Thermostabilization of *Escherichia coli* Ribonuclease HI by Replacing Left-handed Helical Lys	extsuperscript{95} with Gly or Asn*

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From the systematic replacements of amino acid residues of *Escherichia coli* ribonuclease HI with those of its thermophilic counterpart, the basic protrusion domain including region 6 (R	extsubscript{6}) from residues 91 to 95 was found to increase the structural stability of the mutant protein (Kimura, S., Nakamura, H., Hashimoto, T., Oobstake, M., and Kanaya, S. (1992) J. Biol. Chem. 267, 21535–21542). Further mutagenesis concentrating in the R	extsubscript{6} region has revealed that replacements of Lys	extsuperscript{95} at the left-handed structure with Gly or Asn essentially enhances the protein stability. Gly and Asn substitutions stabilize the protein up to 1.9 kcal/mol and 0.9 kcal/mol in the free energy changes of unfolding, respectively. We propose that the amino acid substitution of left-handed non-Gly residue with Gly or Asn residue can be used as one of the general strategies to enhance protein stability, when such a non-Gly residue itself does not seriously contribute to protein stability.

An artificial enhancement of protein stability is one of the main purposes of protein engineering. Various mechanisms stabilizing the native proteins have, so far, been examined and confirmed to increase stabilities of engineered proteins (1).

One strategy to find out such mechanisms is a comparative study investigating the structure-stability relationships between the proteins from mesophilic and thermophilic species. Ribonuclease H (RNase H)\(^1\) (EC 3.1.26.4), a small protein cleaving the RNA strand of a DNA/RNA hybrid (2), is a good system for this purpose for the following reasons: 1) overproduction systems for the enzyme from *Escherichia coli* (E. coli RNase HI, 17.6 kDa) (3) and the associated thermophilic enzyme from *Thermus thermophilus* HB8 (T. thermophilus RNase H, 18.3 kDa) (4) have been established; 2) the x-ray crystal structure of *E. coli* RNase HI has already been solved (5–7); and 3) the amino acid sequence of *T. thermophilus* RNase H has been revealed to have 52% identity with the sequence of *E. coli* RNase HI (4).

The amino acid residues of *E. coli* RNase HI were systematically replaced with those of its thermophilic counterpart by Kimura et al. (8). It has been found that one of the origins of the structural stability of *T. thermophilus* RNase H is the amino acid sequence of the basic protrusion domain including region 6 (R	extsubscript{6}, residues 91–95) as shown in Fig. 1 (8). In *T. thermophilus* RNase H, the amino acid sequence of the R	extsubscript{6} indicated by single-letter codes is RTAEG, while that of *E. coli* RNase HI is KTADK (4). Interestingly, the backbone structure of Lys\textsuperscript{95} in *E. coli* RNase HI is a typical left-handed helical structure. We have made various mutants systematically in the R	extsubscript{6} region of *E. coli* RNase HI, referring the amino acid sequence of *T. thermophilus* RNase H, and examined the changes of their thermostabilities.

**MATERIALS AND METHODS**

Mutant structural genes were constructed by site-directed mutagenesis using polymerase chain reaction as described previously (8). For the construction of R\textsubscript{6}E, E\textsuperscript{96}, G\textsuperscript{96}, and N\textsuperscript{96}-RNase H, the AAA, GAC, AAA, and AAA codons of Lys\textsuperscript{95}, Asp\textsuperscript{96}, Lys\textsuperscript{95}, and Lys\textsuperscript{95} were replaced by CCG, GAA, GGC, and AAC, respectively. A\textsuperscript{96}-RNase H was previously constructed (9). In addition, the ACC codon of Thr\textsuperscript{26} was silently replaced by ACT to introduce a unique *SalI* site within the *rnhA* gene. Construction of the expression plasmids and overproduction and purification of the mutant proteins were also carried out as described previously (8).

Each mutation was confirmed by peptide mapping using *Achromobacter proteus* I and chymotrypsin, followed by amino acid sequence analysis as described previously (10). Protein concentration was determined from UV absorption at 285 nm. The absorption coefficient \(A_{285} = 2.02\) was used assuming that each mutant protein has the same absorption coefficient as that of wild-type *E. coli* RNase HI (10).

RNase H activity was determined by measurement of the radioactivity of the acid-soluble digestion product from the substrate, \(^3\)H-labeled M13 DNA/RNA hybrid, as described previously (11). 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/mg of protein. CD spectra were measured in 10 mM sodium acetate (pH 5.5) containing 0.1 M sodium chloride at 25 °C on a JASCO J-600 spectropolarimeter.

