Stabilization of Ribonuclease HI from *Thermus thermophilus* HB8 by the Spontaneous Formation of an Intramolecular Disulfide Bond†

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Received February 17, 1998; Revised Manuscript Received May 6, 1998

ABSTRACT: To identify factors that contribute to the thermal stability of ribonuclease HI (RNase HI) from *Thermus thermophilus* HB8, protein variants with a series of carboxyl-terminal truncations and Cys → Ala mutations were constructed, and their thermal denaturations were analyzed by CD. The results indicate that Cys^{41} and Cys^{149} contribute to the protein stability, probably through the formation of a disulfide bond. Peptide mapping analysis for the mutant protein with only two cysteine residues, at positions 41 and 149, indicated that this disulfide bond is partially formed in a protein purified from *Escherichia coli* in the absence of a reducing reagent but is fully formed in a thermally denatured protein. These results suggest that the thermal stability of *T. thermophilus* RNase HI, determined in the absence of a reducing reagent, reflects that of an oxidized form of the protein. Comparison of the thermal stabilities and the enzymatic activities of the wild-type and truncated proteins, determined in the presence and absence of a reducing reagent, indicates that the formation of this disulfide bond increases the thermal stability of the protein by 6−7 °C in T_m and ∼3 kcal/mol in ΔG without seriously affecting the enzymatic activity. Since *T. thermophilus* RNase HI is present in a reducing environment in cells, this disulfide bond probably is not formed in vivo but is spontaneously formed in vitro in the absence of a reducing reagent.

Ribonuclease HI (RNase HI),[1] which had been designated as RNase H until a second RNase H (RNase HII) was isolated from *Escherichia coli* (*1*), specifically hydrolyzes the RNA moiety of RNA/DNA hybrids (*2*). We have used the RNases HI from *Thermus thermophilus* HB8 and *E. coli* as a thermophilic/mesophilic protein pair for analyses of the stabilization mechanisms of proteins, because the crystal structures of these enzymes are available (*3*−*6*) and their conformational stabilities can be analyzed thermodynamically (*7*−*9*). The *T. thermophilus* HB8 and *E. coli* RNases HI, which both exist in a monomeric form, are composed of 166 (*9, 10*) and 155 (*11*) amino acid residues, respectively, and the identity of these sequences is 52%. The former is more stable than the latter by 33.9 °C in T_m at pH 5.5 in the presence of 1.2 M GdnHCl, by 11.8 kcal/mol at 25 °C, and by 14.1 kcal/mol at 50 °C in $\Delta G[H_2O]$ (*9*). By replacing the amino acid residues of *E. coli* RNase HI with those from *T. thermophilus* RNase HI (*8, 12, 13*) or by introducing random mutations into the *E. coli* RNase HI sequence (*14*), we have succeeded in identifying four amino acid substitutions that may contribute to the greater stability of *T. thermophilus* RNase HI as compared to that of *E. coli* RNase HI. They are Gly^{23} → Ala, His^{62} → Pro, Val^{74} → Leu, and Lys^{95} → Gly, which increase the stability of *E. coli* RNase HI by 1.8, 4.1, 3.3, and 6.8 °C in T_m, respectively, at pH 5.5 in the presence of 1.0 M GdnHCl. Crystallographic (*13, 15*) and theoretical (*16, 17*) studies of the *E. coli* RNase HI variants with either one of these mutations, except for that with the Gly^{23} → Ala mutation, allowed us to propose that the introduction of the proline residue into the turn region (His^{62} → Pro), the cavity-filling mutation (Val^{74} → Leu), and the replacement of the left-handed helical residue with Gly (Lys^{95} → Gly) are effective to increase the protein stability. In addition, we showed that the thermostabilizing effects of the five mutations, including these four mutations, are additive (*18*). However, the combination of the four stabilizing mutations can increase the stability of *E. coli* RNase HI by at most only 16.0 °C, which is less than half of the difference between the T_m values of the *T. thermophilus* and *E. coli* RNases HI. Additional stabilizing factors, which have yet to be identified, must contribute to the stability of *T. thermophilus* RNase HI.

Our strategy to identify the factors that make *T. thermophilus* RNase HI more stable than *E. coli* RNase HI is to introduce amino acid substitutions into *E. coli* RNase HI and look for thermostabilizing mutants. We have not constructed any mutant *T. thermophilus* RNase HI protein for this purpose. However, because stabilizing forces or interactions are often generated from multiple amino acid interactions, they are not always created by introducing a single amino acid substitution into *E. coli* RNase HI. On the other hand, it may be relatively easier to impair such forces or interactions by introducing a single amino acid substitution into *T. thermophilus* RNase HI. The accumulation of this information will be helpful to identify these stabilizing forces or interactions. In addition, mutational studies of *T. thermophilus* RNase HI may provide an answer to the question as

† This work was supported in part by Grants 08455382 and 09261222 from the Ministry of Education, Science and Culture of Japan.

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[1] Abbreviations: GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; LEP, lysyl endopeptidase; RNase HI, ribonuclease HI; PCR, polymerase chain reaction.

S0006-2960(98)00373-0 CCC: $15.00
Published on Web 08/20/1998

to whether the difference in the in vitro stability between *T. thermophilus* RNase HI and *E. coli* RNase HI reflects the difference in the in vivo stability between them, because protein stability is often affected by modifications in vitro, such as disulfide bond formation.

Proteins from thermophilic sources usually have fewer cysteine residues than their mesophilic counterparts (*19*). However, *T. thermophilus* RNase HI, which contains four cysteine residues, has one more cysteine residue than *E. coli* RNase HI. Therefore, there is a chance that the formation of a disulfide bond enhances the T_m value of this protein. It has been shown that all three cysteine residues in *E. coli* RNase HI exist in reduced forms and that the mutations of these residues to Ala do not seriously affect the enzymatic activity (*20*). Two of the four cysteine residues (Cys^{13} and Cys^{63}) in *T. thermophilus* RNase HI are conserved in the sequences of these enzymes. Cys^{13} is located at the loop between the βA and βB strands and is relatively buried inside the protein molecule. Cys^{63} is located at the loop between the αI helix and the βD strand and is exposed to the solvent. The other two cysteine residues (Cys^{41} and Cys^{149}) are located at the turn between the βC strand and the αI helix and at the C-terminal region, respectively. Since *T. thermophilus* RNase HI is present in a reducing environment, all of the cysteine residues must exist in reduced forms in vivo. Structural and biochemical characterizations of the recombinant protein purified from *E. coli* suggested that these cysteine residues exist in reduced forms in vitro as well (*6*, *9*). Namely, the backbone structure of the protein, which is available for the residues from Arg^2 to Thr^{147}, clearly shows that Cys^{13} and Cys^{63} exist in reduced forms. In addition, because structural disorder in the crystallographic analyses prevented the definition of the carboxyl-terminal (C-terminal) 14 amino acid residues, including Cys^{149}, in the electron density map (*6*), it is unlikely that a disulfide bond is fully formed between Cys^{41} and Cys^{149}. Chemical modification with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) has also suggested that all of the cysteine residues exist in reduced forms (*9*). However, these results cannot rule out the possibility that a disulfide bond is formed between Cys^{41} and Cys^{149} under different conditions, especially those in which thermal denaturation of the protein is examined.

