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Proline Effect on the Thermostability and Slow Unfolding of a Hyperthermophilic Protein

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Ribonuclease HII from hyperthermophile *Thermococcus kodakaraensis* (Tk-RNase HII) is a robust monomeric protein under kinetic control, which possesses some proline residues at the N-terminal of α-helices. Proline residue at the N-terminal of an α-helix is thought to stabilize a protein. In this work, the thermostability and folding kinetics of Tk-RNase HII were measured for mutant proteins in which a proline residue is introduced (Xaa to Pro) or removed (Pro to Ala) at the N-terminal of α-helices. In the folding experiments, the mutant proteins examined exhibit little influence on the remarkably slow unfolding of Tk-RNase HII. In contrast, E111P and K199P exhibit some thermostabilization, whereas P46A, P70A and P174A have some thermodestabilization. E111P/K199P and P46A/P70A double mutations cause cumulative changes in stability. We conclude that the proline effect on protein thermostability is observed in a hyperthermophilic protein, but each proline residue at the N-terminal of an α-helix slightly contributes to the thermostability. The present results also mean that even a natural hyperthermophilic protein can acquire improved thermostability.

Key words: folding, hyperthermophilic protein, proline residue, ribonuclease HII, stability.

Abbreviations: ASA, accessible surface area; CD, circular dichroism; GdnHCl, Guanidine hydrochloride; Pho, Pyrococcus holikoshii; RNase, ribonuclease; Tk, Thermococcus kodakaraensis.

Proteins from hyperthermophiles are usually more stable than those from organisms that grow at moderate temperatures (*1*). It is expected that studies of proteins from hyperthermophiles will provide general or additional insights into forces stabilizing the native conformation of proteins (*2*). Studies on protein stability using hyperthermophilic proteins, however, are far fewer than those using proteins at normal temperature because of the limitation of model proteins that must possess the characteristics of a monomer, as well as reversible and two-state unfolding. It has also been reported that the unfolding is dramatically slower for hyperthermostable proteins than for their mesostable homologs (*3–9*). The molecular mechanism of the slow unfolding is not yet clear. Recently, Dong *et al*. (*10*) demonstrated that the hydrophobic effect is one practical cause of the slow unfolding of ribonuclease HII from the hyperthermophilic archaeon, *Thermococcus kodakaraensis* (Tk-RNase HII) using systematic hydrophobic mutant proteins.

Tk-RNase HII is a monomeric protein with 228 amino acid residues (*11*). The crystal structures of Tk-RNase HII and its several variants have been determined (*12–14*). Tk-RNase HII is highly stable with a reversible unfolding reaction against heat- and chemical-denaturation (*9*). Its stabilization mechanism is characterized by its remarkably slow unfolding (*9*, *10*, *15*, *16*). As compared with amino acid sequences among homologous proteins, there are some proline residues at the N-terminal of the α-helix in RNase HII from hyperthermophiles, including Tk-RNase HII (Fig. 1A). This suggests that the proline residues may also impart robustness to Tk-RNase HII.

Proline residues decrease the conformational entropy of the denatured state and consequently lead to protein stabilization (*17*). Introduction of proline residue sometimes increases protein stability (*18–26*). In particular, proline residue at specific sites such as the N-terminus of an α-helix is often found (*27*) and is more effective for protein thermostabilization (*28–30*). From sequence comparison among homologous proteins with different thermostability, proline residues are more ubiquitous at α-helix N-termini in more stable proteins (*31*). This evidence suggests that proline residue at the N-terminal of an α-helix contributes to the thermostabilization of hyperthermophilic proteins. However, few studies have systematically examined the proline effect in hyperthermophilic protein.

