Kinetically Robust Monomeric Protein from a Hyperthermophile†

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ABSTRACT: Equilibrium and kinetic studies were carried out under denaturation conditions to clarify the energetic features of the high stability of a monomeric protein, ribonuclease HII, from a hyperthermophile, *Thermococcus kodakaraensis* (*Tk*-RNase HII). Guanidine hydrochloride (GdnHCl)-induced unfolding and refolding were measured with circular dichroism at 220 nm, and heat-induced denaturation was studied with differential scanning calorimetry. Both GdnHCl- and heat-induced denaturation are very reversible. It was difficult to obtain the equilibrated unfolding curve of *Tk*-RNase HII below 40 °C, because of the remarkably slow unfolding. The two-state unfolding and refolding reactions attained equilibrium at 50 °C after 2 weeks. The Gibbs energy change of GdnHCl-induced unfolding ($\Delta G(H_2O)$) at 50 °C was 43.6 kJ mol^{-1}. The denaturation temperature in the DSC measurement shifted as a function of the scan rate; the denaturation temperature at a scan rate of 90 °C h^{-1} was higher than at a scan rate of 5 °C h^{-1}. The unfolding and refolding kinetics of *Tk*-RNase HII were approximated as a first-order reaction. The ln k_u and ln k_r values depended linearly on the denaturant concentration between 10 and 50 °C. The $\Delta G(H_2O)$ value obtained from the rate constant in water using the two-state model at 50 °C, 44.5 kJ mol^{-1}, was coincident with that from the equilibrium study, 43.6 kJ mol^{-1}, suggesting the two-state folding of *Tk*-RNase HII. The values for the rate constant in water of the unfolding for *Tk*-RNase HII were much smaller than those of *E. coli* RNase HI and *Thermus thermophilus* RNase HI, which has a denaturation temperature similar to that of *Tk*-RNase HII. In contrast, little difference was observed in the refolding rates among these proteins. These results indicate that the stabilization mechanism of monomeric protein from a hyperthermophile, *Tk*-RNase HII, with reversible two-state folding is characterized by remarkably slow unfolding.

Microorganisms can be classified into psychrophiles, mesophiles, thermophiles, or hyperthermophiles on the basis of the differences in their optimal growth temperatures. The stabilities of proteins from these microorganisms exhibit different properties. Proteins from hyperthermophiles generally reveal greater stability than those from any other microorganisms. Therefore, proteins from hyperthermophiles are expected to provide unique information about protein folding, stability, and function (*1*−*7*).

The stability of proteins in solution is evaluated by the Gibbs energy changes (ΔG) upon denaturation, when they are reversible under experimental conditions. The value of ΔG is obtained from equilibrium and kinetic experiments. Studies concerning the stability of proteins from hyperthermophiles have focused primarily on the equilibrium aspects (*8*−*13*). The results suggested that extremely high stability of these proteins can be achieved by increasing the number of ionic interactions and the extent of hydrophobic surface burial. Several reports have recently appeared in which the equilibrium and kinetic aspects of proteins from hyperthermophiles were analyzed quantitatively; they indicated that hyperthermophilic proteins often possess a more remarkable kinetic stability than proteins from mesophiles (*14*−*18*). Here kinetic stability means that a protein shows unusual slow unfolding upon denaturation. However, the proteins examined were oligomer or monomer with irreversible unfolding or with multistate folding.

Ribonuclease HII from a hyperthermophile, *Thermococcus kodakaraensis* (*Tk*-RNase HII),[1] is a monomer and consists of 228 amino acid residues (with a molecular weight of 26K) (*19*). The crystal structure has already been determined (*20*). RNase H hydrolyzes only the RNA strand of an RNA/DNA hybrid (*21*). The enzyme is ubiquitously present in various organisms and is involved in DNA replication and repair. RNase H is classified into two major types according to the sequence similarity, type 1 and type 2 RNase H (*22*, *23*). *Tk*-RNase HII is a member of type 2 RNase H. A comparison of the crystal structures of *Escherichia coli* RNase HI,

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[1] Abbreviations: RNase, ribonuclease; *Tk*-RNase HII, *Thermococcus kodakaraensis* RNase HII; *Ec*-RNase HI, *Escherichia coli* RNase HI; *Tt*-RNase HI, *Thermus thermophilus* RNase HI; GdnHCl, guanidine hydrochloride.

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Thermus thermophilus RNase HI, and *Tk*-RNase HII indicates that these enzymes share a folding motif, referred to as the RNase H-fold due to its characterization in *E. coli* RNase HI (*24*), although there is no sequence similarity between RNase HI and RNase HII. The stability and folding of RNase HI from the mesophilic bacterium *E. coli* and the thermophilic bacterium *T. thermophilus*, which belong to type 1 RNase H, have been studied in detail (*25*−*32*). In contrast, less information is available for type 2 enzymes. A study of the stability and folding of *Tk*-RNase HII would also produce insights into the structural properties of the entire RNase H family.

We focused on the equilibrium and kinetic aspects of the folding of *Tk*-RNase HII by circular dichroism (CD) and differential scanning calorimetry (DSC) in this paper. We examined guanidine hydrochloride (GdnHCl)-induced unfolding and refolding and heat-induced denaturation. We observed the reversible two-state unfolding of *Tk*-RNase HII in both GdnHCl- and heat-induced denaturation. *Tk*-RNase HII is very stable against denaturant and heat-induced denaturation, and the stabilization mechanism of *Tk*-RNase HII was revealed to originate from the remarkably slow unfolding rate.