In the C-terminal region of *T. thermophilus* RNase HI, four proline residues are clustered at positions 148, 150, 151, and 154, whereas no proline residues are located at the corresponding region of *E. coli* RNase HI. It has been reported that thermophilic proteins have more proline residues than mesophilic proteins (*21*). In fact, proline residues have been shown to contribute to increasing protein stability when they are located at a β-sheet or a turn/loop region, because the entropy of the unfolded state of the protein is decreased (*22*). Therefore, we decided to introduce a series of truncations into the C-terminal region of *T. thermophilus* RNase HI, to analyze the role of the proline and cysteine residues located in this region in the protein stability.

In this report, we show that none of the proline residues clustered in the C-terminal region of *T. thermophilus* RNase HI contributed to the thermal stability of the protein. However, a disulfide bond formed between Cys^{41} and Cys^{149} accounts for 6−7 °C in T_m and ∼3 kcal/mol in ΔG_m.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes and modifying enzymes for recombinant DNA technology were from Takara Shuzo Co., Ltd. Ultrapure guanidine hydrochloride (GdnHCl) was from ICN Biomedicals Inc. Phosphocellulose (P-11) was from Whatman. Lysyl endopeptidase (LEP) was from Wako Chemical Co., Ltd.

Cells and Plasmids. Plasmid pJAL700T, for the overproduction of *T. thermophilus* RNase HI, was constructed previously (*9*). This plasmid bears the wild-type *rnh* gene under the control of the bacteriophage λ promoters P_R and P_L, the cI^{ts857} gene, and the bacteriophage fd transcription terminator. Competent cells of *E. coli* HB101(F^- *hsd S20 recA13 ara-14 proA2 lacY1 galK2 rpsL20(str) xyl-5 mtl-1 supE44 leuB6 thi-1*) were from Takara Shuzo Co., Ltd.

Mutagenesis. The mutant *rnh* genes, encoding the *T. thermophilus* RNase HI variants with a series of C-terminal truncations, were constructed by PCR using a 5′ primer with an *Nde*I site and 3′ mutagenic primers with *Sal*I sites. The sequence of the 5′ primer was 5′-GCGAATTCCAT*AT-G*AATCCATCACCTAGAAAA-3′, where the initiation codon of the *rnh* gene is shown in italic type and the underlined bases show the position of the *Nde*I site. The 3′ mutagenic primers were designed to replace the codon for the amino acid residue 144, 146, 148, 149, 150, 152, 154, or 156 with the TAA termination codon. Their sequences were 5′-GCGTCGAC*TTA*-$(B)_n$-3′, where the sequence complementary to the termination codon of the *rnh* gene is shown in italic type, and the underlined bases show the position of the *Sal*I site. $(B)_n$ represents the 18−19 bases with the sequences that are complementary to the 3′-terminal region of the *rnh* gene. The truncated proteins are designated as TRNH[Δ*X*], where *X* represents the truncated region at the C-terminus. For example, TRNH[Δ150−161] represents the truncated protein lacking the C-terminal 12 residues from positions 150 to 161.

The mutant *rnh* genes, encoding the mutant proteins, in which single or multiple Cys → Ala mutations were introduced into either the wild-type protein or TRNH[Δ150−161], were constructed by PCR using a 5′ primer with an *Nde*I site, a 3′ primer with a *Sal*I site, and 5′ and 3′ mutagenic primers, as described previously for the construction of the mutant *E. coli* RNase HI proteins (*23*). The sequence of the 5′ primer was mentioned above. The sequences of the 3′ primers were 5′-GCGTCGACCTTAAAGGTGGGGC-TTTCCCGGTCC-3′ for the full-size mutant protein and 5′-GCGTCGACTTAGCAGGGCGTTTTGGCCTGGGAC-3′ for the TRNH[Δ150−161] mutant proteins, where the underlined bases show the positions of the *Sal*I sites. The mutagenic primers were designed so that the codons for Cys^{13} and Cys^{41} were changed from TGC to GCC for Ala, and the codon for Cys^{63} was changed from TGC to GCA for Ala. Since the 3′ primer includes the sequence complementary to the codon for Cys^{149}, the TGC codon for Cys^{149} was changed to GCC, for the construction of the TRNH[Δ150−161] derivatives in which Cys^{149} is replaced by Ala. The mutant *rnh* genes, encoding the TRNH[Δ150−161] variants with a series of substitutions at the C-terminus (Cys^{149}), were constructed by PCR using the 5′ primer mentioned above and the 3′ mutagenic primers with the sequences of 5′-GCGTCGACT-TANNNGGGCGTTTTGGCCTGGGAC-3′, where the un-

derlined bases show the position of the *Sal*I site and NNN represents the sequence complementary to the codon for the mutated residue. The 3′ mutagenic primers were designed so that the TGC codon for Cys^{149} was changed to TCC for Ser, ACC for Thr, GTC for Val, and ATC for Ile. The resultant derivatives of the wild-type protein and TRNH-[Δ150−161] are designated as X′-RNase HI and X′-TRNH-[Δ150−161], respectively, in which X′ represent(s) the amino acid residue(s) at position(s) 13, 41, 63, and/or 149 (one-letter notation). For example, A^{149}-RNase HI represents the mutant protein in which Cys^{149} of the wild-type protein is replaced by Ala, and $A^{13}A^{63}$-TRNH[Δ150−161] represents the mutant protein in which Cys^{13} and Cys^{63} of TRNH-[Δ150−161] are replaced by Ala.

All primers were synthesized by Sawady Technology Co., Ltd. PCR was performed for 25 cycles with a Perkin-Elmer GeneAmp PCR System 2400, using Vent DNA polymerase from New England Biolabs, Inc. All of the nucleotide sequences of the mutant *rnh* genes were confirmed by the dideoxy chain-termination method (*24*). After digestion by *Nde*I and *Sal*I, the PCR fragments were ligated into the *Nde*I−*Sal*I sites of plasmid pJAL700T to construct the expression vectors for the mutant proteins. The overproducing strains were constructed by transforming *E. coli* HB101 with the resultant expression vectors.

Overproduction and Purification. Cultivation of the *E. coli* HB101 transformants with the plasmid pJAL700T derivatives, overproduction of the truncated and mutant proteins, and preparations of the crude extracts (lysates) from cells were carried out as described previously for the wild-type protein (*9*). However, the following purification procedures were slightly modified, as described below. For the purification of the wild-type, truncated, and mutant proteins, except for TRNH[Δ148−161], ammonium sulfate was added to the crude lysates to a concentration of 80%, and the resultant precipitates were collected by centrifugation at 15000*g* for 30 min at 4 °C. They were then suspended in 40 mL of 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA (TE buffer), dialyzed against TE buffer with a dialysis membrane (Size 20) from Viskase Sales Corp., and applied to a column (3 mL) of P-11 equilibrated with TE buffer. After the column was washed with TE buffer, the enzyme was eluted from the column with a linear gradient of NaCl from 0 to 0.5 M in TE buffer. The protein fractions that eluted at an NaCl concentration of approximately 0.3 M were pooled. For the purification of TRNH[Δ148−161], 1 M sodium acetate (pH 5.5) was added to the crude lysate to a final concentration of 20 mM. This solution was applied to a column (3 mL) of P-11 equilibrated with 20 mM sodium acetate (pH 5.5) containing 0.2 M NaCl. After the column was washed with 20 mM sodium acetate (pH 5.5) containing 0.2 M NaCl, the enzyme was eluted from the column with a linear gradient of NaCl from 0.2 to 1 M in 20 mM sodium acetate (pH 5.5). The protein fractions that eluted at an NaCl concentration of approximately 0.7 M were pooled.

