilized due to unfavorable electrostatic interactions caused by the increased number of positive charges on the protein molecule and reversibly unfolds in a single cooperative fashion in the absence of denaturant (44). At pH 5.5, the enzyme is most stable and reversibly unfolds in a single cooperative fashion only in the presence of denaturants (25, 44). The thermal unfolding curves of some of the mutant proteins are compared with that of the wild-type protein in Fig. 5, which shows the levels of stability. The parameters characterizing the thermal denaturation of all the mutant proteins are summarized in Table III. Comparison of the Tₘ values of the mutant proteins with single region replacements with that of the wild-type protein indicates that only the replacement of the amino acid sequence (or an amino acid residue) in each of the three regions R₄—R₇ resulted in an increase in the thermal stability at either pH. Replacement of the amino acid sequence in region R₄ increased the thermal stability at pH 5.5, but decreased it at pH 3.0. All the Tₘ values of the other mutant proteins with single region replacements were equal to or below that of the wild-type protein at either pH. Notably, R₄-RNase H became extremely unstable. In addition to the region replacements, a few single amino acid substitutions were also introduced to examine their effects on protein stability. Neither the insertion of Gln²⁰⁰ nor the Gln¹⁷₃→Pro substitution significantly affected protein stability. However, the His¹⁹⁹→Glu substitution substantially affected protein stability. The His¹⁹⁹→Glu mutation in R₄-RNase H gave R₄E¹⁹⁹-RNase H. The Tₘ value of R₄E¹⁹⁹-RNase H remained unchanged at pH 5.5, but increased by 4.6°C at pH 3.0 as compared to that of R₄-RNase H.

For mutant proteins with multiple amino acid sequence replacements, hypothetical ΔTₘ values were obtained by simply adding the ΔTₘ values determined for mutant proteins with a single replacement of the amino acid sequence in each region and are listed in Table III. Only for the mutant proteins in which the amino acid sequences in regions R₄ and R₇ were both replaced were the ΔTₘ values slightly larger (by 0.8–1.8°C) than the hypothetical values. All other observed ΔTₘ values were equal to or below the hypothetical ones.

**Stability against GdnHCl Denaturation**—The stabilities against GdnHCl denaturation of the mutant proteins with higher ΔTₘ values than that of the wild-type protein were analyzed at pH 5.5. GdnHCl-induced denaturation curves and plots of ΔG versus GdnHCl concentration around the midpoint of the denaturation are shown in Fig. 6A and 6B, respectively. The parameters characterizing the GdnHCl denaturation are summarized in Table IV. The [D]₁/₂ values of all the mutant proteins examined, except for that of R₄-RNase H, increased as compared to that of the wild-type protein. The additivity of these [D]₁/₂ values was confirmed by introducing simultaneous region replacements, except for the combination of regions R₄ and R₇. The hypothetical [D]₁/₂ values were obtained by adding the Δ[D]₁/₂ values of the individual mutant proteins with single region replacements and are also listed in Table IV. The [D]₁/₂ values of the mutant proteins in which both amino acid sequences in regions R₄ and R₇ were replaced increased when compared to the hypothetical values, but only by 0.12–0.18 M. In contrast to the differences in the [D]₁/₂ values, the differences in the ΔG⁰⁺⁺ values were not proportional to those in the Tₘ values for the mutant proteins. Such discrepancies were mainly due to variations in the m values for the different mutant proteins. The m value for all of the mutant proteins, except for those of R₄-RNase H and R₄/R₆-RNase H, were almost identical to one another and varied within 4.0% of the mean value of 4.94, whereas the m values for R₄-RNase H and R₄/R₆-RNase H were obviously smaller than those of the wild-type protein (11.8 and 8.0% less than the mean value of 4.94, respectively). Nevertheless, R₄/R₆/R₇-RNase H, which had the highest [D]₁/₂ and Tₘ values, also yielded the highest ΔG⁰⁺⁺ value.