MATERIALS AND METHODS

Preparation of the Protein. Overproduction and purification of Tk-RNase HII were performed as reported by Haruki et al. (*19*). The protein concentration was estimated by assuming an $A_{280\ nm}$ of 0.63 for 1 mg mL^{-1} protein (*19*).

Equilibrium Experiments on GdnHCl-Induced Unfolding and Refolding. CD measurements were carried out on a J-725 automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.). Each CD measurement was an average of the signal at 220 nm for 1 min in a 2 mm path length cuvette. Tk-RNase HII was incubated in GdnHCl at different concentrations and at different temperatures for unfolding. The protein, which is unfolded completely at a 4 M GdnHCl concentration, was diluted with buffer for refolding, and the diluted protein solution was incubated at the selected temperature until refolding reached equilibrium. The fraction of unfolding (f_u) was calculated from eq 1

$$f_u = (b_n^0 + a_n[D] - y)/(b_n^0 + a_n[D] - b_u^0 - a_u[D]) \quad (1)$$

where f_u is the fraction of the unfolded state, y is the CD signal at a given concentration of GdnHCl, and [D] is the concentration of GdnHCl. b_n^0 and b_u^0 are the CD signals for the native and unfolded states. a_n and a_u are the slopes of the pre- and post-transition of the baselines. The Gibbs energy change of GdnHCl-induced unfolding in the absence of GdnHCl [$\Delta G(H_2O)$] was determined by the linear extrapolation model assuming a two-state transition, according to the following equations (*33*).

$$K = f_u/(1 - f_u) \quad (2)$$

$$\Delta G = -RT \ln K \quad (3)$$

$$\Delta G = \Delta G(H_2O) - m[D] \quad (4)$$

$$f_u = 1/(1 + \exp((\Delta G(H_2O) - m[D])/(RT)) \quad (5)$$

Here, K is the apparent equilibrium constant of the unfolding and ΔG is the Gibbs energy change of the unfolding. m is the slope of the linear correlation between ΔG and the GdnHCl concentration [D]. The experimental raw data was directly fit with eqs 1 and 5 using SigmaPlot (Jandel Scientific). All equilibrium experiments were performed in 20 mM Tris-HCl, 2 mM EDTA at pH 9.0. Each Tris-HCl buffer, which would be pH 9 at each temperature examined, was adjusted at 25 °C (*34*). The protein concentration when CD measurements were taken was 0.169 mg mL^{-1}.

Heat-Induced Unfolding Experiments. DSC measurements were carried out on a high-sensitivity VP-DSC controlled by the VPViewer software package (Microcal, Inc.), with a cell volume of 0.51 and at an excess pressure of 30 psi (1 psi ≈ 6.9 kPa) to avoid degassing during heating. Experiments at different heating rates (5, 30, 60, 90 °C h^{-1}) were performed at a protein concentration of 0.629 mg mL^{-1} in 20 mM Tris-HCl, 2 mM EDTA at pH 9. The Tris-HCl buffer, which would be pH 9 at 50 °C, was adjusted at 25 °C (*34*). The excess heat capacity curves for the protein were corrected by subtracting the corresponding buffer baseline at the scan rate and were then normalized by the protein concentration using the Origin software package (Microcal Inc.). The reversibility of thermal denaturation was verified by reheating the samples.

Kinetic Experiments on GdnHCl-Induced Unfolding and Refolding. The unfolding and refolding reactions were followed by CD at 220 nm, using 2 mm and 1 cm path length cuvettes. CD measurements were conducted using a J-725 automatic spectropolarimeter. The unfolding reactions of proteins were induced by GdnHCl jump to various concentrations. The refolding reactions of the proteins were generated by dilution of the GdnHCl concentration of the protein solution, in which the proteins were completely unfolded at a 4 M GdnHCl concentration. *Tk*-RNase HII was incubated in 4 M GdnHCl for 1 day prior to the refolding reaction measurements. The protein solution was stirred using a spinning mixer with a magnetic stirrer in a 1 cm path length cuvette for the unfolding and refolding, and the CD was recorded at 220 nm as a function of time. The dead time of this method was 2 s. The protein solution was stirred manually during use of the 2 mm path length cuvette. The dead time of this method was 10 s. All kinetic experiments were performed in 20 mM Tris-HCl at pH 9.0. Each Tris-HCl buffer, which would be pH 9 at each temperature examined, was adjusted at 25 °C (*34*). The protein concentrations when CD measurements were taken were 0.0338−0.169 mg mL^{-1}. The kinetic data were analyzed using eq 6.

$$A(t) - A(\infty) = \Sigma A_i e^{-kit} \quad (6)$$

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 the ith phase.