The protein concentration was determined from UV absorption, assuming that the mutant proteins obtained in this experiment have the same $A_{280}^{0.1\%}$ value (1.6) as that of the wild-type protein (*9*). The cellular production levels of the mutant (truncated) proteins and their purities were estimated by subjecting whole cell lysates to SDS−PAGE on a 15% polyacrylamide gel (*25*), followed by staining with Coomassie Brilliant Blue. The molecular weight of each truncated protein was estimated by applying an aliquot of the purified sample to a column (1.6 × 100 cm) of Sephacryl S-300 (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The flow rate was 10 mL/h, and 2.5 mL fractions were collected. Bovine serum albumin (67K), ovalbumin (43K), chymotrypsinogen A (25K), and RNase A (13.7K) were also applied individually to the column as standard proteins.

RNase H Activity. The enzymatic activity was basically determined in 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol (2-Me), and 100 μg/mL bovine serum albumin at 30 °C, by measuring the radioactivity of the acid-soluble digestion product from a ^{3}H-labeled M13 RNA/DNA hybrid, as described previously (*7*). The concentration of 2-Me was changed to either 0 or 100 mM when the effect of 2-Me on the enzymatic activity was analyzed.

Circular Dichroism Spectra. The CD spectra were measured on a J-720W automatic spectropolarimeter from Japan Spectroscopic Co., Ltd., at 10 °C in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. For the measurement of the far-ultraviolet (UV) CD spectra (200−260 nm), the protein concentration was approximately 0.13 mg/mL, and a cell with an optical path length of 2 mm was used. For the measurement of the near-UV CD spectra (250−320 nm), the protein concentration was 0.5−1.0 mg/mL and a cell with an optical path length of 10 mm was used. The mean residue ellipticity, θ, which has the units of deg cm^2 dmol^{-1}, was calculated by using an average amino acid molecular weight of 110.

Thermal Denaturation. The thermal denaturation curves and the temperature of the midpoint of the transition, T_m, were determined as described previously (*8*) by monitoring the change in the CD value at 220 nm. The proteins were dissolved in 20 mM sodium acetate (pH 5.5) containing 1 M guanidine hydrochloride (GdnHCl), unless specifically described. The protein concentration was approximately 0.13 mg/mL, and a cell with an optical path length of 2 mm was used. The enthalpy change of unfolding at the T_m (ΔH_m) was calculated by van't Hoff analysis. The difference in the free energy change of unfolding between the mutant and wild-type proteins, at the T_m of the wild-type protein ($\Delta\Delta G_m$), was estimated by the relationship given by Becktel and Schellman (*26*), $\Delta\Delta G_m = \Delta T_m \Delta S_m$. ΔT_m is the change in T_m of a mutant protein relative to that of the wild-type protein. ΔS_m is the entropy change of the wild-type protein at the T_m, which was determined as 0.437 kcal/(mol·K) from four independent experiments with errors of ±0.04 kcal/(mol·K). The theoretical curves for unfolding were drawn on the assumption that the protein unfolds by a two-state mechanism, using the experimentally determined T_m and ΔH_m values, and the ΔCp value of 1.79 kcal/mol determined for *E. coli* RNase HI (*27*).

Peptide-Mapping Analysis. Proteins were digested in 0.1 M Tris-HCl (pH 9.0) at 37 °C for 1 h with lysyl endopeptidase (LEP) at an enzyme/substrate ratio of 1:50. The resultant peptides were separated by reverse-phase HPLC on a Cosmosil ODS column (4.6 mm × 150 mm) from Nacalai tesque, using an HPLC apparatus from Shimadzu LC-10A. The molecular weight of each peptide was analyzed by an on-line mass spectrometer, LCQ from Finnigan MAT, using electrospray ionization.

