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Heat denaturation and cold denaturation of *Escherichia coli* RNase HI investigated by circular dichroism[1]

Tomoko Yamasaki, Shigenori Kanaya, Motohisa Oobatake*

Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan

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Abstract

Circular dichroism has been used to investigate the thermodynamics of the thermal unfolding of *Escherichia coli* ribonuclease HI as a function of pH over the pH range 0–4. This protein undergoes a reversible thermal conformational change from the native state to the denatured state with an isodichroic point. The calculated thermodynamic values at pH 3.0 are as follows: $t_m = 50.2°C$, ΔH_m (t_m) = 93.8 kcal mol^{-1}, ΔG (25°C) = 6.08 kcal mol^{-1}, and ΔG (10°C) = 8.14 kcal mol^{-1}. At pH 4, ΔG (25°C) = 10 kcal mol^{-1} and ΔG (10°C) = 12 kcal mol^{-1}. At a pH below 2, this protein denatures at 25°C with ΔG (25°C) = −1 kcal mol^{-1}, but it is stable at 10°C with ΔG (10°C) = 2 kcal mol^{-1}. The ΔC_p value determined from the $\Delta H_m(T_m)$ versus T_m plot is 1.4 kcal mol^{-1} K^{-1}. The thermal unfolding curves at pH values above 2.18 showed a highly cooperative thermal transition. Cold denaturation was observed at temperatures below 10°C between pH 2.03 and 1.55. A cooperative heat denaturation was also observed over this pH range. At pH values lower than 1.45, cold denaturation was not observed. A broad thermal transition was observed between pH 0.83 and 0.53.

Keywords: Heat denaturation; Cold denaturation; *Escherichia coli* RNase HI; Circular dichroism

1. Introduction

Ribonuclease H (RNase H) (EC 3.1.26.4) is an endo-nuclease that specifically cleaves the RNA strand of a DNA/RNA hybrid. The structure and functional relationship of *Escherichia coli* (*E. coli*) RNase HI have been extensively studied by X-ray analyses

* Corresponding author.

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[1,2], site-directed mutagenesis [3,4], and nuclear magnetic resonance (NMR) [5,6]. This enzyme is composed of a single polypeptide chain of 155 amino acid residues, with an isoelectric point (p*I*) of 9.0 [4].

When the temperature is increased from room temperature, a protein usually denatures because of the increase in the entropy of the polypeptide chain. A protein also unfolds with decreasing temperature. This phenomenon, known as cold denaturation, has been observed in the presence of denaturant or at low pH [7]. Cold denaturation is accounted for mainly by the hydration enthalpy, which favors the denatured state because the amount of hydrated water in the denatured state is greater than that in the native state [8].

The thermal denaturation of a protein reveals the nature of the forces that stabilize the folded conformation of globular proteins in solution. In a previous paper [9], we examined the thermal stability of *E. coli* RNase HI at various pHs. In this study, we have extended these experimental results for RNase HI using circular dichroism (CD) and have observed cold denaturation over the pH range 1.5–2.0.

2. Materials and methods

2.1. Materials

E. coli RNase HI was prepared and purified as described previously [9]. The protein concentration was determined from ultraviolet (UV) absorption with an absorption coefficient of $A_{280}^{0.1\%} = 2.02$ [10]. The molecular weight was calculated from the amino acid sequence as 17 597 Da.

2.2. CD spectra

CD spectra were measured on a Jasco J-720 spectropolarimeter equipped with a water-circulating cell holder. The spectra were obtained in 10 mM glycine–HCl buffer using a 1-mm path-length cell. The protein concentration was 0.12 mg ml^{-1}. The instrument was calibrated with D-pantolactone [11]. CD spectra are expressed as the mean residue ellipticity ($[\theta]$, deg cm^2 $dmol^{-1}$), using a mean residue weight of 113.53. Spectra for CD between 190 and 250 nm were obtained using a scanning speed of 20 nm min^{-1}, a time response of 1 s, a bandwidth of 1 nm, and an average over 10 scans.