In this work, we focused on the proline residues at the α-helix N-termini in Tk-RNase HII. To analyse the contribution of the proline residues to the thermostability and folding of the hyperstable protein, we constructed seven single-mutant proteins of Tk-RNase HII with Xaa to Pro (L80P, E111P, K199P) or Pro to Ala (P46A, P70A, P139A, P174A) and two double-mutant proteins (E111P/K199P, P46A/P70A). The mutation sites are located at

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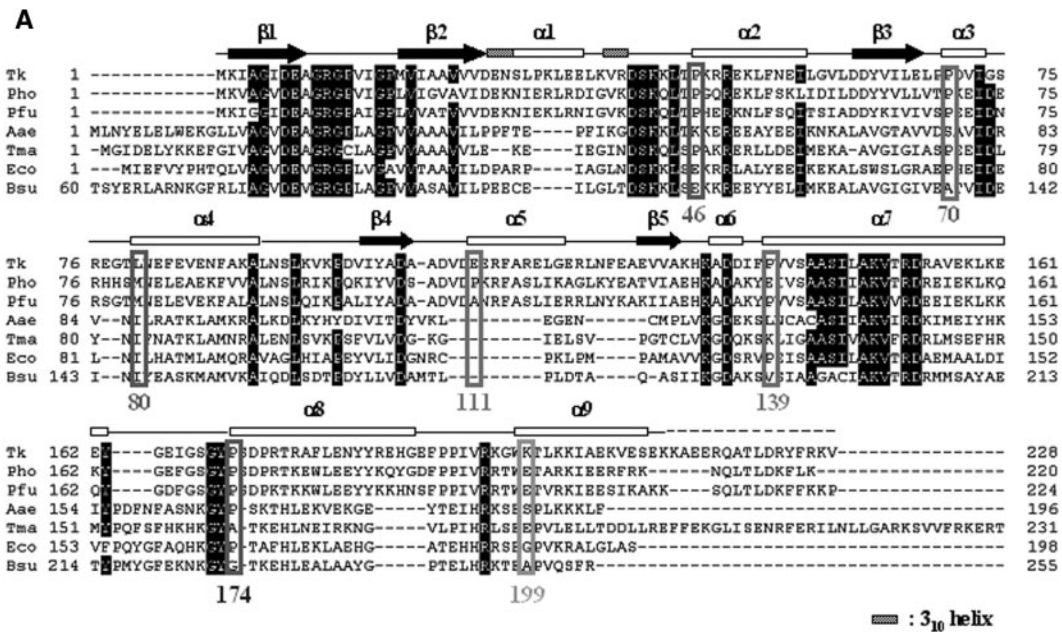

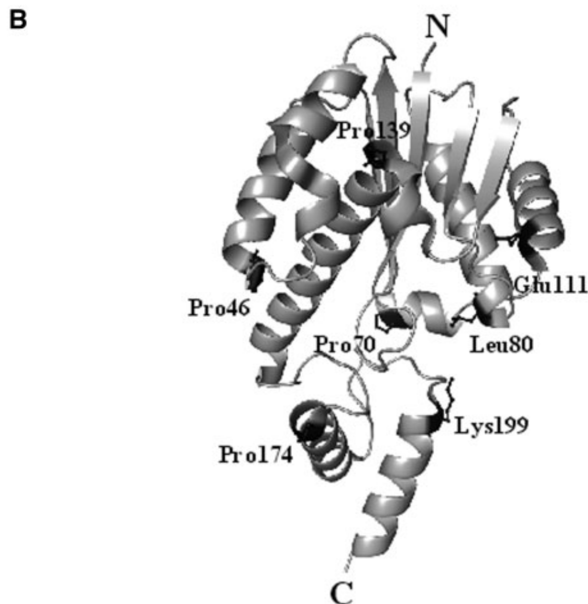

Fig. 1. **(A) Alignment of the amino acid sequences of RNase HII (*42*).** Secondary structures of Tk-RNase HII are depicted above the sequences. The amino acid residues that are conserved in at least six different proteins are highlighted in black. The positions that are replaced in this work are indicated by boxes. Tk, Pho, Pfu, Aae and Tma are hyperthermophiles; Eco and Bsu are mesophiles. (B): Crystal structure of Tk-RNase HII depicting the side-chains of the residues that has been substituted.

the N-terminal of α-helices as summarized in Table 1 and illustrated in Fig. 1B. Thermal- and guanidine hydrochloride (GdnHCl)-induced unfolding and refolding were measured with circular dichroism (CD) at 220 nm. We observed the proline effect on the thermostability but not on the slow unfolding. Using these results, we will discuss the effect of proline residues on the stability and folding of the hyperthermophilic protein and obtain general and new insights into protein stability and folding.

