Stability of ribonuclease T2 from *Aspergillus oryzae*

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Abstract

The stability of ribonuclease T2 (RNase T2) from *Aspergillus oryzae* against guanidine hydrochloride and heat was studied by using CD and fluorescence. RNase T2 unfolded and refolded reversibly concomitant with activity, but the unfolding and refolding rates were very slow (order of hours). The free energy change for unfolding of RNase T2 in water was estimated to be 5.3 kcal·mol⁻¹ at 25 °C by linear extrapolation method. From the thermal unfolding experiment in 20 mM sodium phosphate buffer at pH 7.5, the T_m and the enthalpy change of RNase T2 were found to be 55.3 °C and 119.1 kcal·mol⁻¹, respectively. From these equilibrium and kinetic studies, it was found that the stability of RNase T2 in the native state is predominantly due to the slow rate of unfolding.

Keywords: stability; RNase T2; unfolding and refolding mechanism

Ribonuclease (RNase) T2 was first reported by Sato and Egami (1957) as a ribonuclease in Taka-diestase produced from a culture extract of *Aspergillus oryzae* and purified independently by Rushizky and Sober (1963) and Uchida (1966). RNase T2 exerts its catalytic activity on the phosphodiester bonds of RNA with a preference for adenylic acid residues without an absolute base specificity (Rushizky & Sober, 1963). For the reason of this non-specificity, RNase T2 is often used in molecular genetic studies.

RNase T2 is a 29-kDa monomeric glycoprotein composed of 239 amino acid residues and about 8% oligosaccharides and has five disulfide bonds in its tertiary structure (Kawata et al., 1988). Gene cloning of this enzyme was also performed by Ozeki et al. (1991). The catalytic amino acid residues of RNase T2 were found to be His 53 and His 115 by chemical modification and NMR experiments (Kawata et al., 1990). From the sequence alignment between RNase T2 and other proteins, Kawata et al. (1990) found that high sequence similarities to the regions around the two catalytic histidine residues of RNase T2 could be observed in a 32-kDa S2 stylar glycoprotein (Anderson et al., 1986) from *Nicotiana glauca*, which was expressed in a gametophytic self-incompatible stylar. From this fact, Kawata et al. proposed that the S2 glycoprotein is a kind of RNase. McClure et al. (1989) subsequently determined that the S2 glycoprotein is an RNase by activity measurements. This enzyme, called S-RNase, was suggested to be very important in the mechanism of gametophytic self-incompatibility in flowering plants (Haring et al., 1990; McClure et al., 1990; Clarke & Newbigin, 1993). Consequently, studies of structural stability and the folding

mechanism of RNase T2 may provide valuable information regarding S-RNase, and ultimately, the mechanism of self-incompatibility. Also, detailed structural characterization of RNase T2 is a prerequisite in studies regarding the catalytic properties of this enzyme.

In this paper, we have studied the stability of RNase T2 from *A. oryzae* against guanidine hydrochloride (Gdn-HCl) and heat and found that the unfolding and refolding mechanism of RNase T2 could be expressed by a two-state transition. As the micro rate constants of unfolding and refolding reactions were very slow, it was suggested that RNase T2 in the native state is stable mainly due to the unusual slow rate of unfolding.

Results

CD and fluorescence spectra

Figure 1 shows the near- and far-UV CD spectra of native and unfolded RNase T2. A large negative CD maximum peak at 208 nm with a shoulder peak of 220 nm and a small positive CD maximum peak at 232 nm were observed for native RNase T2. By using the method of Chen et al. (1972), the α -helix content of RNase T2 was estimated to be 18%. In the presence of 4 M Gdn-HCl, the far-UV CD signal decreased, indicating that the tertiary structure of RNase T2 unfolded completely. At 65 °C, RNase T2 was unfolded, although the CD spectrum was slightly different from that in 4 M Gdn-HCl.