RESULTS

GdnHCl-Induced Equilibrium Unfolding of Tk-*RNase HII.* A change of the far-UV CD signal at 220 nm was used to measure the GdnHCl-induced unfolding and refolding of *Tk*-RNase HII at various temperatures at pH 9.0. The denaturation by GdnHCl was completely reversible at 50 °C, and the unfolding and refolding reactions of *Tk*-RNase HII

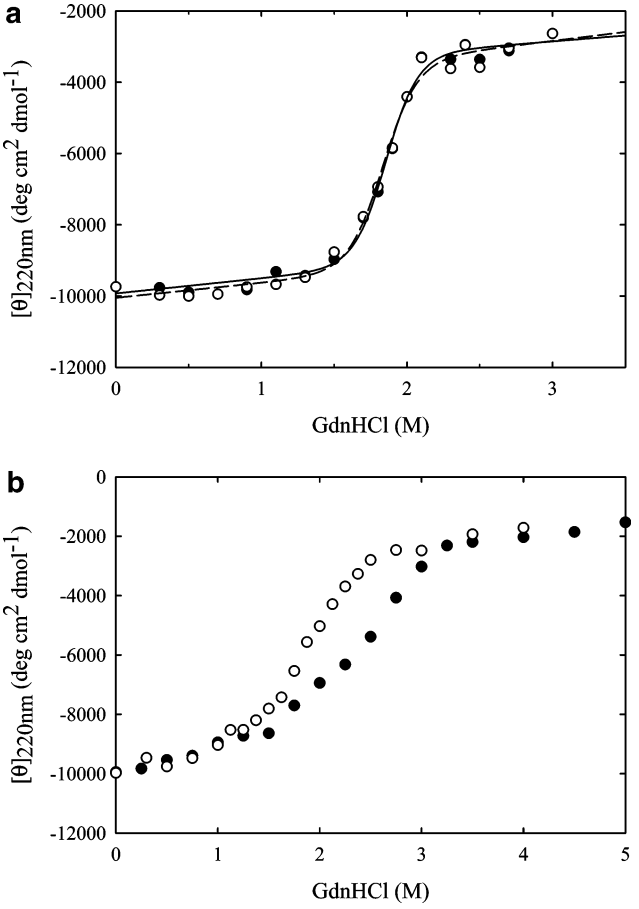

FIGURE 1: GdnHCl-induced unfolding (●) and refolding (○) curves of Tk-RNase HII at (a) 50 °C in 2 weeks and (b) 20 °C in 1 month. The lines represent the fit of eq 5.

attained a two-state equilibrium in 2 weeks, as indicated in Figure 1a. The thermodynamic parameters for GdnHCl-induced equilibrium unfolding were calculated using eqs 1−5. $\Delta G(H_2O)$ and the *m*-value at 50 °C were 43.6 ± 5.1 kJ mol^{-1} and 23.6 ± 2.8 kJ mol^{-1} M^{-1}. These values indicate that *Tk*-RNase HII is very stable at 50 °C compared to mesophilic proteins (*8*). However, the unfolding curve was inconsistent with the refolding curve at 20 °C, even after 30 days, as illustrated in Figure 1b. It was impossible to obtain the equilibrated unfolding curve below 40 °C. This result may be due to the remarkably slow unfolding, refolding, or both of *Tk*-RNase HII.

Heat-Induced Unfolding by DSC. DSC measurements were used to examine the heat-induced denaturation of *Tk*-RNase HII. The reversibility was verified by reheating experiments. Repeated thermal scans revealed similar transition profiles, as illustrated in Figure 2a, indicating the highly reversible heat-induced unfolding of *Tk*-RNase HII. Figure 2b depicts the partial molar heat capacity for *Tk*-RNase HII at pH 9.0 at scan rates of 30 and 60 °C h^{-1}. The transition curves appeared to be a single peak. However, the denaturation temperature of the DSC curve for *Tk*-RNase HII shifted as a function of the scan rate. The denaturation temperature at a scan rate of 90 °C h^{-1} was 89.2 °C, whereas the denaturation temperature at 5 °C h^{-1} was 87.2 °C. This indicates that the heat-induced unfolding of *Tk*-RNase HII did not attain equilibrium in these scan rates because of the remarkably slow unfolding. Heat-induced unfolding of most mesophilic proteins can attain equilibrium at 60 °C h^{-1} (*35*).