METHODS

Materials—GdnHCl was purchased from WAKO (Osaka, Japan). All chemicals were reagent grade.

Preparation of the Protein—Overproduction and purification of the wild-type and mutant proteins were performed as described previously (*11*). The purity of the proteins was analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) on a

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Table 1. **Structural characteristics at the N-terminal mutation sites of α-helices in Tk-RNase HII.**

	Secondary structure[a]	φ[b] (°)	ψ[b] (°)	ASA[c] (%)	B/B$_{av}$[d]
Leu80	H(80–94)	−64.3	−36.7	30.7	0.90
Glu111	H(111–120)	−58.1	−45.3	39.1	1.37
Lys199	H(199–121)	−57.7	−25.3	82.6	1.46
Pro46	H(46–57)	−54.1	−36.3	60.3	1.03
Pro70	H(70–74)	−54.0	−23.2	19.5	0.83
Pro139	H(139–163)	−53.1	−34.8	46.4	0.79
Pro174	H(174–187)	−48.9	−45.0	73.8	1.26

[a]Abbreviation of secondary structure: H, helix. Numbers in parentheses represent the range of the particular element of the secondary structure. [b]Dihedral angle values for the residues in the wild-type protein. [c]Accessible surface area. ASA (%) = (ASA^{fold}/ASA^{extend}) × 100. ASA^{extend} is the ASA value of the extended conformation as the reference value for the dependent state, assuming that these atoms in the denatured state should be fully exposed to solvent. The ASA values are calculated using the procedure described by Connolly (*43*) with probes of 1.4 Å^2 (*44*). [d]Ratio of B-factor of the backbone to the average B-factor of all atoms in the wild-type protein obtained from the crystal structure of Tk-RNase HII.

12% polyacrylamide gel. The protein concentration for the wild-type and mutant proteins was estimated by assuming 280 nm to be 0.63 for 1 mg ml^{-1} protein (*11*).

CD Spectra Measurements—CD spectra of the wild-type and mutant proteins were measured on a J-725 automatic spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). The optical path length was 2 mm. The protein concentration was 0.16 mg ml^{-1}. The buffer we used was 20 mM Tris–HCl at pH 9.0 and 25°C.

Heat-induced Unfolding Experiments—Heat-induced unfolding was examined by monitoring CD values at 220 nm. CD measurements were carried out on a J-725 automatic spectropolarimeter as described previously (*32*). The optical path length was 2 mm. The buffer we used was 20 mM Gly-NaOH at pH 9.0. The protein concentration was 0.16 mg ml^{-1}. All experiments were carried out at a scan rate of 1°C min^{-1}. A nonlinear least-squares analysis (*33*) was used to fit the data to:

$$y=\frac{((b_n+a_n[T])+(b_u+a_u[T])\exp((\Delta H_m/RT)((T-T_m)/T_m)))}{(1+\exp((\Delta H_m/RT)((T-T_m)/T_m)))} \quad (1)$$

where y is the observed CD signal at a given temperature T, b_n is the CD signal for the native state and b_u is the CD signal for the unfolded states. a_n is the slope of the pre-transition of the baseline, and a_u is the slope of the post-transition of the baseline. ΔH_m is the enthalpy of unfolding at the transition midpoint temperature (T_m). T is the temperature and R is the gas constant. Curve fitting was performed using SigmaPlot (Jandel Scientific, CA, USA).