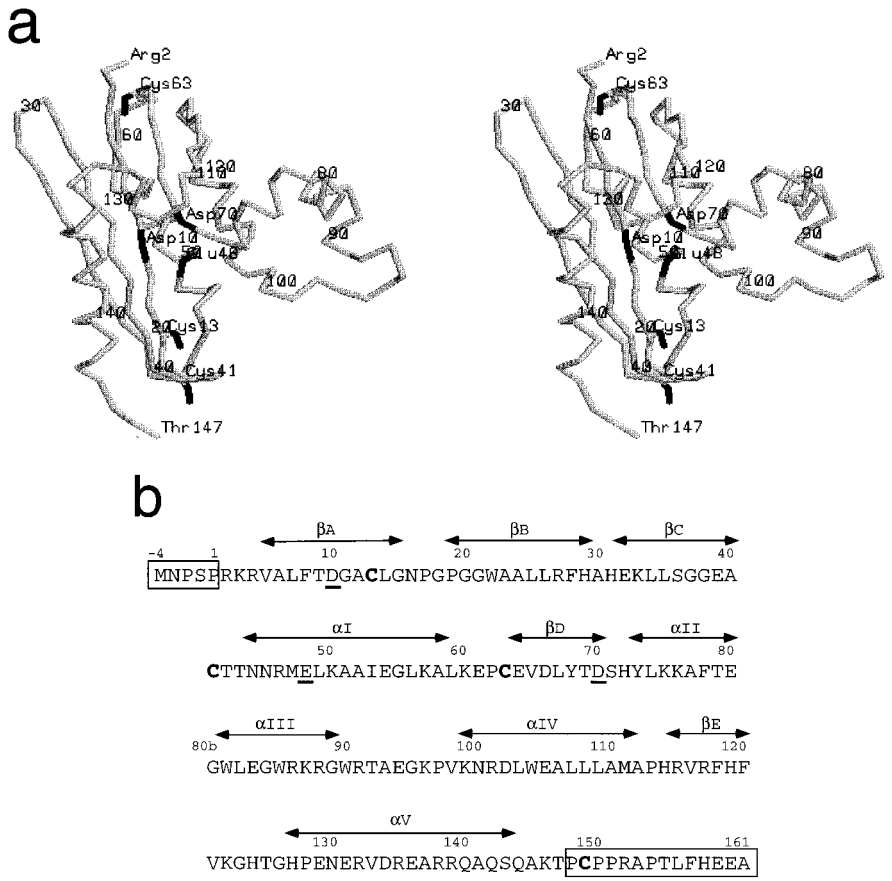

FIGURE 1: Structure of *T. thermophilus* RNase HI. (a) This stereoview of the backbone structure of *T. thermophilus* RNase HI, determined by Ishikawa et al. (*6*), was drawn with the program RasMol. Numbers represent the positions of the amino acid residues, which start from −4 for the initiator methionine of the protein. The initiator methionine starts at −4, because the *T. thermophilus* RNase HI sequence contains an additional four residues at the N-terminus as compared to the *E. coli* RNase HI sequence (*9*). Arg[2] and Thr[147] represent the N- and C-terminal residues in this crystal structure. The side chains of Cys[13], Cys[41], and Cys[63] are indicated by solid lines. The Cα atoms of the active-site residues (Asp[10], Glu[48], and Asp[70]) are also indicated by solid lines. This crystal structure of *T. thermophilus* RNase HI has been deposited in the Brookhaven Protein Data Bank under accession number 1RIL. (b) The amino acid sequence of *T. thermophilus* RNase HI, determined by Kanaya and Itaya (*9*), is shown. The ranges of the five α-helices and the five *β*-strands are shown along the sequence. All four cysteine residues, at positions 13, 41, 63, and 149, are shown in boldface type, and the three catalytically essential carboxylates (Asp[10], Glu[48], and Asp[70]) are underlined. The N-terminal region from Met[−4] to Pro[1] and the C-terminal region from Pro[148] to Ala[161], which have not been defined by crystallographic analyses, probably due to structural disorder, are boxed.

RESULTS

C-Terminal Truncation Design. The backbone structure of *T. thermophilus* RNase HI, which has been determined for the residues from Arg[2] to Thr[147] at 2.8 Å resolution (*6*), as well as the amino acid sequence of the protein, are shown in Figure 1. Since our initial purpose was to understand the role of the C-terminal residues in the protein stability and the enzymatic activity, we decided to construct mutant proteins with a series of C-terminal truncations. The mutant proteins TRNH[Δ148−161]∼TRNH[Δ156−161] were constructed to analyze the roles of the cysteine and proline residues located at the C-terminal region. The mutant proteins TRNH[Δ144−161] and TRNH[Δ146−161] were constructed to analyze the tolerance of the enzyme for the additional C-terminal truncations.

Purification of Truncated Proteins. All of the truncated proteins that were overproduced in *E. coli* HB101 were recovered in a soluble form after sonication lysis, although the more truncated forms showed a tendency to accumulate in an insoluble form (Figure 2). It has previously been shown that the wild-type protein in the crude lysate obtained after sonication lysis binds to P-11 at pH 5.5 in the presence of 8 M urea but does not bind to it in the absence of 8 M urea (*9*). Since the C-terminal truncations may alter a property of the protein, we have examined whether the truncated proteins bound to P-11 in the absence of 8 M urea.

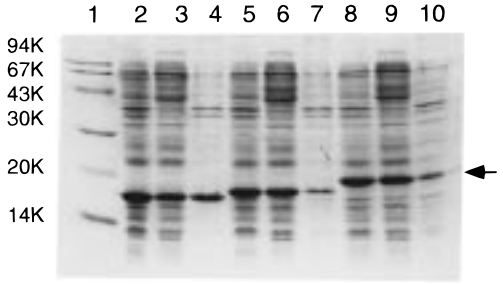

FIGURE 2: Production of truncated proteins in cells. The cellular production levels of the *T. thermophilus* RNase HI variants with the C-terminal truncations were analyzed by SDS−15% PAGE for the wild-type protein (lanes 8−10), TRNH[Δ149−161] (lanes 5−7), and TRNH[Δ144−161] (lanes 2−4). The gel was stained with Coomassie Brilliant Blue. Lanes 2, 5, and 8, whole cell extract; lanes 3, 6, and 9, soluble fraction obtained after sonication lysis; and lanes 4, 7, and 10, insoluble fraction obtained after sonication lysis. Size marker proteins in lane 1 are phosphorylase *b*, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; trypsin inhibitor, 20K; and α-lactalbumin, 14K.

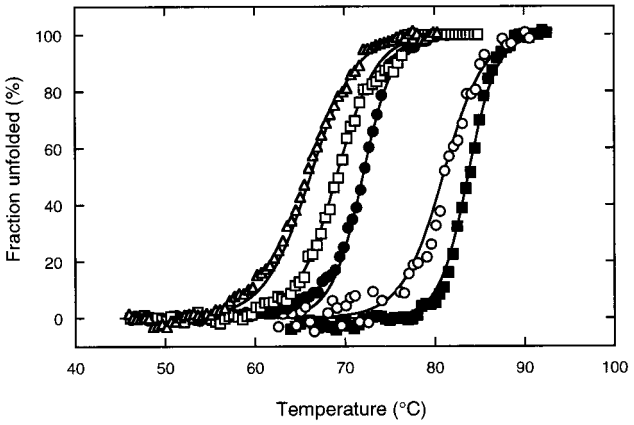

FIGURE 3: Thermal denaturation curves of the wild-type and truncated proteins. The apparent fraction of unfolded protein is shown as a function of temperature. Thermal denaturation curves of all proteins were determined at pH 5.5 in the presence of 1 M GdnHCl by monitoring the change in the CD value at 220 nm, as described in the Experimental Procedures. The curves of the wild-type (■), TRNH[Δ150−161] (○), TRNH[Δ149−161] (●), TRNH-[Δ146−161] (□), and TRNH[Δ144−161] (△) proteins are shown as representatives. The theoretical curves for unfolding were drawn as described in the Experimental Procedures.

Consequently, we found that only TRNH[Δ148−161] bound to P-11 in the absence of 8 M urea. In addition, we found that the wild-type and all of the other truncated proteins bound to it if they had been precipitated by the addition of ammonium sulfate beforehand. The reason TRNH[Δ148−161] effectively bound to P-11, in the absence of 8 M urea and without the ammonium sulfate precipitation, remains to be determined. The purification yields were roughly 25% for the wild-type and all of the truncated proteins. The amounts of the proteins purified from 1-L cultures gradually decreased as the size of the truncated region increased and ranged from 11 to 22 mg for these proteins. Gel filtration with Sephacryl S-300 indicated that all of the truncated proteins existed in a monomeric form, like the wild-type protein.