2.3. CD-detected thermal denaturation

Thermal denaturation curves were determined by monitoring the CD value at 220 nm in a Jasco J-600 spectropolarimeter, with increasing temperature measured by a Takara D641 thermistor, using a 2-mm path-length cell [12]. Proteins were dissolved in 10 mM glycine–HCl buffer at a concentration of 0.12 mg ml^{-1}. The heating rate was about 0.6°C min^{-1}. The thermal unfolding of this protein exhibits poor solubility at pH values greater than 4.4, especially at the isoelectric point (p*I* 9.0). However, at an acidic pH below 4.3, high reversibility (greater than 90%), defined as the recovery of the ellipticity at 220 nm, was observed when the temperature was raised to 90°C and then lowered to the

initial temperature. The experimental errors in the ellipticity due to noise are within about 3%.

2.4. Analysis of denaturation curves

The fraction of native protein was calculated from the CD values by linearly extrapolating the pre- and post-transition baselines, respectively, based on the assumption that the unfolding equilibrium follows a two-state mechanism, i.e. the native state and the denatured state. The temperature of the midpoint of the transition (T_m in K) was calculated from curve fitting of the normalized CD values by a non-linear least-squares method. The enthalpy change $\Delta H_m(T_m)$ at T_m was calculated by van't Hoff analysis. The unfolding Gibbs free energy $\Delta G(T)$, at temperature T, is calculated using the following equation:

$$\Delta G(T) = \Delta H_m(T_m) + \Delta C_p (T - T_m) - T\Delta H_m(T_m)/T_m - T\Delta C_p \ln(T/T_m) \quad (1)$$

where ΔC_p is the heat capacity change upon unfolding. The ΔC_p value was determined from the $\Delta H_m(T_m)$ versus T_m plot, using the CD measurement of this study, as 1.404 kcal mol^{-1} K^{-1} (5.784 kJ mol^{-1} K^{-1}).

3. Results and discussion

3.1. Temperature-dependence of CD spectra

The thermal unfolding spectra at pH 3.0 and 1.4 are shown in Fig. 1A,B, respectively. RNase HI has five α-helices (42% helical content) and a five-stranded β-sheet (28% β-sheet content) [1]. The spectra at low temperature (1°C at pH 1.4 or 10°C at pH 3.0) show the characteristic pattern for proteins with certain secondary structures. The spectrum at pH 3.0 exhibits a broad minimum from 210 to 220 nm; however, the spectrum at pH 1.4 has a single minimum at 207 nm and a shoulder at 220 nm. With increasing temperature, isodichroic points are observed at 205.2 nm with the ellipticity −10 900 deg cm^2 $dmol^{-1}$ (pH 1.4), and at 206.6 nm with the ellipticity −9600 deg cm^2 $dmol^{-1}$ (pH 3.0). The spectra at high temperature (50°C at pH 1.4 or 80°C at pH 3.0) are typical of a random-coil polypeptide. These thermal-denatured states (D_T) have a CD value (at 220 nm) of −5000 deg cm^2 $dmol^{-1}$, which is independent of the pH, as shown in Fig. 2A,B. The guanidine hydrochloride (GdnHCl)-denatured (D_G) states also exhibit the typical spectra of a random-coil polypeptide. The CD values in the D_G state at pH 1.8 (10°C) are −3900, −1700, and −800 deg cm^2 $dmol^{-1}$ in 3, 4, and 6 M GdnHCl, respectively. The CD values in the D_G state at pH 2.9 and 5.5 (10°C) are −600 deg cm^2 $dmol^{-1}$ in 6 M GdnHCl. These values are lower in magnitude than the −5000 of the D_T state, indicating that the GdnHCl-denatured state is different from the thermal-denatured state.