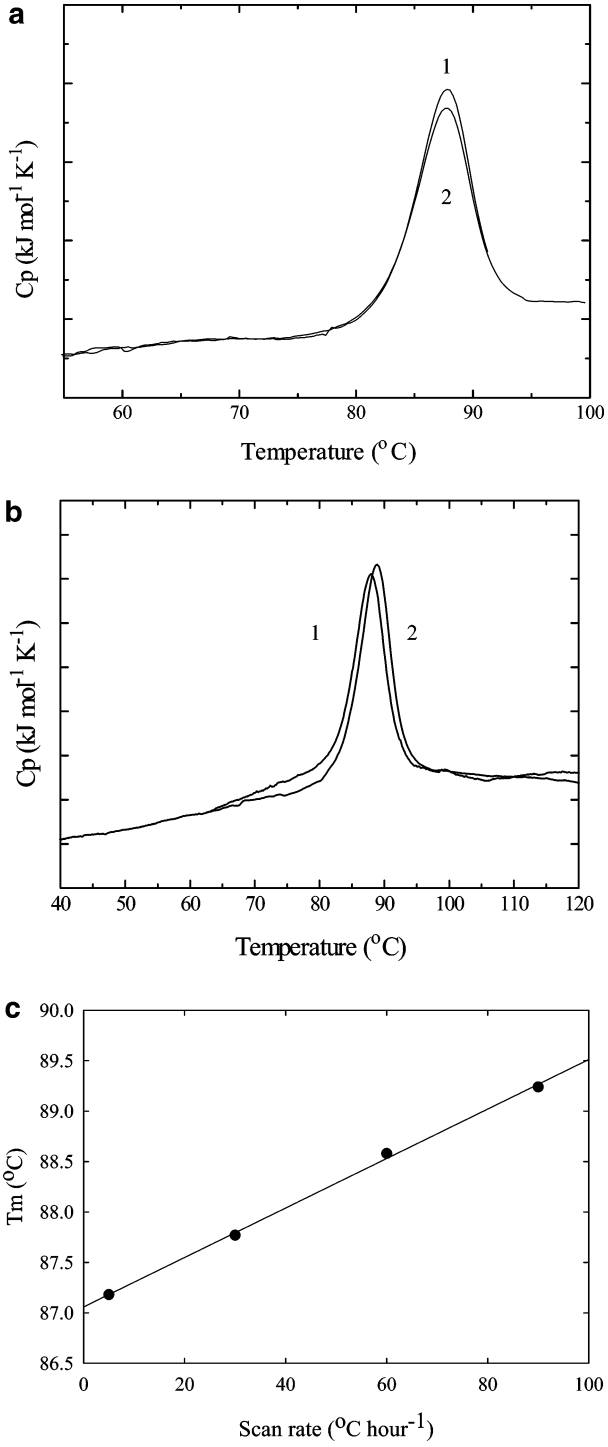

FIGURE 2: (a) Reversibility of heat denaturation of *Tk*-RNase HII. Curves 1 and 2 represent the first and second scans, respectively. (b) Dependence of excess heat curves of *Tk*-RNase HII on the scan rates. Samples with the same protein concentration were measured at different scan rates. Curves 1 and 2 represent the DSC curve at scan rates of 30 and 60 °C/h, respectively. (c) The denaturation temperature was plotted as a function of the scan rate. The line represents a linear fit.

The denaturation temperature is plotted as a function of the scan rate in Figure 2c and they fit linearly. The extrapolated value was 87.1 °C. Although the linear fit is not theoretical, this value indicates that *Tk*-RNase HII is very stable against heat-induced denaturation. The calorimetric enthalpy, ΔH_{cal}, and van't Hoff enthalpy, ΔH_{vH}, at 88.6 °C at scan rate of 60 °C h^{-1} were 511 and 683 kJ mol^{-1}.