Stability and Activity of Truncated Proteins. To analyze the effects of the C-terminal truncations on the protein stability, the thermal denaturation curves were measured by monitoring the change in the CD values at 220 nm (Figure 3). Since we constructed the truncated proteins of *T. thermophilus* RNase HI to identify the factors that make it more stable than *E. coli* RNase HI, and we have analyzed the stabilities of a variety of the thermostabilizing mutants of *E. coli* RNase HI in the presence of 1 M GdnHCl at pH 5.5, we analyzed the stabilities of the *T. thermophilus* RNase HI mutants under these conditions. The enzyme was shown to reversibly unfold in a single cooperative fashion under these conditions, with a T_m value of 83.7 °C. This value was 1.6 °C higher than that previously reported, because the thermal denaturation curve of the enzyme was previously measured at pH 5.5 in the presence of 1.2 M GdnHCl, instead of 1 M GdnHCl (*9*). The thermodynamic parameters of the truncated proteins, as well as their enzymatic activities relative to that of the wild-type protein, are summarized in Table 1. The truncated protein TRNH[Δ150−161] was less stable than the wild-type protein by only 2.8 °C in T_m and 1.2 kcal/mol in ΔG_m, whereas TRNH[Δ149−161] was less stable than the wild-type protein by 11.7 °C in T_m and 5.1 kcal/mol in ΔG_m. The enzymatic activities of these truncated proteins were 92% and 74% of that of the wild-type enzyme. These results indicated that C-terminal truncations of up to 12 residues did not seriously affect either the protein stability or the enzymatic activity, but the additional truncation of Cys^{149} considerably decreased the protein stability without seriously affecting the enzymatic activity. Since the stabilities and the enzymatic activities of the truncated proteins gradually decreased as the number of truncated residues increased beyond 13, but those of TRNH[Δ144−161] were comparable with those of TRNH[Δ149−161], further truncations of up to five residues again did not affect either the protein stability or the enzymatic activity. The far- and near-UV CD spectra of the truncated proteins suggested that C-terminal truncations of up to 18 residues did not seriously influence the secondary and tertiary structures of the protein (Figure 4). These results indicate that none of the four proline residues clustered in the C-terminal region contributes to either the enzymatic activity or the thermal stability of the protein. In contrast, Cys^{149} contributes greatly to the protein stability.

Table 1: Enzymatic Activities and Thermodynamic Parameters of Mutant Proteins with C-Terminal Truncations[a]

protein	relative activity (%)	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	$\Delta\Delta G_m$ (kcal/mol)
WT	100	83.7		155.3	
TRNH[Δ156−161]	100	83.3	−0.4	94.5	−0.17
TRNH[Δ154−161]	100	82.0	−1.7	105.2	−0.74
TRNH[Δ152−161]	92	80.0	−3.7	89.6	−1.62
TRNH[Δ150−161]	92	80.9	−2.8	109.0	−1.22
TRNH[Δ149−161]	74	72.0	−11.7	135.0	−5.11
TRNH[Δ148−161]	69	68.1	−15.6	87.4	−6.82
TRNH[Δ146−161]	70	69.1	−14.6	104.4	−6.38
TRNH[Δ144−161]	45	66.7	−17.0	86.4	−7.43

[a] The enzymatic activity was determined at 30 °C for 15 min, in 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl, 100 μg/mL bovine serum albumin, and 1 mM 2-mercaptoethanol, by using an M13 RNA/DNA hybrid as a substrate. The specific activity of the wild-type protein (WT) was ∼2.0 units/mg under these conditions. The relative activity was calculated by dividing the enzymatic activity of the mutant protein by that of the wild-type protein. The melting temperature, T_m, is the temperature of the midpoint of the thermal-denaturation transition. The difference in the melting temperature between the wild-type and mutant proteins (ΔT_m) was calculated as T_m(mutant) − T_m(WT). ΔH_m is the enthalpy change of unfolding at T_m, which was calculated by van't Hoff analysis. The difference in the free energy change of unfolding of the mutant proteins and that of the wild-type protein, at the T_m of the wild-type protein ($\Delta\Delta G_m$), was estimated by the relationship given by Becktel and Schellman (*26*), as described in the Experimental Procedures. The ΔS_m value of 0.437 kcal/(mol·K), which was determined from four independent experiments with errors of ±0.04 kcal/(mol·K), was used for the calculation of the $\Delta\Delta G_m$ values. Errors are within 20% for the enzymatic activity, ±0.5 °C for T_m, ± 15 kcal/mol for ΔH_m, and ±0.2 kcal/mol for $\Delta\Delta G_m$.

Stabilities and Activities of Cys Mutants. To examine whether Cys^{149} contributes to the protein stability by forming a disulfide bond, the TRNH[Δ150−161] variants with single or multiple Cys → Ala mutations, as well as A^{149}-RNase HI, in which Cys^{149} of the wild-type protein is replaced by Ala, were constructed. If a disulfide bond involving Cys^{149} as one partner contributed to the protein stability, then the mutation of a cysteine residue that is the other partner would reduce the protein stability. The purification procedures for the Cys mutants were identical to those for the parent proteins, and the amounts of the mutant proteins purified

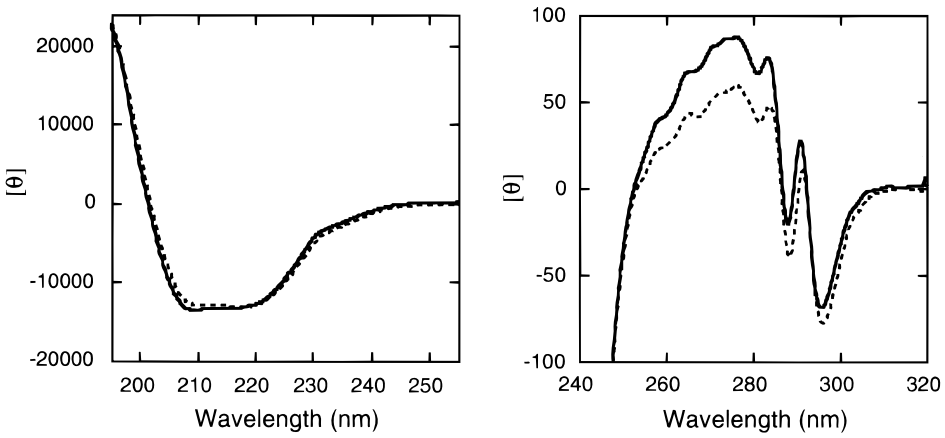

FIGURE 4: CD spectra of the wild-type and truncated proteins. The CD spectra of the wild-type and all of the truncated proteins were measured as described in the Experimental Procedures. The far-UV (left) and near-UV (right) CD spectra of the wild-type protein (solid line) and TRNH[Δ144−161] (broken line) are shown. The spectra of all of the other truncated proteins are slightly different from those of the wild-type protein, but to a lesser extent than those of TRNH[Δ144−161].

Table 2: Enzymatic Activities and Thermodynamic Parameters of the *T. thermophilus* RNase HI and TRNH[Δ150−161] Variants[a]

protein	relative activity (%)	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	$\Delta\Delta G_m$ (kcal/mol)
WT	100	83.7		155.3	
WT (reduced)[b]	100	77.4	−6.3	94.3	−2.75
A^{149}-RNase HI	100	74.7	−9.0	117.0	−3.93
TRNH[Δ150−161]	100	80.9		109.0	
A^{13}-TRNH[Δ150−161]	17	80.1	−0.8	106.8	−0.35
A^{41}-TRNH[Δ150−161]	92	71.7	−9.2	143.4	−4.02
A^{63}-TRNH[Δ150−161]	99	78.8	−2.1	112.4	−0.92
A^{149}-TRNH[Δ150−161]	87	72.5	−8.4	135.6	−3.67
$A^{13}A^{63}$-TRNH[Δ150−161]	11	78.5	−2.4	110.2	−1.05
$A^{13}A^{63}$-TRNH[Δ150−161] (oxidized)[c]	9	78.5	−2.4	112.3	−1.05
$A^{13}A^{63}$-TRNH[Δ150−161] (reduced)[b]	11	71.6	−9.3	93.4	−4.06
$A^{13}A^{41}A^{63}A^{149}$-TRNH[Δ150−161]	18	68.9	−12.0	124.6	−5.24
S^{149}-TRNH[Δ150−161]	129	73.1	−7.8	121.1	−3.41
I^{149}-TRNH[Δ150−161]	115	74.6	−6.3	125.1	−2.75
T^{149}-TRNH[Δ150−161]	168	75.0	−5.9	126.5	−2.58
V^{149}-TRNH[Δ150−161]	139	75.8	−5.1	139.0	−2.23