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2 S. Kanaya, personal communication.
TABLE III
Parameters characterizing the thermal denaturation of the mutant E. coli RNase HI proteins

| Protein               | pH 3.0 | pH 5.5 |
|-----------------------|--------|--------|
|                       | ΔHg  | ΔSg  | Tg  | ΔTm,°C  | ΔAG  | ΔHg  | ΔSg  | Tg  | ΔTm,°C  | ΔAG  |
| Wild type             |      |      |     |        |      |      |      |     |        |      |
| R1-RNase H            | 98.1  | 0.304| 49.8| -2.0   | -0.5 | 89.0 | 0.275| 52.0|        |      |
| R2-RNase H            | 93.4  | 0.291| 47.8| -2.0   | -0.5 | 78.7 | 0.244| 50.3| -1.7   | -0.5 |
| R3-RNase H            | 92.5  | 0.289| 47.5| -2.3   | -0.7 | 80.5 | 0.250| 48.8| -2.2   | -0.9 |
| R4-RNase H            | 100.5 | 0.335| 52.2| 3.4    | 1.0  | 102.0| 0.310| 56.1| 4.1    | 1.1  |
| R5-RNase H            | 108.6 | 0.333| 52.5| 2.7    | 0.8  | 107.0| 0.325| 56.5| 4.5    | 1.2  |
| G45-RNase H           | 98.0  | 0.295| 51.0| 1.2    | 0.4  | 97.5 | 0.294| 52.8|        |      |
| R6-RNase H            | 104.8 | 0.321| 53.8| 4.0    | 1.2  | 88.6 | 0.263| 57.6| 5.6    | 1.5  |
| R7-RNase H            | 94.7  | 0.299| 44.1| -5.7   | -1.7 | 90.2 | 0.276| 54.4| 2.4    | 0.6  |
| P112-RNase H          | 100.4 | 0.311| 49.2| -0.6   | -0.2 | 81.0 | 0.251| 49.9| -2.1   | -0.6 |
| R25-RNase H           | 77.5  | 0.240| 48.7| -1.1   | -0.3 | 102.9| 0.315| 58.9| 1.9    | 0.5  |
| R3-RNase H            | 96.4  | 0.298| 50.2| 0.4    | 0.0  | 88.8 | 0.264| 52.7|        |      |
| R4-RNase H            | 50.7  | 0.167| 30.9| -18.9  | -5.7 | 45.3 | 0.146| 38.2| -13.8  | -3.7 |
| R5-RNase H            | 98.7  | 0.310| 45.3| -4.5   | -4.3 | 70.2 | 0.219| 47.1| -4.9   | -1.3 |
| R6-RNase H            | 105.4 | 0.325| 50.2| 0.4    | 1.4  | 88.7 | 0.271| 53.9| 1.9    | 2.4  |
| R7-RNase H            | 98.5  | 0.306| 47.7| -2.1   | -0.9 | 105.2| 0.323| 51.9| -0.1   | -0.8 |
| R8-RNase H            | 106.6 | 0.323| 47.8| -2.0   | -2.3 | 107.0| 0.322| 56.9| 6.9    | 1.9  |
| R9-RNase H            | 94.4  | 0.294| 47.9| -1.9   | -3.0 | 108.0| 0.324| 60.2| 8.2    | 6.9  |
| R10-RNase H           | 112.6 | 0.342| 55.8| 6.0    | 1.8  | 108.5| 0.325| 61.2| 9.2    | 10.1 |
| R11-RNase H           | 106.6 | 0.323| 56.5| 6.7    | 7.4  | 122.1| 0.365| 68.8| 9.7    | 2.4  |
| R12-RNase H           | 110.3 | 0.339| 51.8| 2.0    | 0.4  | 114.8| 0.340| 64.5| 12.5   | 11.0 |
| R13-RNase H           | 102.3 | 0.314| 51.7| 1.9    | 1.0  | 107.0| 0.317| 64.4| 12.4   | 12.5 |
| R14/R15/R16-RNase H   | 113.4 | 0.344| 56.0| 6.2    | 4.4  | 114.9| 0.336| 68.7| 16.7   | 16.6 |