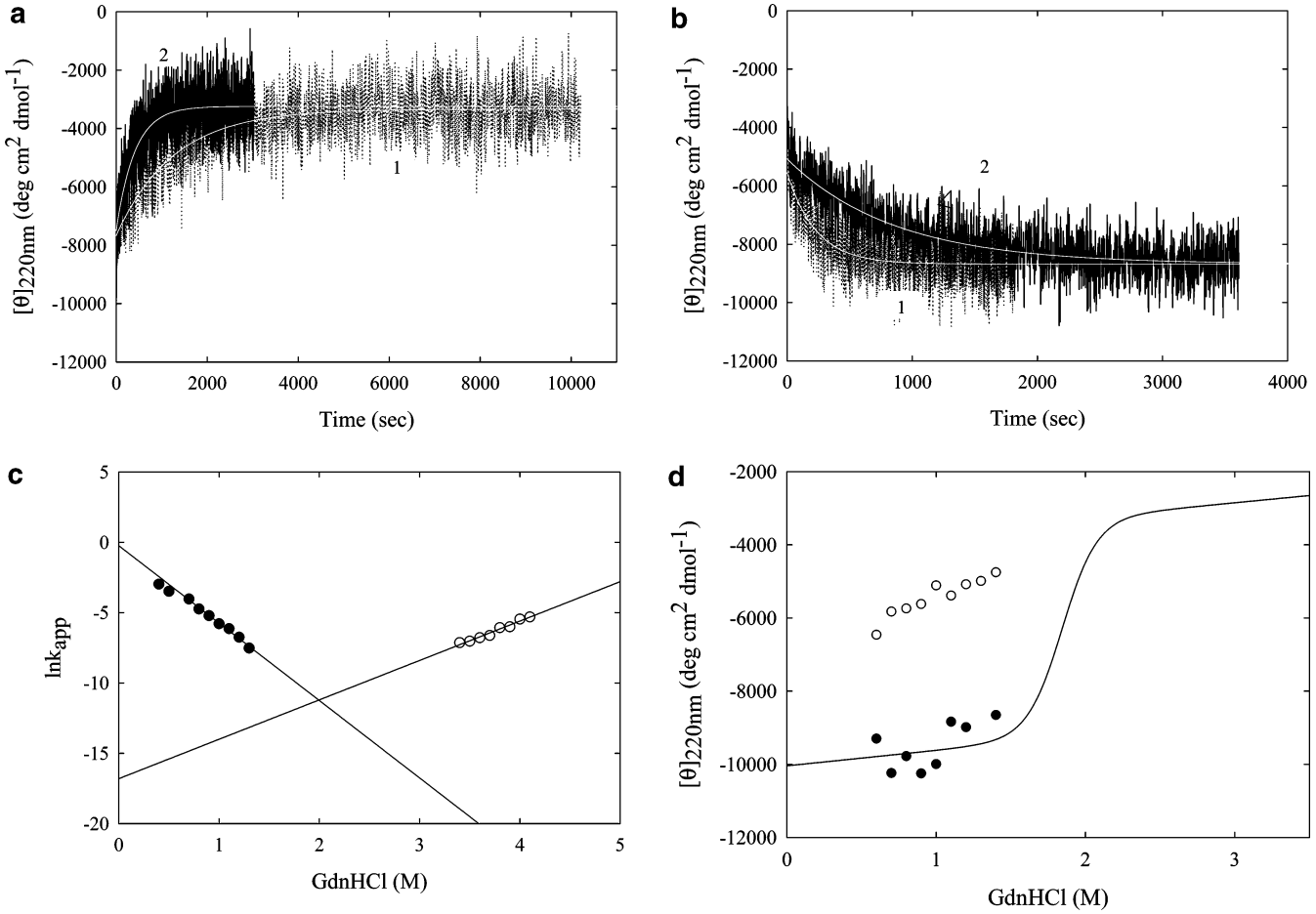

FIGURE 3: (a and b) Representative kinetic curves for unfolding and refolding of *Tk*-RNase HII at 50 °C. The reaction progress was followed by a change in CD at 220 nm. The solid lines represent the fit of eq 6. (a) Kinetic traces of unfolding to a final concentration of (1) 3.4 and (2) 3.9 M GdnHCl. (b) Kinetic traces of refolding to a final concentration of (1) 1.0 and (2) 1.2 M GdnHCl. (c) GdnHCl concentration dependence of the apparent rate constants (k_{app}) of the unfolding (○) and refolding (●) kinetics of *Tk*-RNase HII at 50 °C. The solid line represents the fit of eqs 7 and 8. (d) Burst phase (○) and final (●) signals of the refolding experiments at 50 °C. The solid line represents the fit of equilibrium unfolding at 50 °C (Figure 1a).

GdnHCl-Induced Unfolding and Refolding Kinetics of Tk-RNase HII. Both GdnHCl-induced and heat-induced denaturation studies suggest that the unfolding of *Tk*-RNase HII is extremely slow. The kinetics of GdnHCl-induced unfolding and refolding were therefore next examined at 50 °C and pH 9.0. Representative kinetic curves for the unfolding and refolding are provided in Figure 3a,b. All kinetic traces were approximated as first-order reactions.

Figure 3c indicates the GdnHCl concentration dependence of the logarithms of the apparent rate constant (k_{app}) of the unfolding and refolding for *Tk*-RNase HII at 50 °C. ln k_{app} linearly correlated with the GdnHCl concentration in both unfolding and refolding reactions, and therefore, the rate constants of unfolding, k_u, and refolding, k_r, were analyzed using eqs 7 and 8.

$$\ln k_u = \ln k_u(H_2O) + m_u[D] \quad (7)$$

$$\ln k_r = \ln k_r(H_2O) + m_r[D] \quad (8)$$

Here, $k_u(H_2O)$ and $k_r(H_2O)$ are the rate constants of unfolding and refolding in the absence of GdnHCl. m_u and m_r are the slopes of the linear correlation of ln k_u and ln k_r with the GdnHCl concentration. k_{app} ($k_u + k_r$) should be close to k_u in the unfolding zone, since $k_u \gg k_r$. Conversely, k_{app} ($k_u + k_r$) should be close to k_r in the refolding zone. The unfolding and refolding of *Tk*-RNase HII were measured only in the unfolding and refolding zone. The $k_u(H_2O)$ and $k_r(H_2O)$ values were estimated by extrapolation to 0 M GdnHCl using eqs 7 and 8. The $ku(H_2O)$ and $kr(H_2O)$ values at 50 °C were $(5.0 \pm 4.1) \times 10^{-8}$ s^{-1} and 0.78 ± 0.2 s^{-1}, and m_u and m_r at 50 °C were 2.8 ± 0.2 M^{-1} s^{-1} and 5.5 ± 0.2 M^{-1} s^{-1}. The equilibrium constant, K, in water can be calculated from $k_u(H_2O)/k_r(H_2O)$ if the unfolding−refolding reaction of *Tk*-RNase HII in water is a two-state folding. $\Delta G(H_2O)$ of *Tk*-RNase HII at 50 °C was found to be 44.5 kJ mol^{-1} from the rate constants. This value is similar to the $\Delta G(H_2O)$ from the equilibrium experiment, 43.6 kJ mol^{-1}. Figure 3d shows the burst phase and final signals of the refolding experiments. The denaturant-dependence burst phase signals were linear. These results suggest the two-state unfolding of *Tk*-RNase HII. The equilibrium and kinetic parameters of the unfolding and refolding of *Tk*-RNase HII at pH 9.0 at 50 °C are summarized in Table 1.

Temperature Dependence of $\Delta G(H_2O)$. The unfolding and refolding kinetics were measured between 10 and 50 °C to evaluate the temperature dependence of $\Delta G(H_2O)$ from the kinetic study of *Tk*-RNase HII. GdnHCl-induced denaturation is very reversible, and the unfolding and refolding kinetics was approximated as a first-order reaction. The ln k_u and ln k_r values depended linearly on the denaturant concentration

Table 1: Equilibrium and Kinetic Parameters of Unfolding and Refolding of *Tk*-RNase HII at 50 °C

method	$k_u(H_2O)$ (s^{-1})	m_u, (M^{-1} s^{-1})	$k_r(H_2O)$ (s^{-1})	m_r (M^{-1} s^{-1})	m^a (kJ mol^{-1} M^{-1})	K	$\Delta G(H_2O)$ (kJ/mol)
equilibrium					23.6 ± 2.8	8.8×10^{-8}	43.6 ± 5.1
kinetics	$(5.0 \pm 4.1) \times 10^{-8}$	2.8 ± 0.2	0.78 ± 0.2	-5.5 ± 0.2	22.3	6.4×10^{-8}	44.5

[a] The kinetic *m*-value was calculated by $m = RT(m_u - m_r)$.