[a] The enzymatic activities and the thermal stabilities of the *T. thermophilus* RNase HI and TRNH[Δ150−161] variants were determined as described for Table 1. The relative activity of the *T. thermophilus* RNase HI variant (A^{149}-RNase HI) was calculated by dividing the enzymatic activity of this mutant protein by that of the wild-type protein. The relative activities of the TRNH[Δ150−161] variants were calculated by dividing the enzymatic activity of the mutant protein by that of TRNH[Δ150−161]. Likewise, the ΔT_m value was calculated as T_m(mutant) − T_m(WT) for A^{149}-RNase HI and as T_m(mutant) − T_m(TRNH[Δ150−161]) for the TRNH[Δ150−161] variants. The $\Delta\Delta G_m$ values were calculated by multiplying the ΔS_m value of 0.437 kcal/(mol·K) with the ΔT_m values. Errors are the same as those described in the legend for Table 1. [b] The enzymatic activity was determined in the presence of 100 mM 2-Me and the thermal denaturation curve was measured in the presence of 20 mM 2-Me.[c] The protein was thermally denatured and then renatured at room temperature. The enzymatic activity was determined in the absence of 2-Me.

from 1-L cultures were roughly the same as those of the parent proteins. The stabilities and the activities of these Cys mutants are summarized in Table 2. The mutation of Cys^{41} or Cys^{149} in TRNH[Δ150−161] decreased the protein stability by 8.4−9.2 °C in T_m (3.7−4.0 kcal/mol in ΔG_m), whereas the mutation of either Cys^{13} or Cys^{63} decreased it by only 0.8−2.1 °C in T_m (0.4−0.9 kcal/mol in ΔG_m). Similar phenomena were observed for the TRNH[Δ150−161] variants with multiple Cys → Ala mutations. Namely, the double mutant protein $A^{13}A^{63}$-TRNH[Δ150−161], which contains only two cysteine residues (Cys^{41} and Cys^{149}) was less stable than TRNH[Δ150−161] by only 2.4 °C in T_m (1.1 kcal/mol in ΔG_m), whereas the Cys-free mutant protein $A^{13}A^{41}A^{63}A^{149}$-TRNH[Δ150−161] was less stable than it by 12.0 °C in T_m (5.2 kcal/mol in ΔG_m). The difference in T_m between $A^{13}A^{63}$-TRNH[Δ150−161] and $A^{13}A^{41}A^{63}A^{149}$-TRNH[Δ150−161] (9.6 °C) was comparable to that between TRNH[Δ150−161] and A^{41}-TRNH[Δ150−161] or A^{149}-TRNH[Δ150−161]. In addition, A^{149}-RNase HI was less stable than the wild-type protein by 9.0 °C in T_m (3.9 kcal/mol in ΔG_m). These results strongly suggest that the disulfide bond formed between Cys^{41} and Cys^{149} contributes equally to increasing the stabilities of both TRNH[Δ150−161] and the wild-type protein. Among the various Cys mutants, only those in which Cys^{13} was replaced by Ala had considerably less enzymatic activity than the wild-type protein, suggesting that only Cys^{13} is important for the enzymatic activity.

Disulfide Bond Identification. To identify a putative disulfide bond in either the wild-type protein or TRNH-[Δ150−161], peptide-mapping analyses were carried out. When these proteins were digested by lysyl endopeptidase (LEP) at pH 9.0, peptides with a variety of disulfide bonds, including Cys^{13}−Cys^{13}, Cys^{41}−Cys^{41}, Cys^{63}−Cys^{63}, and Cys^{149}−Cys^{149}, were produced (data not shown). In addition, all four kinds of peptides with reduced cysteine residues were produced. These results indicate that the wild-type protein and the TRNH[Δ150−161] mutant protein contain a disulfide bond, but the peptides with this disulfide bond are not stable, because of the acceleration of the sulfhydryl/disulfide exchange reactions upon proteolysis. Attempts to digest the protein with LEP or other proteases at a pH lower than 7, which would suppress the sulfhydryl/disulfide exchange reactions, have thus far been unsuccessful, because the protein is not effectively cleaved under these conditions. Therefore, we decided to digest $A^{13}A^{63}$−TRNH[Δ150−161] with LEP. Since $A^{13}A^{63}$−TRNH[Δ150−161] contains only two cysteine residues, at positions 41 and 149, the sulfhydryl/disulfide exchange reactions occur only when the disulfide bond is partially formed between these cysteine residues.

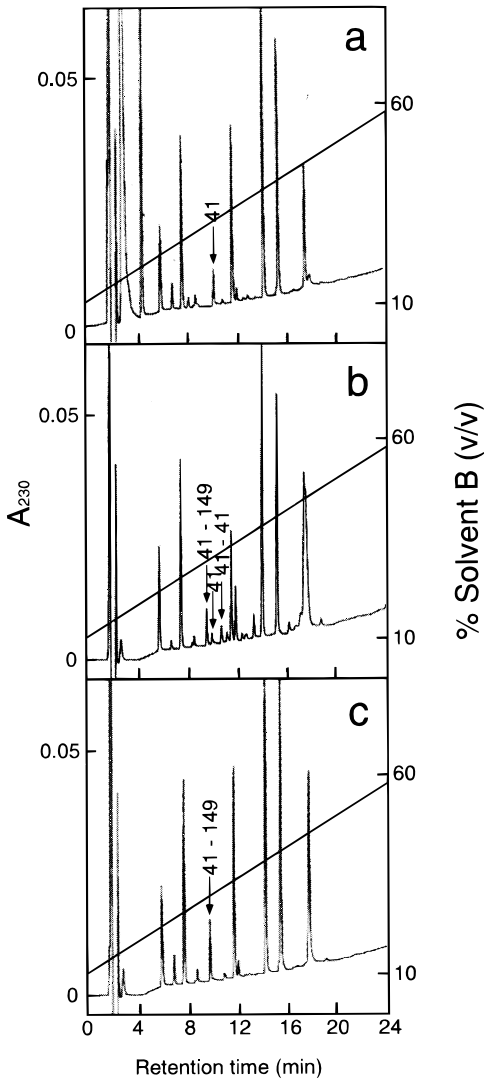

FIGURE 5: Separation of lysyl endopeptidase digests of $A^{13}A^{63}$-TRNH[Δ150−161] in reduced and oxidized forms by reverse-phase HPLC. The mutant protein $A^{13}A^{63}$-TRNH[Δ150−161], purified from *E. coli*, was digested by lysyl endopeptidase (LEP) in the presence (a) or absence (b) of 1 mM 2-Me, as described in the Experimental Procedures. Likewise, $A^{13}A^{63}$-TRNH[Δ150−161], which was thermally denatured and then renatured at room temperature, was digested by LEP (c). The resultant LEP digests (0.5 nmol) were applied to a Cosmosil ODS column (4.6 × 150 mm) equilibrated with 10% (v/v) solvent B in solvent A. Elution was performed by a linear increase of the concentration of solvent B in solvent A from 10% to 60% (v/v) in 25 min. Solvent A was aqueous 0.1% (v/v) trifluoroacetic acid, and solvent B was acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1.0 mL/min. The peptides were detected by UV at 230 nm. The molecular weight of each peptide was determined by on-line mass spectrometry. The straight line represents the concentration of solvent B. All of the peptides that were eluted from the column were identified, and only the peptides with a reduced Cys^{41}, a disulfide bond between two Cys^{41}, and that between Cys^{41} and Cys^{149} are shown by arrows, which are labeled 41, 41−41, and 41−149, respectively.