3.2. Thermal denaturation

The thermal transitions of secondary structures at various pHs were examined, as

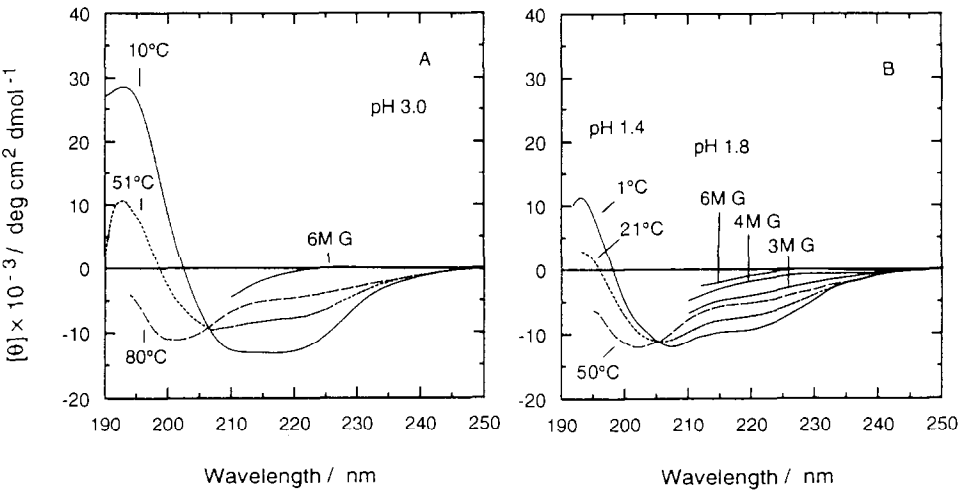

Fig. 1. (A) CD spectra of RNase HI at pH 2.95 and at 10°C (native state, ——), 51°C (·····), and 80°C (denatured state, – – –) in 10 mM glycine–HCl buffer with a protein concentration of 0.12 mg ml^{-1}. GdnHCl-denatured CD spectra (6 M G) at 10°C and at pH 2.9 in 10 mM glycine–HCl and 6 M GdnHCl, containing 0.23 mg ml^{-1} protein. (B) CD spectra of RNase HI (0.12 mg ml^{-1}) at 1°C (native state, ——), 21°C (·····), and 50°C (denatured state, – – –) and at pH 1.4 in 10 mM glycine–HCl buffer. GdnHCl-denatured CD spectra (0.12 mg ml^{-1}; 3 M, 4 M, and 6 M G) at 10°C and at pH 1.8 in 10 mM glycine–HCl and 3 M, 4 M, and 6 M GdnHCl, respectively.

shown in Fig. 2A,B. The thermal unfolding curves at pH values above 2.18 show a highly cooperative thermal transition. Cold denaturation is observed at temperatures below 10°C between pH 2.03 and 1.55. A cooperative heat denaturation is also observed over this pH range [13]. At a pH lower than 1.45, cold denaturation is not observed, as shown in Fig. 2B. A broad thermal transition is observed between pH 0.83 and 0.53. This broad transition has also been observed recently for a mutant of *E. coli* RNase HI with the three native cysteines replaced by alanines [14]. At pH 0.25, the protein denatures at a low temperature and does not show the thermal transition. Interestingly, the CD values at thermal denaturation temperatures show the same value (–5000 deg cm^2 $dmol^{-1}$) over the pH range 0.53–3.66 (Fig. 2A,B), indicating that the thermal-denatured state is not dependent on either the pH or the initial state.