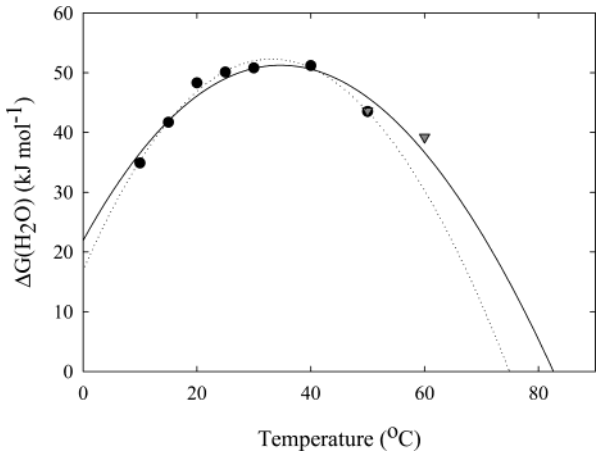

FIGURE 4: Thermodynamic stability profile of *Tk*-RNase HII. The circles and triangles represent the data from kinetic and equilibrium methods. The dotted and solid lines represent fits to eq 9 using kinetic and both kinetic and equilibrium data.

at all temperatures. The $\Delta G(H_2O)$ value at each temperature was calculated from $k_u(H_2O)/k_r(H_2O)$. $\Delta G(H_2O)$ was plotted as a function of temperature, resulting in the stability profile of *Tk*-RNase HII depicted in Figure 4. The stability profile displayed a maximum around 40 °C and was fit to a two-state model using eq 9.

$$\Delta G(H_2O) = \Delta H_m - T\Delta H_m/T_m + \Delta C_p[T - T_m - T\ln(T/T_m)] \quad (9)$$

Here, ΔH_m is the enthalpy of unfolding at the transition midpoint temperature and ΔC_p is the difference in heat capacity between the native and unfolded states. The values of T_m, ΔH_m, and ΔC_p of *Tk*-RNase HII using all the data were 82.8 °C, 745 ± 49 kJ mol^{-1}, and 14.5 ± 1.8 kJ mol^{-1} K^{-1}. The expected ΔC_p value for the size of the protein is 16.5 kJ mol^{-1} K^{-1} (*36*). The enthalpy change as a function of temperature, $\Delta H(T)$, was calculated using these data and eq 10.

$$\Delta H(T) = \Delta H(T_m) - \Delta C_p\,(T_m - T) \quad (10)$$

At 88.6 °C, which is the denaturation temperature at a scan rate of 60 °C h^{-1} in DSC, the calculated ΔH value was 829 $\pm$ 116 kJ mol^{-1}. This value was larger than the ΔH_{cal} (511 kJ mol^{-1}) and ΔH_{vH} (683 kJ mol^{-1}) values at 88.6 °C at scan rate of 60 °C h^{-1} from DSC. This may be due to the nonequilibrium unfolding at this scan rate in DSC or the temperature dependence of ΔC_p at high temperature.

DISCUSSION

Reversible Denaturation of Tk-RNase HII, a Monomeric Protein from Hyperthermophile. Barnase, chymotrypsin inhibitor 2, and lysozyme have previously been extensively studied as model proteins for investigations of protein stability and folding (*37*−*39*). They are all proteins from organisms that grow at moderate temperatures, so they do not suffice for clarifying the mechanism of adaptation to high temperature. The thermodynamic stability and folding of hyperthermophilic proteins have been also reported. However, many hyperthermophilic proteins exhibit irreversible unfolding against heat denaturation because of their high stability. *Tk*-RNase HII is very reversible against both GdnHCl and heat denaturation, even if it unfolds at around 90 °C (Figure 2a). Moreover, *Tk*-RNase HII exists as a monomeric form in vitro (*19*), so it is not stabilized by oligomerization (*16*, *18*). Therefore, *Tk*-RNase HII is considered to be one of the best models for clarifying the mechanism that stabilizes hyperthermophilic proteins.

Two-State Folding of Tk-RNase HII. An equilibrated unfolding curve was obtained at 50 °C in an equilibrium experiment of *Tk*-RNase HII, and the thermodynamic parameters were determined according to a two-state model. All kinetic traces were approximated as first-order reactions in the kinetic experiment. The ln k_u and ln k_r values depended linearly on the denaturant concentration. The $\Delta G(H_2O)$ value obtained from $k_u(H_2O)$ and $k_r(H_2O)$ using a two-state model at 50 °C, 44.5 kJ mol^{-1}, is coincident with that from the equilibrium study, 43.6 kJ mol^{-1} (Table 1). The *m* value from the kinetic study also agreed with that derived from the equilibrium study (Table 1). On the other hand, the $\Delta G(H_2O)$ value obtained from $k_u(H_2O)$ and $k_r(H_2O)$ using a three-state model at 50 °C was 52.1 kJ mol^{-1}, which was different from that of the equilibrium study, 43.6 kJ mol^{-1} (Table 1). These results indicate that *Tk*-RNase HII folds as a two-state model and there is no stable intermediate in the folding process.