Kinetic Experiments on GdnHCl-induced Unfolding and Refolding—The unfolding and refolding reactions were followed by a CD measurement at 220 nm as described previously (*9*). The optical path length was 2 mm. The kinetic data were analysed using Eq. (*2*).

$$A(t)-A(\infty)=\Sigma A_i^{e-kit} \quad (2)$$

Here, $A(t)$ is the value of the CD signal at a given time t, $A(\infty)$ is the value when no further change is observed, k_i is the apparent rate constant of the ith kinetic phase and A_i is the amplitude of the ith phase. The GdnHCl concentration dependence of the logarithms of the apparent rate constant (k_{app}) for unfolding and refolding was also examined. The rate constants for unfolding and refolding in the absence of GdnHCl [$k_u(H_2O)$ and $k_r(H_2O)$] were calculated by fitting to Eq. (*3*).

$$\ln k_{app}=\ln(k_r(H_2O)\exp(-m_r[D])+k_u(H_2O)\exp(-m_u[D])) \quad (3)$$

Here, m_u and m_r are the slopes of the linear correlations of ln k_u and ln k_r with the GdnHCl concentration. The fitting was performed using SigmaPlot. All kinetic experiments were performed in 20 mM Tris–HCl at pH 9.0 and 50°C. The Tris–HCl buffer, which reaches pH 9.0 at 50°C, was adjusted to 25°C (*34*). Two or three replicates were measured for each condition.

Equilibrium Experiments on GdnHCl-induced unfolding and refolding—GdnHCl-induced unfolding was examined by monitoring the CD signals at 220 nm as described previously (*9*). The GdnHCl-induced unfolding curves were determined, and a non-linear least-squares analysis (*35*) was used to fit the data to

$$y=\frac{((b_n^0+a_n[D])+(b_u^0+a_u[D])\exp((\Delta G(H_2O)-m[D])/RT))}{(1+\exp((\Delta G(H_2O)-m[D])/RT))} \quad (4)$$

$$C_m=\frac{\Delta G(H_2O)}{m} \quad (5)$$

where y is the observed CD signal at a given concentration of GdnHCl, and $[D]$ is the concentration of GdnHCl. b_n^0 is the CD signal for the native state, and b_u^0 is the CD signal for the unfolded states. a_n is the slope of the pre-transition of the baseline, and a_u is the slope of the post-transition of the baseline. $\Delta G(H_2O)$ is the Gibbs energy change (ΔG) of the unfolding in the absence of GdnHCl, m is the slope of the linear correlation between ΔG and the GdnHCl concentration [D], and C_m is the GdnHCl concentration at the midpoint of the curve. The raw experiment data were directly fitted to Eq. (*4*) using SigmaPlot. All equilibrium experiments were performed in 20 mM Tris–HCl at pH 9.0 and 50°C. The Tris–HCl buffer, which reaches pH 9.0 at 50°C, was adjusted to 25°C (*34*). Two or three replicates were measured for each condition.

RESULTS

CD Spectra Measurements—The secondary structural features of the wild-type and mutant proteins of Tk-RNase HII were studied by far-UV CD as depicted in Fig. 2A. CD spectra analysis reveals that the mutations do not affect the overall structure of Tk-RNase HII.

Heat-induced Unfolding—A change in the far-UV CD signal at 220 nm was used to detect heat-induced unfolding of the wild-type and nine mutant proteins of Tk-RNase HII. Heat-induced unfolding is highly reversible and exhibits a two-state transition regardless of the mutation. Fig. 2B presents representative heat-induced unfolding curves for the wild-type and mutant proteins. T_m values of the mutant proteins change by −1.8 to 3.0°C as compared with the wild-type protein, as listed in Table 2.