The fluorescence spectrum of RNase T2 excited at 280 nm in 0 M Gdn-HCl had a maximum at 335 nm, as shown in Figure 2. With increasing concentrations of Gdn-HCl, the maximum fluorescence wavelength was shifted from 335 nm to 350 nm and the intensity was lowered, indicating that the fluorescent resi-

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Fig. 1. CD spectra of RNase T2: in 20 mM Tris-HCl buffer at pH 7.5 and 25 °C (curve 1); in 20 mM Tris-HCl buffer at pH 7.5 and 25 °C containing 4 M Gdn-HCl (2); in 20 mM sodium phosphate buffer at pH 7.5 at 65 °C (3). Protein concentrations were 5 μ M.

dues (Tyr and Trp) of the RNase T2 were exposed to the solvent upon unfolding. In 4 M Gdn-HCl, the fluorescence intensity was about 50% of native RNase T2.

Unfolding equilibrium

In order to estimate the structural stability of RNase T2, we have studied unfolding and refolding equilibrium reactions by increasing the concentration of Gdn-HCl. Figure 3 shows the unfolding curves obtained with Gdn-HCl of RNase T2 in terms of the ellipticity at 222 nm and the fluorescence at 335 nm. Because identical curves were obtained by using the enzyme unfolded in 4 M Gdn-HCl as starting material, the unfolding transition was found to be reversible. The transition curves were normalized by assuming that ellipticities or fluorescence for the native and unfolded proteins that were observed before and after the tran-

Fig. 2. Fluorescence spectra of RNase T2 at 0 M (curve 1), 1.85 M (2), 2 M (3), and 3 M (4) Gdn-HCl in 20 mM Tris-HCl buffer at pH 7.5 and 25 °C. Ordinate represents the fluorescence relative to that in the absence of Gdn-HCl. Excitation was at 280 nm. Spectra were corrected for solvent Raman scattering. Protein concentrations were 2 μ M.

Fig. 3. Unfolding transitions by Gdn-HCl of RNase T2 measured in terms of the change in CD at 222 nm (●) and fluorescence intensity at 335 nm (▲). Fluorescence was measured with 280-nm light for excitation. Inset shows the unfolding transition represented in terms of the fraction of unfolded protein (f_D). Solid curves in the transition region are theoretical curves based on the parameters given in the text.

sition zone, respectively, can be extrapolated linearly into the transition zone. As can be seen in the inset of Figure 3, the unfolding transition curve measured by CD agreed well with the curve measured by fluorescence. The Gdn-HCl concentration at the midpoint of RNase T2 unfolding was 1.9 M.

Kinetics of unfolding and refolding

The kinetics of unfolding by Gdn-HCl of RNase T2 were measured by the change in fluorescence. Surprisingly, the unfolding rate of RNase T2 was so slow that we could measure it by manual mixing even at 6 M Gdn-HCl. The total kinetic changes in fluorescence measured for unfolding in a Gdn-HCl concentration range from 1.8 to 6 M were equal to the equilibrium fluorescence changes and were described by a single-exponential decay term.

The kinetics of refolding of RNase T2 were also measured by monitoring the fluorescence. The refolding reactions from 4 M Gdn-HCl were also slow enough to be able to be measured by manual mixing. The kinetic and equilibrium changes in fluorescence were equal also for refolding. The refolding kinetics inside the transition zone (1.0–1.9 M Gdn-HCl) were also described by a single-exponential decay term. Because the intensity at zero time obtained by extrapolation was identical to the fluorescence of the RNase T2 in 4 M Gdn-HCl (data not shown), it may be said that any fast phase that escaped observation was not present in the refolding kinetics in the transition zone. For the refolding kinetics below the denaturant concentrations of 0.4 M Gdn-HCl, however, a fast phase was detected. This phase accounted for about 30% of the amplitude of the total fluorescence change and an apparent rate constant greater than the main phase. Double-jump experiments (Brandts et al., 1975; Nall et al., 1978) have often been used under these circumstances to examine whether two forms of the unfolded species exist in the unfolded state. In the case of RNase T2, however, because the unfolding rate was very slow (complete unfolding requires 2 or 3 min even in 6 M Gdn-HCl), we could not perform the double-jump experiment.