TABLE IV
Parameters characterizing GdnHCl denaturation at pH 5.5 and 25°C

The midpoint of the GdnHCl denaturation curve (ΔTm), the measurement of the dependence of ΔG on the GdnHCl concentration (m), and the free energy change of unfolding in H2O (ΔG°H2O) were calculated from the unfolding data shown in Fig. 6. The differences in [D]m (Δ [D]m) and that in ΔG°H2O (ΔΔG°H2O) between the wild-type and mutant proteins were calculated by subtracting the values of the wild-type protein from those of the mutant proteins. The hypothetical Δ (D)m value (ΔΔG°H2O) of the mutant protein with multiple region replacements was calculated by adding the Δ [D]m values of the mutant proteins with each single region replacement. Errors, which represent the 67% confidence limits, are within ±0.03 M for [D]m, ±0.08 kcal/mol/M for m, and ±0.25 kcal/mol/M for ΔG°H2O.

| Protein               | [D]m  | Δ [D]m | ΔG°H2O | m    | ΔΔG°H2O |
|-----------------------|-------|--------|--------|------|---------|
| Wild type             | 1.83  | 0.01   | 5.01   | 9.15 |
| R1-RNase H            | 1.98  | 0.15   | 4.98   | 9.89 | 0.74    |
| R2-RNase H            | 2.07  | 0.24   | 5.06   | 10.47| 0.72    |
| R3-RNase H            | 2.10  | 0.27   | 4.36   | 9.14 | 0.03    |
| R4-RNase H            | 1.83  | 0.00   | 4.74   | 8.69 | -0.46   |
| R5-R6-RNase H         | 2.02  | 0.19   | 5.07   | 10.25| 1.10    |
| R7-R8-RNase H         | 2.19  | 0.36   | 4.87   | 10.67| 1.52    |
| R9-R10-RNase H        | 2.35  | 0.52   | 4.54   | 10.88| 1.83    |
| R11-R12-RNase H       | 2.27  | 0.44   | 4.91   | 11.14| 1.99    |
| R13-R14-RNase H       | 2.38  | 0.55   | 4.91   | 11.69| 2.54    |
| R15-R16-RNase H       | 2.49  | 0.65   | 5.09   | 12.68| 3.53    |
| R17-R18-RNase H       | 2.67  | 0.84   | 4.88   | 12.81| 3.66    |

Heat Inactivation—To examine whether the mutant proteins with higher Tm and [D]m values than those of the wild-type protein are more stable against irreversible heat inactivation than the wild-type protein, we have measured the residual activities of R1/R2/R3/R4-RNase H after heating at various temperatures for 10 min as described previously (23).
The temperatures at which the wild-type and mutant proteins lost 50% of their activities ($T_{1/2}$) were ~50 and 65 °C, respectively. The difference in these $T_{1/2}$ values is comparable with the difference in the $T_m$ values, indicating that this mutant protein is more stable against irreversible heat inactivation than the wild-type protein as well.

**DISCUSSION**

**Strategy to Identify Structural Elements Responsible for Thermal Stabilization**—The construction of chimeric genes between the structural genes of the thermophilic and mesophilic proteins followed by the determination of the stabilities of these gene products have been successfully employed as a general strategy to determine the structural elements responsible for the unusual stability of thermophilic proteins (14, 16, 45). Such a strategy should be effective if no information on the three-dimensional structure is available for either the thermophilic protein or the mesophilic counterpart. However, the possibility exists that a decrease in protein stability, which is caused by an unfavorable interaction between the amino acid residues from the mesophilic and thermophilic origins, could offset an increase in protein stability, which is caused by the introduction of a structural element responsible for thermal stabilization. Larger replaced sequences within the chimeric protein increase the possibility of an unfavorable interaction due to the larger proportion of amino acid residues of different origin. Such unfavorable interactions could be reduced if the size of the amino acid sequence to be replaced was limited. We have therefore selected nine different amino acid sequences of E. coli RNase HI with limited sizes, including those which form the loops, and replaced them individually or in combination with the corresponding T. thermophilus RNase H sequences (Fig. 2). This approach, while rational, may not necessarily be the only one; and because of the enormous possible amino acid substitutions at any given position, there could be many proteins of equivalent stabilities generated using a different approach.