When it was digested by LEP and the resultant peptides were analyzed by reverse-phase HPLC, the peptide with reduced Cys^{41} and the peptides with the disulfide bond between Cys^{41} and Cys^{41} and between Cys^{41} and Cys^{149} were detected (Figure 5b). In this case, the peptide with reduced Cys^{149} and that with the disulfide bond between Cys^{149} and Cys^{149} should be generated by the LEP digestion, but they must elute in the flowthrough fraction of the reverse-phase column, probably because of their poor hydrophobicities. In contrast, when $A^{13}A^{63}$-TRNH[Δ150−161], which was thermally denatured and then renatured at mild temperatures, was digested by LEP, only the peptide with the disulfide bond between Cys^{41} and Cys^{149} was detected (Figure 5c). These results suggest that the disulfide bond between Cys^{41} and Cys^{149} was partially (∼80%) formed in $A^{13}A^{63}$-TRNH-[Δ150−161], when the protein was purified in the absence of a reducing reagent, but was fully formed once the protein was thermally denatured. When $A^{13}A^{63}$-TRNH[Δ150−161] was digested by LEP in the presence of a reducing reagent, only the peptide with reduced Cys^{41} was detected, as expected (Figure 5a). Attempts to isolate $A^{13}A^{63}$-TRNH-[Δ150−161] in a reduced form have thus far been unsuccessful, because it is unstable in the absence of a reducing reagent and is gradually converted to an oxidized form when the reducing reagent is removed. Since the enzymatic activities of the reduced and oxidized forms of $A^{13}A^{63}$-TRNH[Δ150−161], relative to that of the parent truncated protein, were similar to each other (Table 2), the formation of the disulfide bond between Cys^{41} and Cys^{149} did not seriously affect the enzymatic activity.

Stability in the Presence of a Reducing Reagent. To examine whether the cleavage of the disulfide bond formed between Cys^{41} and Cys^{149} influences the protein stability, thermal denaturation curves for the wild-type protein, $A^{13}A^{63}$-TRNH[Δ150−161], and $A^{13}A^{41}A^{63}A^{149}$-TRNH[Δ150−161] were measured in the presence of 20 mM 2-Me. The addition of 2-Me decreased the stabilities of the wild-type protein and $A^{13}A^{63}$-TRNH[Δ150−161] by 6.3 and 6.9 °C in T_m (2.8 and 3.0 kcal/mol in ΔG_m), respectively (Table 2), but did not seriously affect the stability of $A^{13}A^{41}A^{63}A^{149}$-TRNH[Δ150−161]. These results suggest that the disulfide bond formed between Cys^{41} and Cys^{149} increases the protein stability by 6−7 °C in T_m and ∼3 kcal/mol in ΔG_m.

Stabilities of the TRNH[Δ150−161] Variants at Cys^{149}. The mutation of Cys^{41} or Cys^{149} to Ala decreased the protein stability by ∼9 °C in T_m and ∼4 kcal/mol in ΔG_m, whereas the cleavage of the disulfide bond by the addition of 2-Me decreased it by only 6−7 °C in T_m and ∼3 kcal/mol in ΔG_m. These results suggest that the thiol groups of these cysteine residues contribute to the protein stability by 2−3 °C in T_m and ∼1 kcal/mol in ΔG_m. To clarify the mechanism by which the thiol group increases the protein stability, mutant proteins of TRNH[Δ150−161], in which Cys^{149} was replaced by several other amino acid residues, were constructed and their thermal denaturations were analyzed. The results are summarized in Table 2. The decrease in the protein stability by the mutation of Cys^{149} to Val, Thr, or Ile (5−6 °C in T_m and 2.2−2.6 kcal/mol in ΔG_m) was comparable to that due to the cleavage of the disulfide bond, whereas the decrease in the protein stability by the mutation of Cys^{149} to Ser (7.8 °C in T_m and 3.4 kcal/mol in ΔG_m) was comparable to that due to the mutation of Cys^{149} to Ala. A characteristic common to the structures of Val, Thr, and Ile is that they have nonpolar methyl groups at the γ position. Therefore, these results suggest that the thiol group of Cys^{149} can be replaced by a methyl group without seriously affecting the protein stability. The thiol group of Cys^{149} may contribute to the protein stability by 2−3 °C in T_m and ∼1 kcal/mol in ΔG_m by a hydrophobic effect. The enzymatic activities of

these TRNH[Δ150−161] derivatives ranged from 87% to 168% of that of TRNH[Δ150−161]. It remains to be determined why all of these mutant proteins, except for A^{149}-TRNH[Δ150−161], are more active than TRNH[Δ150−161].

DISCUSSION

Stabilization Mechanisms of T. thermophilus RNase HI. We have previously shown that the sum of the local stabilizing forces or interactions, which are independent of one another, accounts for half of the difference in the in vitro stability between the *T. thermophilus* and *E. coli* RNases HI (*8, 18*). In this study, we showed that a disulfide bond formed between Cys^{41} and Cys^{149} accounts for 6−7 °C in T_m and ∼3 kcal/mol in ΔG_m, which is roughly one-fifth of the difference in the in vitro stability between these proteins. Since the T_m value of *E. coli* RNase HI is 52.0 °C at pH 5.5 in the presence of 1 M GdnHCl (*8*), the difference in the in vitro stability between *T. thermophilus* RNase HI and *E. coli* RNase HI is 31.7 °C in T_m under these conditions. In addition, it has been reported that *T. thermophilus* RNase HI is more stable than *E. coli* RNase HI, by 11.8 kcal/mol in $\Delta G[H_2O]$ at 25 °C (*9*). Therefore, the difference in the in vivo stability between these proteins must be ∼25 °C in T_m and 8.8 kcal/mol in $\Delta G[H_2O]$ at 25 °C. This means that sum of the stabilizing factors identified thus far (16 °C in T_m and 4.4 kcal/mol in ΔG_m) (*18*) already accounts for two-thirds of the difference in the in vivo stability between these proteins, if the difference in the T_m values is compared. The difference in the ΔG_m value between *E. coli* RNase HI and its variant with the higher T_m value of 16 °C (4.4 kcal/mol) may be underestimated, because it was calculated by using the ΔS_m value of 0.275 kcal/mol determined for *E. coli* RNase HI (*18*), which is much lower than that (0.437 kcal/mol) determined for *T. thermophilus* RNase HI. Further mutagenesis studies may allow us to identify additional interactions that can account for the remaining difference in the in vivo stability between these proteins, and would thereby facilitate an understanding of the stabilization mechanisms of thermophilic proteins.