3.3. Characterization of thermodynamic quantities

We normalized the denaturation curves expressed in $[\theta]$ and fitted the normalized curves by a non-linear least-squares method to obtain the thermodynamic quantities. Table 1 summarizes the results from the CD measurements. The notable point is that high reversibility (Rev in Table 1), greater than 90%, was observed over the pH range 0.53–4.34 when the temperature was raised to, e.g. 90°C, defined as t_{final} in Table 1, and lowered to the initial temperature. However, the reversibility of the thermal unfolding was not always 100%. It has been reported that one type of irreversible inactivation of lyso-

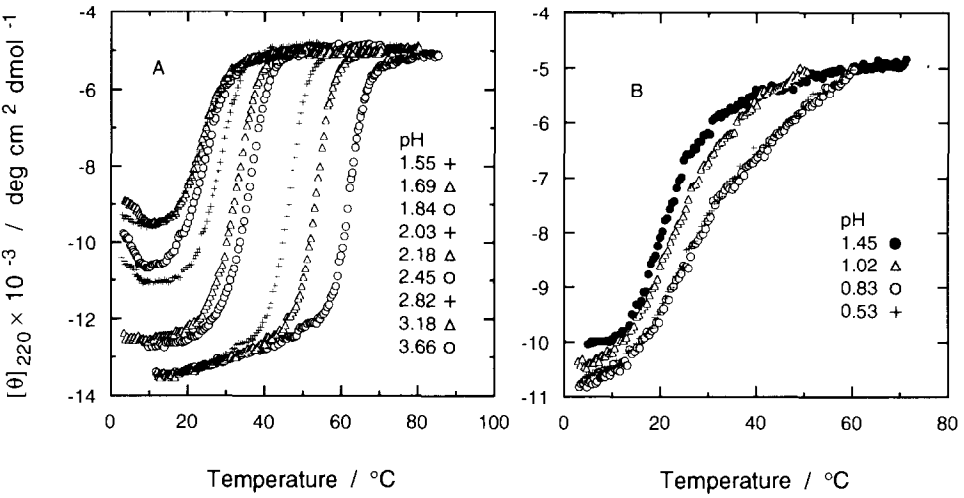

Fig. 2. Temperature dependence of the ellipticity at 220 nm and at several pHs in 10 mM glycine–HCl buffer with a protein concentration of 0.12 mg ml^{-1}. Figures indicate pHs with increasing thermal stability. (A) The pHs were 1.55 (+), 1.69 (△), 1.84 (○), 2.03 (+), 2.18 (△), 2.45 (○), 2.82 (+), 3.18 (△), and 3.66 (○). (B) The pHs were 1.45 (+), 1.02 (△), 0.83 (○), and 0.53 (+).

zyme is due to the hydrolysis of the peptide bonds at the aspartic acid residues at pH 4 and 100°C [15]. We have therefore examined whether such a hydrolysis resulted in an incomplete reversibility, using SDS-PAGE as described previously [9]. No peptide fragment was observed at either pH 3 or 1.5 when the t_{final} was 60°C. However, only a small amount of cleavage was observed at the t_{final} of 70°C, but the amount of cleavage increased as the increase of t_{final} values greater than 70°C. Thus, the incomplete reversibility of the thermal unfolding, which is dependent on the final temperature and the incubation time from t_m to t_{final}, was shown to be due to the hydrolysis of the peptide bonds. Next, the value of the reversibility at pH 1.84 was 110%, which exceeds the expected maximum value of 100%. A similar phenomenon was also observed over the pH range 1.52–2.03 for t_{final} values lower than 60°C. In these cases, the type of initial spectrum at pH 1.4 (1°C) in Fig. 1B changed to the type of spectrum at pH 3 (10°C) on cooling, shown in Fig. 1A, which resulted in an increase in the absolute CD value at 220 nm. The exceptional decrease in the absolute CD value, by 7–12% over this pH range, was probably due to acid hydrolysis for t_{final} values greater than 70°C (ex. at pH 1.94, Rev = 88%, and t_{final} = 85°C).