Analyses for the stabilities of the enzymes generated in this study allow us to suggest that four of the nine sequences (those in regions R1–R5) almost independently contribute to the unusual stability of the thermophilic RNase H, but that the amino acid sequences in regions R6–R9 do not (Table III). To identify the amino acid substitutions that determine the unusual stability of the thermophilic protein, the strategy presented here, which involves the systematic replacement of the amino acid sequences with limited sizes, might therefore be more useful than one that involves the construction of a chimeric protein with larger replaced segments. A slight cooperativity was seen between regions R1–R4 for stabilization against thermal (Table III) and GdnHCl (Table IV) denaturations. Such a cooperativity might result from the reinforcement of the hydrophobic interactions between a1II and a3E.

The difference in the $T_m$ values between the wild-type protein and R1/R2/R3–RNase H is approximately half of the value between E. coli and T. thermophilus proteins. If it is assumed that the difference in stability between E. coli RNase HI and T. thermophilus RNase H reflects the sum of the individual local interactions that stabilize a protein, approximately half of such local interactions could be introduced by the amino acid substitutions in regions R1–R5. Simultaneous replacement of the amino acid sequences in regions R1–R3 of E. coli RNase HI with the corresponding T. thermophilus RNase H sequences increases the identity between these sequences from 52 to 65%. Additional replacement of the amino acid sequences in regions R4–R9 (which have no affect on stability) increases it to almost 70%. This suggests that the difference in stability between R4/R5/R6–RNase H and T. thermophilus RNase H might originate from the 30% difference in the remaining amino acid sequence. It is possible then to consider the "70%" R4/R5/R6–RNase H as a modified T. thermophilus RNase H in which E. coli RNase HI amino acid residues destabilize the enzyme. We are looking outside regions R2–R5 for other amino acid substitutions that increase the stability of E. coli RNase HI.

**Role of Proline Residues—** T. thermophilus RNase H has more Pro residues than E. coli RNase HI. Of the 12 present in the T. thermophilus protein, there are only four that correspond to the five present in the E. coli protein. Of the remaining eight, four are in the COOH terminus, and two are in the NH2 terminus. Both the COOH-terminal and NH2-terminal regions seem to have no positive influence on the stability (Table III). Two Pro residues (Pro159 and Pro176) are found in loop regions and may help in increasing the stability of the protein. Others have suggested that Pro residues in loops contribute to protein stability probably by decreasing the entropy of unfolding (7, 46–48). In fact, each replacement of His65 with Pro or the amino acid sequence in region R7, with the corresponding T. thermophilus RNase H sequence in which Pro113 is involved increased the stability of E. coli RNase HI (Table III). The single Gln123 → Pro substitution slightly decreased the stability (Table III), probably because the conformation of the loop between a1IV and a3E in E. coli RNase HI might be considerably different from that in T. thermophilus RNase H, and the Gln123 → Pro substitution alone may not be sufficient to alter the nature of this loop.

**Effect of Gly90 Insertion and His155 → Gln**—G90–RNase H was constructed to examine the effect of the insertion of Gly90 on the stability of E. coli RNase HI. Since the increase in the $T_m$ value of G120–RNase H was much less than that of R5–RNase H as compared to the $T_m$ value of the wild-type protein (Table III), the insertion of Gly90 alone cannot account for the increase in the stability of R5–RNase H. The great reduction in the enzymatic activity caused by the insertion of Gly90 may be due to some alteration in the geometry around a1II and a3E, which are proposed to contribute to the formation of the substrate-binding site (28). The reverse substitution from His155 to Gln in R4–RNase H resulted in an increase in the $T_m$ value by 4.6 °C at pH 3.0, but not at pH 5.5, as compared to the $T_m$ value of R4–RNase H. Since the O of Gln155 forms a hydrogen bond with the N of a1II in E. coli RNase HI (28), this result suggests that the decrease in the stability of R4–RNase H at pH 3.0 is due to an electrostatic repulsion between His155 and Gln155. The relatively low enzymatic activity of R4–E155–RNase H and R4–RNase H (Table II) is probably due to a slight deformation in the active site geometry resulting from the modification of the hydrophobic interactions between a1II and a3E in these mutant proteins. Further mutagenesis experiments will be required to limit the amino acid substitutions to those that are responsible for the increase in the stability of R2–R5, R7, or R7–RNase H.