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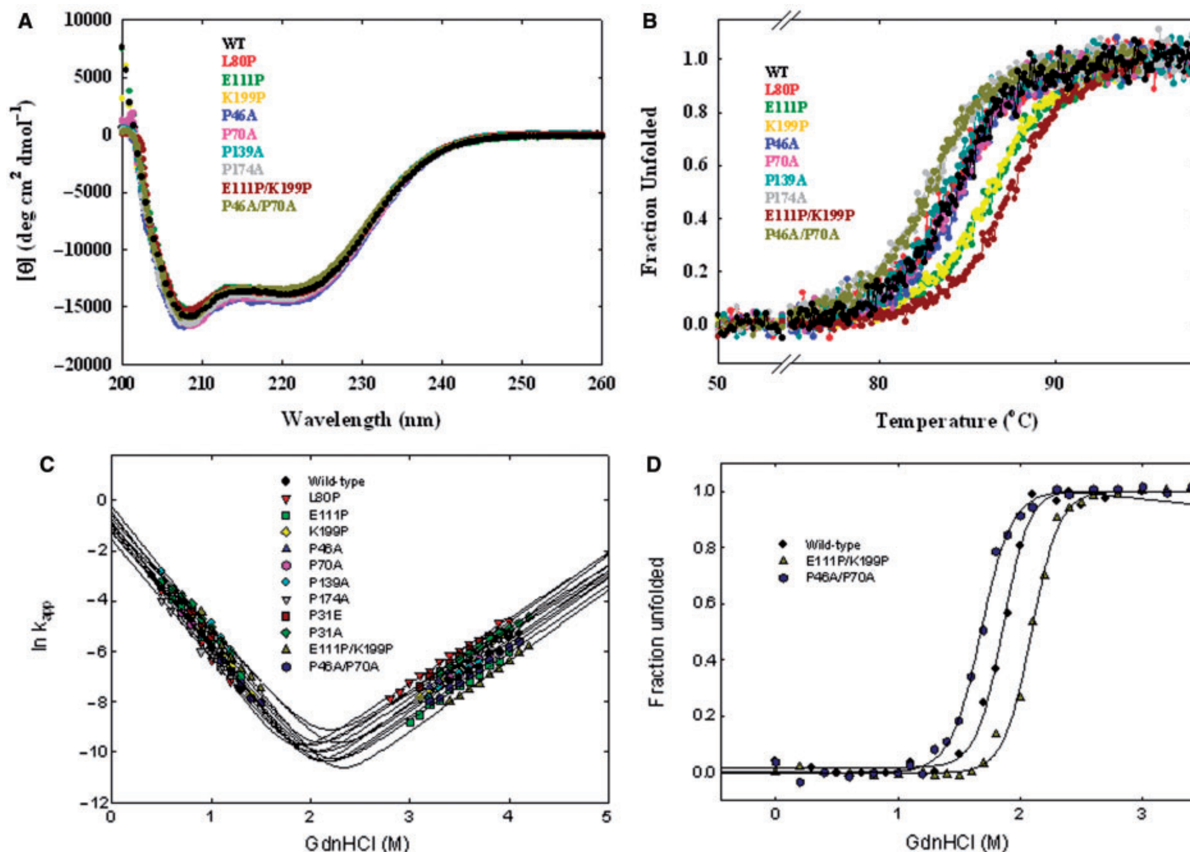

Fig. 2. (**A**) **CD spectra of the wild-type (WT) and mutant proteins of Tk-RNase HII at pH 9.0 and 25°C.** (B) Heat-induced unfolding curves of the WT and mutant proteins of Tk-RNase HII at pH 9.0. (C): GdnHCl concentration dependence of the logarithms of the apparent rate constant (k_{app}) of unfolding and refolding kinetics of the WT and mutant proteins of Tk-RNase HII at pH 9.0 and 50°C. The lines are best fits to Eq. (*3*). (D) GdnHCl-induced unfolding curves of the WT and mutant proteins of Tk-RNase HII at pH 9.0 and 50°C. The lines represent the fit of Eq. (*4*).

The T_m value was found to depend on the scan rate of heating because of the high kinetic stability of Tk-RNase HII (*9*), but the difference in scan rate dependence of the T_m value between the wild-type protein and a various protein is small (*15*). For single mutations, the mutant proteins with proline residue, E111P and K199P, stabilize whereas those without proline residue, P46A, P70A and P174A, destabilize. Although L80P and P139A have little effect on the thermostability (neutral to stability), the results support the proline effect on protein stability (*30*). Furthermore, the double-mutant proteins enhance the effect additively.