Fig. 3 shows t_m as a function of pH from the CD results. Using a least-squares analysis between pH 2 and 3.5, t_m is given by

$$t_m\,(°C) = a\,\mathrm{pH} - b \tag{2}$$

where a is 22.97 ± 0.49°C pH^{-1}, b is 18.69 ± 1.34°C, and the correlation coefficient is 0.99. The stability decreases as the pH decreases. Assuming a simple two-state transition

Table 1

Thermodynamic parameters from far-UV CD thermal denaturation curves for the unfolding of *E. coli* RNase HI[a]

pH	t_m /°C	$\Delta H_m(t_m)$[b] /kcal mol^{-1}	$\Delta H_m(t_m)$ /kJ mol^{-1}d	Rev[c] (in %)	t_{final} /°C
0.25	No[e]				
0.53	23.6	41.2	172.3	95	60
0.83	23.9	37.3	156.1	96	60
1.02	20.4	34.7	145.0	98	50
1.07	24.9	35.3	147.9	88	74
1.08	22.3	49.6	207.4	102	50
1.14	21.5	51.5	215.4	101	60
1.24	21.5	48.7	203.7	103	60
1.28	23.0	52.7	220.4	100	60
1.30	21.2	56.9	237.9	101	55
1.31	20.9	45.5	190.4	94	74
1.40	20.3	52.9	221.3	91	80
1.41	21.0	46.1	192.7	98	50
1.45	21.8	52.0	217.6	101	71
1.52	21.5	55.3	231.4	107	50
1.55	20.8	54.4	227.7	104	50
1.55	21.7	53.4	223.4	93	80
1.62	20.6	56.4	236.1	100	70
1.63	21.9	55.7	232.9	108	50
1.67	23.6	70.6	295.3	108	60
1.69	22.9	63.3	265.0	110	50
1.71	21.1	58.3	243.9	98	75
1.75	21.7	72.5	303.1	90	70
1.76	25.2	78.5	328.3	101	50
1.79	23.9	60.7	254.0	104	80
1.84	23.9	64.8	270.9	110	50
1.94	24.8	74.6	312.3	88	85
2.00	26.5	61.3	256.4	98	71
2.03	28.7	67.9	284.2	109	55
2.04	28.5	70.6	295.5	95	50
2.15	29.9	67.1	280.7	92	70
2.18	32.5	63.5	265.6	90	70
2.22	33.0	65.8	275.2	95	70
2.26	33.0	68.5	286.4	90	70
2.39	35.5	66.7	367.1	95	70
2.45	35.4	71.8	300.3	96	70
2.53	39.8	76.0	317.9	92	70
2.65	42.3	81.3	340.0	91	75
2.79	46.1	89.0	372.3	92	70
2.82	46.7	93.0	389.2	92	75
2.86	46.4	80.5	336.7	97	75
2.95	50.1	91.6	383.3	92	80
3.06	50.7	101.6	425.1	90	90
3.10	53.9	105.6	441.8	85	85
3.11	52.2	100.7	421.5	91	85
3.18	54.0	101.9	426.5	93	80

Table 1 (*continued*)

pH	t_m /°C	$\Delta H_m(t_m)$[b] /kcal mol^{-1}	$\Delta H_m(t_m)$ /kJ mol^{-1}[d]	Rev[c] (in %)	t_{final} /°C
3.25	55.1	109.4	457.7	94	85
3.26	58.9	96.6	404.3	95	90
3.34	57.6	100.0	418.3	94	80
3.38	57.4	101.2	423.3	96	80
3.64	62.1	106.2	444.3	93	85
3.66	62.3	103.4	432.6	94	85
3.98	66.0	125.2	523.8	93	90
4.00	66.6	112.0	468.4	94	90
4.34	67.7	112.2	469.4	96	93

[a]Experimental conditions include 10 mM glycine–HCl buffer and a protein concentration of 0.12 mg ml^{-1}. Average errors are ±1.5°C less than pH 2 and ±0.7°C above pH 2 in t_m and ±10% in ΔH_m.
[b]$\Delta H_m(t_m)$ is the enthalpy change of unfolding at the transition temperature (t_m).
[c]Rev is the reversibility defined as 100 $[\theta]_{220}$ (t'_i)/$[\theta]_{220}$ (t_i), using the ellipticity $[\theta]_{220}$ at 220 nm, where t_i is the initial temperature, from which the thermal denaturation starts, and t'_i is the same temperature cooled after the attainment of the final temperature (t_{final}).
[d]1 kcal mol^{-1} = 4.184 kJ mol^{-1}.
[e]No observation of thermal transition.