The ΔH_{cal} value (511 kJ mol^{-1}) was smaller than the ΔH_{vH} value (683 kJ mol^{-1}) at 88.6 °C at a scan rate of 60 °C h^{-1}. If these values are equal, it indicates a two-state folding (*40*). The difference between them for *Tk*-RNase HII may be due to the nonequilibrium unfolding at this scan rate.

Kamagata et al. (*41*) summarized the kinetic folding data for two-state proteins. The chain length distribution for two-state folders is narrow, between 40 and 110. *Tk*-RNase HII has 228 amino acid residues and may be one of the biggest proteins with two-state folding.

Slow Unfolding of Tk-RNase HII. The unfolding curve was inconsistent with the refolding curve below 40 °C in the equilibrium experiment of *Tk*-RNase HII (Figure 1b), as a result of the remarkably slow unfolding, refolding, or both. The denaturation temperature of the DSC curve shifted as a function of the scan rate (Figure 2c), indicating that the heat-induced unfolding did not attain equilibrium because of the remarkably slow unfolding. The $k_u(H_2O)$ values at 50 and 40 °C were 5.0×10^{-8} and 2.2×10^{-9} s^{-1} in the kinetic experiments. The $k_f(H_2O)$ values at 50 and 40 °C were 0.78 and 0.78 s^{-1}. The $k_u(H_2O)$ values were unusual for protein folding, whereas the $k_r(H_2O)$ values were not (*15*, *17*, *18*, *41*). These results indicate that *Tk*-RNase HII has a kinetically robust native structure in water. The equilibrium half-time at the denaturation midpoint in GdnHCl denaturation could

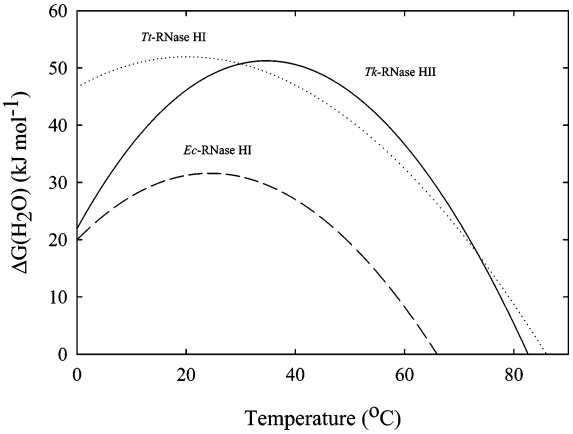

FIGURE 5: Thermodynamic stability profiles of *Tk*-RNase HII, *Ec*-RNase HI, and *Tt*-RNase HI (*18*). The solid, dashed, and dotted lines represent *Tk*-RNase HII, *Ec*-RNase HI, and *Tt*-RNase HI.

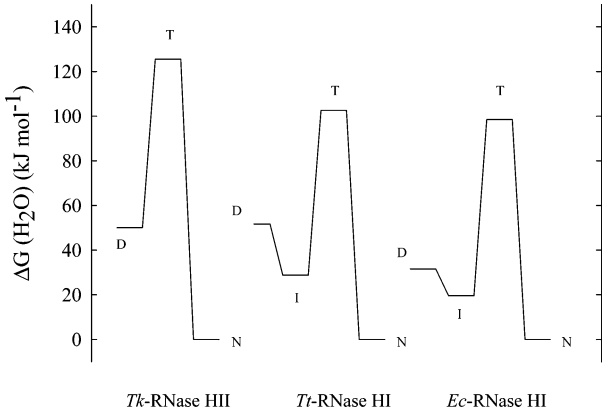

FIGURE 6: Energy diagrams of the folding processes of *Tk*-RNase HII, *Ec*-RNase HI (*17*), and *Tt*-RNase HI (*20*) at 25 °C. N, I, T, and D represent the native, intermediate, transition, and denatured states, respectively. The activation energies of the transition states were determined from Eyring's equation (*51*).

be estimated by the apparent rate constant values (k_{app}) of the unfolding and refolding experiments, i.e., 0.9 days at 50 °C, 4.1 days at 40 °C, and 31.2 days at 20 °C.

Stability Profile of Tk-RNase HII. The stability profile of *Tk*-RNase HII was determined from the results of both equilibrium and kinetic experiments for GdnHCl-induced unfolding (Figure 4). The stability profile displayed a maximum around 40 °C. The $\Delta G(H_2O)$ value at 40 °C is 51.2 kJ mol^{-1}, indicating the superior stability of *Tk*-RNase HII (*8*, *42*). A DSC experiment also indicated that *Tk*-RNase HII is very stable against heat-induced denaturation. These results, together with the slow unfolding of *Tk*-RNase HII, demonstrate that the increased stability of *Tk*-RNase HII originates from the remarkably slow unfolding rate.

However, *Tk*-RNase HII unfolds completely in vitro at 90 °C, which is the optimal growth temperature of *T. kodakaraensis* (*43*). *Tk*-RNase HII may be stabilized by metal ion, osmolyte, and macromolecular crowding effects in vivo (*44*). This suggests that proteins are only marginally stable at living temperatures, regardless of whether they are mesophilic or hyperthermophilic proteins (*42*). On the contrary, *Tk*-RNase HII may fold to the native state with chaperons in vivo and then unfold slowly. It has been reported that the native conformation of α-lytic protease is less stable than its unfolded state (*45*). A pro-region facilitates the folding of α-lytic protease, and the large kinetic barrier to unfolding prevents unfolding over the protein's functional lifetime.