Kinetic experiments on GdnHCl-induced unfolding and refolding—The kinetics of GdnHCl-induced unfolding and refolding for the wild-type and mutant proteins was examined at 50°C, because the unfolding of Tk-RNase HII is extremely slow at lower temperatures. All kinetic traces are approximated as first-order reactions. Fig. 2C presents the GdnHCl concentration dependence of the logarithms of the apparent rate constant (k_{app}) for unfolding and refolding of the wild-type and mutant proteins. The kinetic parameters estimated using Eq. (*3*) are listed in Table 3. The refolding rate of the mutant proteins is similar to that of the wild-type protein. The unfolding rate for the mutant proteins also changes less than one order of magnitude compared with that of the wild-type protein. These results suggest that the proline residues have little effect on the slow unfolding of Tk-RNase HII.

Table 2. **T_m value of the wild-type and mutant proteins of Tk-RNase HII at the scan rate of 1°C min^{-1} at pH 9.0.**

	T_m[a] (°C)	ΔT_m[b] (°C)
Wild-type	84.5	–
L80P	84.1	−0.4
E111P	86.4	1.9
K199P	86.3	1.8
P46A	83.7	−0.8
P70A	84.0	−0.5
P139A	84.6	0.1
P174A	82.8	−1.7
E111P/K199P	87.5	3.0
P46A/P70A	82.7	−1.8

[a]The error is ±0.2°C. [b]$\Delta T_m = T_m$(mutant) − T_m(wild-type).

Equilibrium Experiments on GdnHCl-induced Unfolding and Refolding—To confirm the stability change of the mutant proteins and to analyse *m* value effect, we performed GdnHCl-induced equilibrium unfolding experiments. Because the change in T_m values of the

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Table 3. **Kinetic parameters for GdnHCl-induced unfolding and refolding of the wild-type and mutant proteins of Tk-RNase HII at 50°C and pH 9.0.**

	$k_u(H_2O)$ (s^{-1})	m_u[a] ($M^{-1}s^{-1}$)	$k_r(H_2O)$ (s^{-1})	m_r[a] ($M^{-1}s^{-1}$)
Wild-type	5.0×10^{-8}	2.8	0.78	5.5
L80P	3.0×10^{-7}	2.6	0.37	5.2
E111P	5.4×10^{-8}	2.7	0.80	4.5
K199P	4.7×10^{-8}	2.9	0.61	5.2
P46A	1.5×10^{-7}	2.6	0.46	4.5
P70A	6.2×10^{-8}	2.7	0.65	4.4
P139A	6.3×10^{-8}	2.8	0.72	4.3
P174A	3.3×10^{-7}	2.4	0.21	3.3
E111P/K199P	2.3×10^{-8}	2.8	0.77	4.9
P46A/P70A	5.1×10^{-8}	2.7	0.32	4.7

[a]The error is $\pm 0.1\ M^{-1}s^{-1}$.

Table 4. **Thermodynamic parameters for GdnHCl-induced unfolding of the wild-type and mutant proteins of Tk-RNase HII at 50°C and pH 9.0.**

	C_m[a] (M)	m (kJ $mol^{-1} M^{-1}$)	$\Delta G(H_2O)$ (kJ mol^{-1})	$\Delta\Delta G(H_2O)$[b] (kJ mol^{-1})
Wild-type	1.85	23.6 ± 2.8	43.6 ± 5.1	
E111P/K119P	2.09	23.7 ± 1.1	49.6 ± 2.4	5.5
P46A/P70A	1.68	21.5 ± 1.2	36.1 ± 2.8	−3.9

[a]The error is ± 0.10 M. [b]$\Delta\Delta G(H_2O) = m_{av}\ (C_m[\text{mutant}]) - C_m[\text{wild-type}])$. $m_{av} = 22.9$ kJ $mol^{-1} M^{-1}$.