**Effect of COOH-terminal Sequence Replacement**—The COOH termini of the E. coli and T. thermophilus proteins, which show no sequence similarities (Fig. 2), are likely to fold uniquely and interact with different parts of the protein molecule. The dramatic decrease in both the enzymatic activity (Table II) and the protein stability (Table III) of R4–RNase H therefore strongly suggests that the replacement of the COOH-terminal sequence introduces significant strain into the COOH-terminal peptide or within the region with which the COOH-terminal peptide interacts. In fact, the mutant
protein in which the larger COOH-terminal peptide of E. coli RNase HI (Met\(^{142}-\text{Val}^{149}\)) was replaced with Gln\(^{142}-\text{Ala}^{150}\) of \(T.\) thermophilus RNase H was recovered in an insoluble form in cells.\(^3\) An increase in the unfavorable interactions caused by the replacement of the large COOH-terminal peptide may disturb the proper folding of the protein molecule.

**Correlation between Stabilities against Heat and GdnHCl**—
The increase in the \([D]_{19}^2\) values (Table IV) determined from the GdnHCl denaturation curves at pH 5.5 was comparable to the increase in the \(T_m\) values at pH 5.5 (Table III) for all mutant proteins, except for R_R-NH\(2\), which gave a \([D]_{19}^2\) value similar to that of the wild-type protein. Additivity in the \([D]_{19}^2\) values was also observed for the mutant proteins with multiple region replacements, as was seen in the \(T_m\) values. The differences in the \(\Delta G^{140}\) values were not proportional to the differences in the \([D]_{19}^2\) values for R_R-NH\(2\) and R_R-NH\(2\) (Table IV). These two mutant proteins had relatively lower \(\Delta G^{140}\) values than expected because the values of the dependence of \(\Delta G\) on the GdnHCl concentration (\(m\)) of these mutant proteins were smaller than those of the wild-type and other mutant proteins. This result suggests that the cooperativity of the GdnHCl-induced unfolding for these mutant proteins is weaker than that of the wild-type protein. Shortle et al. (49) have shown that the absolute value of the change in \(m\) is correlated with the loss of protein stability. In contrast, R_R-NH\(2\) and R_R-NH\(2\) were more stable even though the \(m\) values of these mutant proteins were decreased. A mechanism different from that proposed by Shortle et al. may be involved in the stability of these mutant proteins. Careful calorimetric measurements will be required to precisely determine the thermodynamic values for these mutant proteins.

**Stability-Activity Relationship—**Controversy still remains as to whether an increase in the thermal stability is caused by a reduction in the enzymatic activity due to a decrease in a conformational flexibility. In fact, \(T.\) thermophilus RNase H exhibited lower enzymatic activity than \(E.\) coli RNase HI under physiologically mild conditions (23). In this study, we have shown that some mutants of \(E.\) coli RNase HI could be stabilized without losing enzymatic activity. A typical example can be seen with R_R-NH\(2\). Whereas this mutant protein has a \(T_m\) value that is 8.5 °C higher than that of \(E.\) coli RNase HI at pH 5.5, it retained 75% of the enzymatic activity of the \(E.\) coli protein. This result supports the previous finding that enzymes from thermophilic origins are catalytically indistinguishable from their mesophilic counterparts (50). It also suggests that the thermostability of mesophilic enzymes can be enhanced by protein engineering technology without loss of enzymatic activity.

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