Figure 4 (closed symbols) shows the apparent rate constants for the unfolding and refolding at various concentrations of

Fig. 4. Dependence on Gdn-HCl concentration of the apparent rate constant (●, ▲) and the microscopic rate constants (○, △) estimated on the basis of Mechanism 1 for folding of RNase T2: ●, from unfolding kinetics; ▲, from refolding kinetics; ○, microscopic unfolding rate constant, k_1 ; △, microscopic refolding rate constant, k_2 . Initial concentrations of Gdn-HCl for unfolding and refolding measurements were 0 and 4 M, respectively. Final protein concentration was about 2 μ M.

Gdn-HCl. Between 1.6 M and 2.0 M of Gdn-HCl, the apparent rate constants obtained from the unfolding experiments agreed well with those obtained from the refolding experiments. There was a minimum at 1.9 M Gdn-HCl, which was the same as the midpoint concentration of the unfolding and refolding transition curve.

Thermal unfolding

In order to obtain more detailed characteristics for structural stability of RNase T2, we have also studied the thermal unfolding of the enzyme. Figure 5 (closed circles) shows the thermal unfolding curves of RNase T2 in terms of the ellipticity at 222 nm in 20 mM sodium phosphate buffer at pH 7.5. As can be seen in the case of the unfolding by Gdn-HCl, the thermal unfolding was also reversible. Identical thermal transition curves were obtained for up and down thermal scans under the conditions described in the Materials and methods. The midpoint of the transition curve was 55.3 ± 0.2 °C.

Functional stability

Next, to also examine the functional stability of RNase T2 against heat, we measured the activities of the samples that were incubated at various temperatures for 5 min and then refolded at room temperature for 1 h. The refolding buffer used was the same as that of the thermal unfolding experiment. Residual activities are shown in Figure 5 (closed triangles). Up to 60 °C, full enzyme activities were observed after refolding. The sample that was refolded from 4 M Gdn-HCl solution by dilution also had more than 90% of its original activity. Thus, it was found that RNase T2 was capable of refolding almost completely from the unfolded state produced not only by Gdn-HCl but also by heat.

Discussion

Structural stability

Assuming a two-state transition, we have analyzed the unfolding curve shown in Figure 3 and obtained the free energy of un-

Fig. 5. Thermal unfolding transitions of RNase T2 (●) and residual activities (▲) after incubation for 5 min at various temperatures. Thermal unfolding transition was measured in terms of the change in CD at 222 nm. Protein concentrations were about 2 μ M. Note that residual activities were measured after refolding for 1 h at room temperature.

folding at a given concentration of Gdn-HCl (ΔG_D) from the equation:

$$\Delta G_D = -RT \cdot \ln[f_D / (1 - f_D)], \quad (1)$$

where f_D is the fraction of the unfolded molecule. ΔG_D was found to vary linearly with Gdn-HCl concentration (data not shown), and a least-squares analysis was used to fit the data to the equation (Pace et al., 1989):

$$\Delta G_D = \Delta G_D^{\text{H}_2\text{O}} - m[\text{Gdn-HCl}], \quad (2)$$

where $\Delta G_D^{\text{H}_2\text{O}}$ is the value of ΔG_D in the absence of Gdn-HCl and m is a measure of the dependence of ΔG_D on Gdn-HCl concentration. The values of $\Delta G_D^{\text{H}_2\text{O}}$ and m were found to be 5.3 kcal·mol⁻¹ and 2.9 kcal·mol⁻¹·M⁻¹, respectively. These values were not anomalous compared to other proteins (Hamaguchi, 1992).

When we estimated $\Delta G_D^{\text{H}_2\text{O}}$ by using the equation proposed by Tanford (1970):

$$\Delta G_D = \Delta G_D^{\text{H}_2\text{O}} - \Delta n \cdot RT \cdot \ln(1 + ka_{\pm}), \quad (3)$$

where Δn is the difference in the number of binding sites between the unfolded and folded states, k (0.6 M⁻¹; Pace et al., 1989) is the average binding constant of the sites, and $a_{\pm}$ is the activity of Gdn-HCl, we obtained the values of $\Delta G_D^{\text{H}_2\text{O}} = 8.0$ kcal·mol⁻¹ and $\Delta n = 31$. It is generally observed that the value of $\Delta G_D^{\text{H}_2\text{O}}$ estimated by the ligand binding model is larger than the value estimated by linear extrapolation (Pace et al., 1989; Okajima et al., 1990).