mutant proteins is not so large, only the double-mutant proteins (E111P/K199P and P46A/P70A) in which the stability changes are larger were examined. The denaturation by GdnHCl was completely reversible at 50°C and the unfolding and refolding reactions of the proteins attained a two-state equilibrium in three weeks. Figure 2D plots GdnHCl-induced unfolding curves for the wild-type and mutant proteins of Tk-RNase HII. The thermodynamic parameters for GdnHCl-induced unfolding listed in Table 4 were calculated using Eqs. (*4*) and (*5*). The effect of the mutation on the conformational stability of Tk-RNase HII, $\Delta\Delta G(H_2O)$ was calculated by using the averaged m value (m_{av}). E111P/K199P is stabilized by 5.5 KJ mol^{-1} whereas P46A/P70A is destabilized by 3.9 KJ mol^{-1} as compared with the wild-type proteins. The result corresponds to that from the heat-induced unfolding experiment.

Proline residues decrease the conformational entropy of the denatured state. The m value is an excellent index describing the denatured state (*36*, *37*). The changes in m value of the double-mutant proteins were, however, within the experimental errors. This means that the conformational change of denatured state by proline substitution may be too small to detect in m value analysis.

DISCUSSION

It is expected that studies of proteins from hyperthermophiles will provide general and novel knowledge about protein folding and stability. For example, new insights included the slow unfolding property (*9*) and hydrophobic effect on the slow unfolding of hyperthermophilic proteins (*10*), and generalities were confirmed in an activity-stability trade-off (*15*) and osmolyte and hydrophobic effects on protein stability (*10*, *16*). In this work, we focus on proline residue at the N-terminal of the α-helix in Tk-RNase HII. From the results, we could obtain the generality for proline effect on protein thermostability, and additional information on slow unfolding of hyperstable proteins and on protein properties from the viewpoint of thermostability.

Proline Effect on the Thermostability of a Hyperthermophilic Protein—It has been reported that proline residues introduced at a critical site in an α-helix contribute most efficiently to enhance protein thermostability (*30*). For *Bucillus cereus* oligo-1,6-glucosidase, the increase in T_m value by introduction of one proline residue ranges from 0.4°C to 1.4°C (*28*). Because proline residue has a pyrrolidine ring, the backbone conformation of proline residue is constrained. In the denatured state of a protein, proline residue decreases the conformational entropy, resulting in protein stabilization. However, in the native state, proline residue sometimes imposes some conformational strains, resulting in protein destabilization. To stabilize a protein, proline residue should be located at certain sites in the native state that can accommodate it without any conformational strains. The N-terminal site of an α-helix is a candidate with the preferred conformation for proline residue, together with the second position of a β-turn (*30*). The thermostabilization by proline residue at the N-terminal of an α-helix is also confirmed by increase in the number of proline residues at the N-terminal of α-helices in thermostable proteins (*31*). There is, however, little positive proof that thermostable proteins are actually stabilized by proline residues.

For Tk-RNase HII, introducing one proline residue increases T_m within the range of 1.8–1.9°C, and the removal decreases within the range of 0.5–1.7°C, except for L80P and P139A. Furthermore, the double-mutant proteins, E111P/K199P and P46A/P70A, provide an additive effect. These results demonstrate that proline residue at the N-terminal of an α-helix in hyperthermophile proteins favourably contributes to the thermostability. The effects observed in Tk-RNase HII are slightly greater than those in *B. cereus* oligo-1,6-glucosidase, possibly because the effects become greater at higher temperature, due to the temperature dependence of the entropic effect (*26*). The contribution of each proline residue, even that of all the proline residues, to the stabilization of a hyperthermostable protein, however, is not so large. This means that proline residues at the N-terminal of an α-helix in hyperthermophile proteins make a partial contribution to the hyper-thermostability.