Reversible thermal denaturation curves were determined as previously described (12), by monitoring the CD value at 220 nm as temperature increased by 0.7 °C per min. The buffer conditions were 10 mM glycine-HCl buffer (pH 3.0) containing 1 mM dithiothreitol or 10 mM sodium chloride at 25 °C on a JASCO J-600 spectropolarimeter. Enzymatic activities of mutants varied from 90 to 140% of that of the wild-type *E. coli* RNase HI, as shown in Table I. They were almost indistinguishable within 67% confidence limits (±30% of the mean of measurement). It means that no current mutations affect the enzymatic activity seriously. The CD spectra of all mutant proteins were almost identical to that of the wild-type *E. coli* RNase HI, suggesting that the whole protein structures of mutant proteins remain the same as the wild-type protein. Since the R\textsubscript{6} region is a separated loop apart from the active site as shown

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\textsuperscript{1} The abbreviations used are: RNase H, ribonuclease H; R\textsubscript{6}, region 6 of *E. coli* RNase HI (residues 91–95) defined in Ref. 8.
The enzymatic activities and parameters characterizing the thermal denaturation of wild-type and mutant E. coli RNase H1

The hydrolysis of the M13 DNA/RNA hybrid with the wild-type and mutant E. coli RNase H1 proteins was carried out at 37 °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 within 30%. Relative activity represents the specific activity of each mutant protein relative to that of the wild-type protein.

TABLE I

| Protein          | Relative activity | pH 3.0        | pH 5.5        |
|------------------|-------------------|---------------|---------------|
|                  | %                 | Tm  c         | ΔTm  c        | DH c          | ΔDG  c        | Tm  c         | ΔTm  c        | DH c          | ΔDG  c        |
| Wild-type*       | 100               | 49.8 °C       | 98.1 kcal/mol | 52.0 °C       | 89.0 kcal/mol |
| Rg' RNase H      | 95                | 50.3 °C       | 119.1 kcal/mol| 52.1 °C       | 96.2 kcal/mol |
| E' RNase H       | 100               | 48.6 °C       | 95.9 kcal/mol | 50.4 °C       | 92.9 kcal/mol |
| G' RNase H       | 120               | 55.5 °C       | 102.4 kcal/mol| 58.8 °C       | 90.4 kcal/mol |
| A' RNase H       | 95                | 50.1 °C       | 114.2 kcal/mol| 52.4 °C       | 96.4 kcal/mol |
| N' RNase H       | 90                | 52.7 °C       | 108.7 kcal/mol| 55.2 °C       | 90.0 kcal/mol |
| Rg'/G' RNase H   | 130               | 47.8 °C       | 93.8 kcal/mol | 50.2 °C       | 87.5 kcal/mol |
| Rg'/Gk' RNase H  | 120               | 53.4 °C       | 126.7 kcal/mol| 57.5 °C       | 102.4 kcal/mol|
| Rg'/Gk' RNase H  | 140               | 55.0 °C       | 114.1 kcal/mol| 57.8 °C       | 92.0 kcal/mol |
| Rg'/RNase H      | 90                | 53.8 °C       | 104.8 kcal/mol| 57.6 °C       | 95.9 kcal/mol |

* The melting temperature, Tm, is the midpoint of the thermal denaturation curve.

ΔTm is the difference in the melting temperature between the wild-type and mutant proteins.

ΔH is the difference in the free energy change of unfolding of the mutant protein and that of the wild-type protein at Tm.

ΔΔG is enthalpy change of unfolding, which was derived from van't Hoff analysis. The entropy changes of wild-type protein at Tm were 0.304 kcal/(mol.K) and 0.275 kcal/(mol.K), at pH 3.0 and 5.5, respectively.

These results indicate that among the three amino acid substitutions at the positions of 91, 94, and 95, Lys to Gly is the determinant of high thermostability of the mutant protein Rg' RNase H, which has the same basic protrusion domain as that of T. thermophilus RNase H. In E. coli RNase HI, Lys has the left-handed α-helical structure forming the typical β-hairpin composed of a type-1 β-turn followed by a β-hinge (7) (named as 3:5-type; see Ref. 14). Since the Rg region in E. coli RNase HI protrudes into the solution without any intra-molecular interaction with the other part of the protein, it was considered that this region of each mutant protein with Gly has a left-handed structure similar to that of the wild-type protein, named as %-RNase H (8). However, the double mutant protein Rg'/E' RNase H with the amino acid substitutions of both Lys to Arg and Asp to Glu, decreased thermostability.