Kinetics of unfolding and refolding

Although in the region below 0.4 M Gdn-HCl the refolding kinetics could not be described by a single-exponential term, the unfolding and refolding kinetics inside and above the transition zone were described by a single-exponential term. Then, we assume that the unfolding and refolding kinetics of RNase T2 in this region follow the two-state transition:

where N is the native protein, U is the unfolded one, and k_1 and k_2 are the unfolding and refolding rate constants, respectively. The apparent rate constant was minimal at 1.9 M Gdn-HCl, which corresponds to the midpoint of the unfolding and refolding transition (Fig. 3). This fact also suggests that the unfolding and refolding kinetics follow the two-state transition (Tanaka et al., 1989). The values of k_1 and k_2 were estimated as follows. Inside the transition zone, the apparent rate constant and equilibrium constant ($K = [U]/[N]$) are expressed by:

$$k_{app} = k_1 + k_2 \quad (4)$$

$$K = k_1/k_2. \quad (5)$$

From the values of K estimated from the transition curve (Fig. 3) and the values of k_{app} (Fig. 4), the values of k_1 and k_2 were determined and plotted also in Figure 4 (open symbols). As can be seen in this result, the unfolding and refolding rates of RNase T2 by Gdn-HCl are very slow (in the order of hours to days). Although the same observation was also found in an immunoglobulin C_{H2} domain (Sumi & Hamaguchi, 1982), these very slow unfolding and refolding kinetics have rarely been found for monomeric proteins.

Thermal stability

The thermal unfolding curve was analyzed by assuming a two-state approximation. The enthalpy change (ΔH_m) upon unfolding was evaluated by using the van't Hoff equation (data not shown). Because $\Delta G_D = 0$ at $T = T_m$, the entropy change (ΔS_m) can be estimated. The values of ΔH_m and ΔS_m of RNase T2 were 119.1 ± 0.8 kcal·mol⁻¹ and 363 ± 2 e.u., respectively. At a given temperature (T), $\Delta G_{(T)}$ can be obtained by the Gibbs-Helmholtz equation:

$$\Delta G_{(T)} = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \cdot \ln(T/T_m)], \quad (6)$$

where ΔC_p is the difference in heat capacity between the folded and unfolded conformations. Pace and Laurents (1989) showed that ΔC_p can be estimated with considerable accuracy by using Equation 6 when ΔG is measured at temperatures considerably below T_m . We assumed that this estimation method is also applicable to the present case of RNase T2 and estimated the value of ΔC_p . The value was calculated to be 3.95 kcal·mol⁻¹·deg⁻¹ using $T_m = 55.3$ °C, $\Delta H_m = 119.1$ kcal·mol⁻¹, and $\Delta G = 5.3$ kcal·mol⁻¹ at 25.0 °C.

By using these values, the experimental data from the Gdn-HCl and thermal unfolding curves for RNase T2 are shown in Figure 6 along with the theoretical curve calculated with Equation 6. The temperature of maximum stability, T_s , where $\Delta S = 0$, can be calculated by using $T_s = T_m \exp(-\Delta H_m \cdot T_m^{-1} \cdot \Delta C_p^{-1})$. The calculated value of T_s for RNase T2 was 26.5 °C. The T_s values of proteins generally fall between -10 °C for a relatively hydrophilic protein and 35 °C for a relatively hydrophobic protein (Pace & Laurents, 1989). Thus, RNase T2 seems to be a rel-

Fig. 6. ΔG as a function of temperature for the unfolding of RNase T2 in 20 mM sodium phosphate buffer at pH 7.5. The point at 25 °C (●) is the $\Delta G_D^{\text{H}_2\text{O}}$ value from the Gdn-HCl unfolding curve (Fig. 3), and those above 50 °C (○) are from the thermal unfolding curve used to determine the values of T_m and ΔH_m . Solid curve is a theoretical curve from Equation 6 using the ΔT_m , ΔH_m , and ΔC_p values cited in the text.

atively hydrophobic protein and could be most stable at room temperature.