Stabilization by Disulfide Bond. The disulfide bond is one of the structural elements of proteins. It is usually involved in protein stabilization (*28*), predominantly by reducing the entropy of the unfolded state of proteins (chain entropy effect) (*29*−*31*). It has been reported that the conformational entropy of the unfolded protein is decreased in proportion to the increase in the natural logarithm of the number of residues in the loop forming the disulfide bond (*32, 33*). We showed that the formation of the disulfide bond between Cys^{41} and Cys^{149} increased the protein stability by 6−7 °C in T_m. This value is comparable to those reported for the mutant proteins of T4 lysozyme, in which a single artificial disulfide bond was introduced into various positions, so that the sizes of the loop formed by the cross-link ranged from 27 to 155 amino acid residues (*33*). However, we should note that the introduction of an artificial intramolecular disulfide bond can even stabilize a protein isolated from a hyperthermophile, which is originally highly resistant to thermal denaturation. Since the formation of the disulfide bond between Cys^{41} and Cys^{149} in *T. thermophilus* RNase HI did not seriously affect the enzymatic activity, the oxidized form of this protein might be more useful than the reduced form as a tool for recombinant DNA and RNA technologies.

Identification of a Disulfide Bond in $A^{13}A^{63}$-TRNH[Δ150−161]. Peptide mapping analysis is often used to identify a disulfide bond in a protein molecule. However, it is not straightforward if the protein contains both reduced and oxidized cysteine residues, as *T. thermophilus* RNase HI does, because the cysteine residues involved in the disulfide bond are rapidly exchanged with the reduced cysteine residues upon proteolysis. In this case, the reduced cysteine residues should be chemically modified by a thiol-blocking reagent to prevent the sulfhydryl/disulfide exchange reaction. However, Cys^{13} of *T. thermophilus* RNase HI in the native state, which should be a reduced form, was not effectively modified, probably because this residue is relatively well buried inside the protein molecule (data not shown). Therefore, we constructed $A^{13}A^{63}$-TRNH[Δ150−161], which contains only two cysteine residues at positions 41 and 149, to provide conclusive evidence that a disulfide bond is formed between these residues. Identification of both the reduced and oxidized forms of $A^{13}A^{63}$-TRNH[Δ150−161] by peptide mapping analysis (Figure 5b) suggests that the disulfide bond between Cys^{41} and Cys^{149} is not formed in vivo but is spontaneously formed in vitro during the purification process in the absence of a reducing reagent. A similar phenomenon has been observed for elongation factor Ts from *T. thermophilus* (*34, 35*). An intermolecular disulfide bond, which contributes to increasing the thermal stability of this protein without seriously affecting the enzymatic activity (*34*), is not formed in vivo but is formed in vitro in the absence of a reducing reagent (*35*).

Possible Involvement of cis−trans Isomerization of Pro^{148} in the Formation of a Non-Cross-Linked Protein. We have previously shown that *T. thermophilus* RNase HI exists in a reduced form in the absence of a reducing reagent and urea, when it was purified in the presence of 8 M urea (*9*). As analyzed by CD, the secondary structure of this protein is not denatured in the presence of 8 M urea (*9*). However, because the backbone structure of the C-terminal region of this protein has not been defined by crystallographic analyses (*6*), this region may be locally unfolded in the presence of 8 M urea and kept unfolded even when urea is removed. Since this region starts from Pro^{148}, Pro^{148} may assume a *cis* conformation, and *cis−trans* isomerization of this residue may create structural disorder in this region, thereby disturbing the formation of a disulfide bond between Cys^{41} and Cys^{149}. It has been reported that *cis−trans* isomerization of Pro often creates an additional, very slow phase in the kinetic processes of protein unfolding and refolding (*36*−*38*). Crystallographic studies of the wild-type protein in an oxidized form, with a single disulfide bond between Cys^{41} and Cys^{149}, will be necessary to prove this hypothesis.

The existence of two forms of *T. thermophilus* RNase HI evokes the question as to whether the previously measured thermal denaturation curve represents that of the protein in a reduced or oxidized form. However, because the thermal denaturation curve of the protein in a reduced form was nearly identical to that of the protein in an oxidized form (data not shown), the thermal denaturation curve of the protein in a reduced form must reflect that of the protein in an oxidized form. The thermal denaturation curve was measured by monitoring the change in the CD values as the

temperature was increased. Therefore, the formation of the disulfide bond between Cys^{41} and Cys^{149} may be accelerated at high temperatures and completed prior to the initiation of thermal denaturation, probably due to a dramatic increase in the rate of *cis*−*trans* isomerization of Pro^{148}. In fact, the peptide mapping analysis for $A^{13}A^{63}$-TRNH[Δ150−161] indicated that the disulfide bond was fully formed between Cys^{41} and Cys^{149} in a thermally denatured protein (Figure 5c).

Enzymatic Activities of Cys Mutants. The mutation of Cys^{13} to Ala considerably decreased the enzymatic activity of *T. thermophilus* RNase HI. It has previously been shown that this mutation increased the K_m value of *E. coli* RNase HI by 13.5-fold but did not seriously alter its k_{cat} value (*39*), suggesting that this mutation impairs the substrate binding of *E. coli* RNase HI without seriously affecting the catalytic efficiency. Therefore, it is unlikely that Cys^{13} is involved in the catalytic function of *T. thermophilus* RNase HI. Instead, as proposed for Cys^{13} of *E. coli* RNase HI (*39*), the main-chain carbonyl oxygen of this residue may be engaged in substrate binding through hydrogen bonding. The mutation of Cys^{13} to Ala may affect the substrate binding of *T. thermophilus* RNase HI more profoundly than that of *E. coli* RNase HI and thereby decrease its enzymatic activity. It is notable that $A^{13}A^{41}A^{63}A^{149}$-TRNH[Δ150−161] is more active than the reduced form of $A^{13}A^{63}$-TRNH[Δ150−161] by ∼50% (Table 2). This observation was surprising, because both of the single mutations of Cys^{41} to Ala and Cys^{149} to Ala slightly decreased the enzymatic activity of TRNH-[Δ150−161]. Since Cys^{41} is located relatively close to Cys^{13}, the decrease in the enzymatic activity caused by the mutation of Cys^{13} to Ala may be compensated, to some extent, by the conformational change caused by the mutation of Cys^{41} to Ala. Further mutagenesis studies will be required to understand the mechanism by which $A^{13}A^{41}A^{63}A^{149}$-TRNH-[Δ150−161] exhibited the unexpectedly high enzymatic activity.

ACKNOWLEDGMENT

We thank Dr. M. Oobatake for helpful discussions.

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BI9803731