Comparison of E. coli RNase HI and T. thermophilus RNase HI. The stability and folding of RNase HI from the mesophilic bacterium *E. coli* (*Ec*-RNase HI) and the thermophilic bacterium *T. thermophilus* (*Tt*-RNase HI) have been studied in detail (*25*−*32*). Figure 5 depicts the temperature dependence of $\Delta G(H_2O)$ for three proteins. Both thermophilic proteins have a similar stability profile and greater stability than *Ec*-RNase HI at most temperatures. It has been reported that the folding and unfolding rates of *Tt*-RNase HI are similar to those of *Ec*-RNase HI (*28*). However, the unfolding rate of *Tk*-RNase HII is extremely slow compared to *Ec*-RNase HI and *Tt*-RNase HI; the unfolding reaction of *Tk*-RNase HII in water at 25 °C (6.0×10^{-10} s^{-1} at pH 9) is 10^5 times slower than that of *Ec*-RNase HI (1.1×10^{-5} s^{-1} at pH 5.5) and 10^4 times slower than that of *Tt*-RNase HI (4.0×10^{-6} s^{-1} at pH 5.5). In contrast, little difference was observed among these proteins in the refolding rates at 25 °C (*27*, *28*). These results indicate that the stabilization mechanisms of *Tk*-RNase HII and *Tt*-RNase HI are very different. Figure 6 displays Gibbs energy diagrams of the folding pathways for the three proteins at 25 °C. *Tt*-RNase HI and *Ec*-RNase HI have a partially folded intermediate (*25*, *28*), whereas *Tk*-RNase HII does not.

The difference in unfolding reaction between *Tk*-RNase HII and *Tt*-RNase HI may be due to a difference in the type of RNase H or a difference in the organism kingdom. *Tk*-RNase HII is a member of type 2 RNase H, and *Tt*-RNase HI belongs to type 1 RNase H (*22*, *23*). *T. kodakaraensis* belongs to Archaea, but *T. thermophilus* belongs to Bacteria (*23*).

The Remarkably Slow Unfolding Kinetics of Hyperthermophile Proteins. The unfolding reaction of *Tk*-RNase HII in water at 25 °C is 10^5 times slower than that of *Ec*-RNase HI, as described above. Similar remarkably slow unfolding rates by several orders of magnitude have been reported for other hyperthermophilic proteins. The unfolding rate of pyrrolidone carboxyl peptidase from *Pyrococcus furiosus* is 10^7 times slower at 25 °C and 10^5 times at 60 °C compared to that of mesophilic pyrrolidone carboxyl peptidase from *Bacillus amyloliquefaciens* (*15*). Similar results were reported for rubredoxin, dihydrofolate reductase, glycosidase, α-amylase, and ORF56 (*14*, *16*, *18*, *46*, *47*). However, these proteins are oligomer or monomer with irreversible unfolding or with multistate folding. Our results for *Tk*-RNase HII indicate that even a monomeric protein with reversible two-state folding unfolds extremely slowly. It has also been reported that the unfolding rate constant of cold shock protein from *Thermotoga maritima*, which is a monomer with reversible two-state folding, is 2 orders of magnitude slower than that of its mesophilic homologue (*48*, *49*). These results presented here, together with other reports, indicate that hyperthermophilic proteins often possess a more remarkable kinetic stability against denaturation than proteins from mesophiles.

The unfolding rate of oligomeric proteins from hyperthermophilic organisms was also found to be much slower than that of mesophilic proteins (*16*−*18*). However, the refolding rates are almost the same for homologous proteins. These results indicate that the slow unfolding rate, not fast folding, is a major strategy for hyperthermophilic proteins to function at higher temperatures, regardless of whether the protein is

a monomer or oligomer. It has also been reported that a mesophilic protease from *Streptomyces griseus*, which is an extracellular protein, shows unusual, slow unfolding upon denaturation (*50*).

What factors contribute to unusually slow unfolding? Machius et al. (*47*) demonstrated that the introduction of hydrophobic residues at the surface of mesophilic α-amylase results in kinetic stabilization. Schuler et al. (*49*) reported the important role of entropic factors for the slow unfolding of cold shock proteins. The mechanism of "remarkably" slow unfolding is presently unclear. Further studies of *Tk*-RNase HII will provide greater understanding.

CONCLUSIONS

We examined GdnHCl-induced unfolding and refolding and heat-induced denaturation of *Tk*-RNase HII to clarify the energetic features of monomeric protein from a hyperthermophile. *Tk*-RNase HII exhibits very stable and reversible two-state folding against a denaturant and heat-induced denaturation. The stabilization mechanism of *Tk*-RNase HII was revealed to originate from the remarkably slow unfolding rate. This extremely slow unfolding rate has been observed in other oligomeric proteins from hyperthermophiles. This appears to be one of common characteristics of hyperthermophilic proteins. Therefore, *Tk*-RNase HII is an excellent model for investigations into the stability and folding of hyperthermophilic proteins.

NOTE ADDED AFTER ASAP POSTING

This paper was inadvertently published 10/12/04. ΔG, K, T_m, ΔH_m, and ΔC_p values were changed in the abstract, the paragraph following eqs 7 and 8, Table 1, and the paragraph following eq 9. The correct version was published 10/14/04.

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