Finally, with regard to the functional stability of RNase T2, we found that the activity of this enzyme regained almost completely after refolding from the unfolded state in 4 M Gdn-HCl or at 65 °C (Fig. 5). This high reversibility enabled us to purify RNase T2 effectively by heat treatment (78–80 °C, for 5 min) in the preparation procedure (Kawata et al., 1988). Moreover, as shown in Figure 4, RNase T2 has considerably slow unfolding rate constants. This unusually slow rate of unfolding is probably the main reason for the structural high stability in the native state of RNase T2, and hence, is very important for its functional characteristics.

Materials and methods

Materials

RNase T2 was purified from Taka-diastase powder (Sanzyme R, a commercial product of Sankyo Co., Ltd.) as described previously (Kawata et al., 1988). Gdn-HCl (specially purified grade) was obtained from Nacalai Tesque (Kyoto). Other reagents were of the highest grade commercially available.

CD measurement

CD measurements were carried out with a Jasco spectropolarimeter, model J-500A, equipped with a data processor (DP-501). The CD instrument was calibrated with *d*-10-camphorsulfonic acid. The results were expressed as mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = (100 \times \theta_{\text{obs}})/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the residue molar concentration of the protein, and l is the length of the light path in centimeters. For calculation of c , a value of 108 was used as the mean residue molecular weight. The temperature was controlled at 25 ± 0.2 °C with a thermostatically controlled cell holder.

Fluorescence measurements

Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, model MPF-4, equipped with a spectral corrector. The temperature was controlled at 25 °C with a thermostatically controlled cell holder.

Unfolding equilibrium by Gdn-HCl

All the measurements in the present studies were carried out in 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer at pH 7.5 and $25 \pm 0.1^\circ\text{C}$. Prepared samples were allowed to stand for 24 h at 25°C before measurements. Unfolding transition curves were measured by fluorescence at 335 nm with 280-nm light for excitation and by CD at 222 nm at various concentrations of Gdn-HCl. Protein concentrations were $5\ \mu\text{M}$ for the CD measurements and $2\ \mu\text{M}$ for the fluorescence measurements.

Thermal unfolding

Thermal unfolding curve was measured in terms of the ellipticity at 222 nm. The temperature was increased continuously at a rate of $0.5^\circ\text{C}/\text{min}$ and was monitored with a Sentelek Model BAT-12 thermometer. The reversibility was examined by measuring the ellipticity by lowering the temperature at a rate of $1.5^\circ\text{C}/\text{min}$. The buffer used was 20 mM sodium phosphate buffer at pH 7.5. Protein concentration was about $2\ \mu\text{M}$.

Kinetics of unfolding and refolding by Gdn-HCl

Unfolding and refolding kinetics were measured in terms of the fluorescence at 335 nm. The excitation wavelength was set at 280 nm for both measurements. A protein solution (0.1 mL) at pH 7.5 was mixed with 2.9 mL of the buffer at pH 7.5 and Gdn-HCl at a given concentration. Initial concentrations of Gdn-HCl for the unfolding and refolding measurements were 0 and 4 M, respectively.

All the kinetic data can be described by an equation of the form:

$$F(t) - F(\infty) = \Sigma \alpha_i \exp(-k_{app,i} \cdot t), \quad (7)$$

where $F(t)$ is the fluorescence at time t , $F(\infty)$ is the fluorescence after equilibration, $k_{app,i}$ is the apparent rate constant of phase i , and α_i is the amplitude of the phase.

Assay for RNase T2

RNase activity was assayed at 37°C with yeast RNA as a substrate, as reported previously (Kawata et al., 1988).

Protein concentration

The protein concentration of RNase T2 was determined spectrophotometrically by using an absorption coefficient of 2.16 at 280 nm for a 1-mg/mL solution in a 1.0-cm cell (Kawata et al., 1988).

pH measurement

pH was measured with a Radiometer PHM26c meter at 25°C .

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