The effects of the amino acid substitutions at three different positions, 91, 94, and 95, on Tm are almost cumulative and essentially the same both at pH 3.0 and pH 5.5. Since the structural stability due to the basic protrusion domain inde-

K. Ishikawa, personal communication.
Fig. 2. Thermal denaturation curves of the wild-type and mutant proteins replacing its basic protrusion domain. The apparent fraction of native protein is shown as a function of the temperature. Proteins are as follows: wild-type E. coli RNase HI (●), R\textsuperscript{T} RNase H (□), E\textsuperscript{O} RNase H (+), G\textsuperscript{O} RNase H (○), A\textsuperscript{O} RNase H (×), N\textsuperscript{O} RNase H (△), and R\textsuperscript{O} RNase H (■).

### Table II

Non-glycine and glycine residues at the left-handed conformations in proteins from mesophilic and thermophilic species

| Protein; resolution (Å) and PDB code* | Source* | Sequence† | (θ, ψ)‡° (degree) |
|--------------------------------------|---------|-----------|------------------|
| Alkaline serine protease (21) 1.8 2PRK (22) (protease K) | Triticum album Limber (M) | EFEGR | (58, 33) |
| Neutral protease (23) (aqualysin I) | Thermus aquaticus YT-1 (E) | EFGR | 28 |
| (aqualysin I) | Bacillus cereus (M) | GATIF | |
| Neutral protease (23) | Bacillus subtilis (M) | GTQII | |
| Neutral protease (23) | Bacillus amyloliquefaciens (M) | GTQII | |
| Neutral protease (23) | Bacillus thermoproteolyticus (T) | GSGIF | (106, 160) |
| Neutral protease (23) | Bacillus steatorrhophilus (T) | GSGIF | |
| Malate dehydrogenase (25) 2.5 4MDH (26) | Porcine heart (M) | ARKLS...KDKTY | (65, 11)* (59, 12)* |
| Malate dehydrogenase (25) 2.5 4MDH (26) | Thermus flavus (E) | ARGAS...KDKAY | 15 |
| Malate dehydrogenase (25) 2.5 4MDH (26) | Salmonella typhimurium (M) | DREEG | (41, 68) |
| Malate dehydrogenase (25) 2.5 4MDH (26) | E. coli (M) | EREEG | |
| Malate dehydrogenase (25) 2.5 4MDH (26) | B. steatorrhophilus (T) | SVNPP | |
| Malate dehydrogenase (25) 2.5 4MDH (26) | T. thermophilus HB87 (E) | SEGRA | |

* Only for those proteins for which the crystal structures are available. The numbers on the aligned residues refer to these proteins.
† Characters in parentheses indicate that the source organisms are mesophilic (M), moderate thermophilic (T), or extreme thermophilic (E) species.
‡ Successive 5 amino acid residues indicated by one-letter codes, the center of which has the left-handed conformation in the X-ray structure (indicated by ↓). These sequences are aligned following references (21, 23, 25, 27).
§ The backbone dihedral angles at the center of the 5th residue indicated at the Sequence column.

While for the symmetric wild-type protein (16), it was expected that amino acid substitution Lys\textsuperscript{60} → Asn can stabilize E. coli RNase HI, too. As expected, the thermostability of the single mutant protein N\textsuperscript{O} RNase H was increased by 2.9 and 3.2 °C in T\textsubscript{m} at pH 3.0 and 5.5, respectively. On the contrary, the thermostability of A\textsuperscript{O} RNase H was almost identical to that of the wild-type protein, as shown in Fig. 2 and Table I. The role of the short side chain of Asn in stabilizing the left-handed structure can be its ability to form hydrogen bonding (15).

Nicholson et al. (16) reported results different from our current study. They replaced two left-handed residues in phage T4 lysozyme with Gly, and their mutant proteins showed essentially identical stability to the wild-type protein. In their mutant proteins, some destabilization might occur by losing the intra-molecular hydrogen bonds existing in the wild-type protein (16).

From the present results, the preferences of Gly and Asn for the left-handed conformation can be understood as one of the mechanisms stabilizing protein native conformations. As
shown in Table II, similar putative examples are found in other proteins, where each non-Gly residue in the left-handed structure of a mesophilic species is substituted with Gly in that of thermophilic species. These examples seem to support Nakamura, H., dehara, M., Matsuzaki, T., and Morikawa, K. (1992) J. Mol. Biol. 223, 1029-1052.

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