Although the proline effect was observed in Tk-RNase HII as described above, there are some differences in the effect among the mutant proteins. These differences result from the differences of environments at the mutation sites. Table 1 summarizes the structural characteristics at the mutation sites of Tk-RNase HII. All sites are located in a favourable region for a proline residue with $\varphi = -63 \pm 15°$ and $\psi = -30 \pm 20°$ (*38*, *39*). For the Xaa to Pro mutations, the accessibility and B-factor in the crystal structure at the mutation site of the proteins stabilized, E111P and K199P, are higher than

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those of the neutral protein, L80P. The effect of proline at the residue 80 must be canceled out by the conformational strains of proline residue introduced in the rigid site or the decrease in hydrophobic interaction due to the loss of leucine side-chain because Leu80 forms a hydrophobic core with the surrounding hydrophobic residues. For Pro to Ala, the neutral protein, P139A, has the lowest B-factor value at the mutation site. The proline residue at position 139 may have unfavourable interactions in the wild-type structure or the substitution may cause structural change in the mutant structure, compensating for the destabilization. In contrast, P174A, which destabilized significantly, has high accessibility and B-factor values at the site. The high accessibility and B-factor values correspond to the solvent-exposed and flexible sites. These sites are thought to be much more able to accommodate a proline residue without any conformational strains. Our results well support this concept.

Proline Effect on the Slow Unfolding of a Hyperthermophilic Protein—For hyperthermostable proteins, the unfolding rate is dramatically slower than for their mesostable homologs (*6–8*). The molecular mechanism of the slow unfolding is not clear yet. Recently, it has been reported that the unfolding rate of the hydrophobic mutant proteins of Tk-RNase HII is one to three orders of magnitude faster than that of the wild-type protein, indicating that the slow unfolding originates from the hydrophobic effect (*10*). Other factors that may affect the slow unfolding, however, have not been examined yet. Here, we tested proline residues at the N-terminal of α-helices of Tk-RNase HII because the proline residues seemed to contribute to the thermostability. The results suggest that proline residues have little effect on the slow unfolding. The reason may be that a proline residue mainly influences the conformation of the denatured state, resulting in no significant effect on the unfolding speed. This suggests that enthalpic effects in the native state of a protein such as the hydrophobic effect and hydrogen bonding are causes of slow unfolding of hyperthermostable proteins, not entropic effects in the denatured state such as proline residues and disulfide bonding.

Stabilization of Hyperthermophilic Protein—In this work, we could also observe another piece of critical evidence for protein stability that the mutant proteins of Tk-RNase HII, E111P, K199P and E111P/K199P are more stable than the wild-type protein. This means that hyperthermophilic proteins do not possess the highest stability. In other words, a protein fine tunes the stability to the environment in which the organism lives.

The mutation E111P stabilizes Tk-RNase HII. The residue corresponding to Glu111 in Tk-RNase HII is proline (Pro111) in RNase HII from *Pyrococcus holikoshii* (Pho-RNase HII) (Fig. 1). Pho-RNase HII would be more stable than Tk-RNase HII because the optimal growth temperature of *P. holikoshii* is higher than that of *T. kodakaraensis* (*40*, *41*). The proline residue (Pro111) in Pho-RNase HII seems to be a candidate residue stabilizing the protein Pho-RNase HII. In contrast, the residue corresponding to Pro139 in Tk-RNase HII is glutamic acid (Glu139) in Pho-RNase HII. Because P139A of Tk-RNase HII is neutral to the stability change, the site (residue 139) in Pho-RNase HII could not be related to thermostabilization. For position 199 in Tk-RNase HII, other RNase HIIs from hyperthermophiles also have nonproline residues. We found the stabilization of K199P of Tk-RNase HII. These suggest that other RNase HIIs could be stabilized by replacing this position with proline residue.

CONCLUSION

In this work, we examined the proline effect at the N-terminal of an α-helix in hyperthermophile protein Tk-RNase HII on thermostability and folding using systematic mutant proteins. The results indicate that proteins are universally stabilized by proline residues at the N-terminal of an α-helix, regardless of their robustness. In particular, an exposed and flexible site is more conducive to proline effect on protein thermostability. However, proline residues contribute little to the slow unfolding, unlike the hydrophobic effect. Accumulation of analyses of other factors will reveal the molecular mechanism of slow unfolding in hyperthermophile proteins in the future. Furthermore, we found a fresh property of the thermostability of proteins: that a hyperthermophilic protein is not perfectly stable.

CONFLICT OF INTEREST

None declared.

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