for denaturation, the change in the number of protons $\Delta\nu_m$ bound between the native and the denatured states at the transition temperature is expressed by the following equation [16]:

$$\Delta\nu_m = \frac{\Delta H_m(T_m)}{2.303RT_m^2}\frac{dT_m}{dpH} \tag{3}$$

where R is the gas constant. The values $\Delta\nu_m$ at pH 2.22, 2.95, and 3.34 are calculated to be 3.5, 4.4, and 4.6, using the t_m values of 33.0, 50.1, and 57.6°C and the ΔH_m values of 65.8, 91.6, and 100.0 kcal mol^{-1} (Table 1), respectively. The ionization constants (pK_a) of the carboxyl groups in *E. coli* RNase HI at 27°C in 0.1 M NaCl solution were determined from NMR [17]. The residues with pK_a values between 2.0 and 3.3, calculated from pH dependence of the chemical shifts of the carboxyl carbon resonances, are Asp 70 (pK_a, 2.6), Asp 94 (3.2), Asp 108 (3.2), and Glu 57 (3.2). The residues with pK_a values between 3.3 and 3.8 are Glu 32 (pK_a, 3.6), Glu 129 (3.6), and Val 155 (3.4). The former set of carboxyl groups corresponds to the change in the number of protons between pH 2 and 3.5 on unfolding. The latter set of carboxyl groups partly corresponds to $\Delta\nu_m$.

Fig. 4 shows the enthalpy change ΔH_m (t_m) at t_m as a function of t_m. Using a least-squares analysis between pH 2 and 3.5, ΔH_m is given by

$$\Delta H_m \text{ (kcal mol}^{-1}) = at_m \text{ (°C)} + b \tag{4}$$

where a is 1.404 ± 0.1 kcal mol^{-1} °C^{-1}, b is 23.27 ± 4.6 kcal mol^{-1}, and the correlation coefficient is 0.90. The ΔC_p value of 1.404 kcal mol^{-1} K^{-1} (5.784 kJ mol^{-1} K^{-1}) is lower

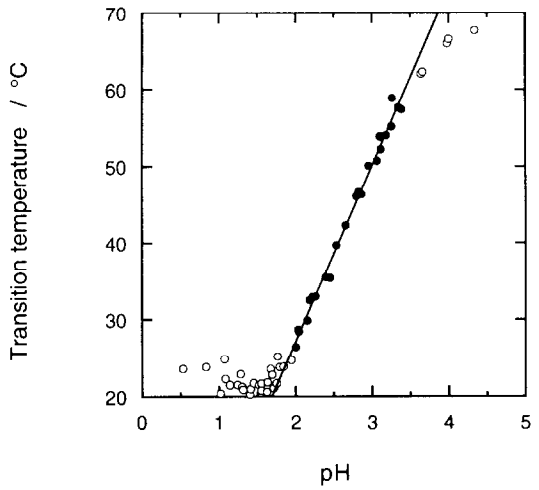

Fig. 3. Transition temperature as a function of pH for the thermal unfolding of RNase HI. (●) From the pH range 2–3.5; (O) from less than pH 2 and above 3.5. The line represents a least-squares analysis of the values from pH 2 to 3.5.

than that obtained from differential scanning calorimetry (DSC), 1.7 kcal mol^{-1} K^{-1} (Oobatake, unpublished result). Fig. 5 shows the unfolding Gibbs free energy ΔG values at 10°C and 25°C, as a function of pH, using Eq. (1). Since the ΔC_p value is obtained between pH 2 and 3.5, the calculation to obtain the ΔG (10°C) value should use a ΔC_p value determined at a pH below pH 2 (about 1.2 kcal mol^{-1} K^{-1} from DSC measure-

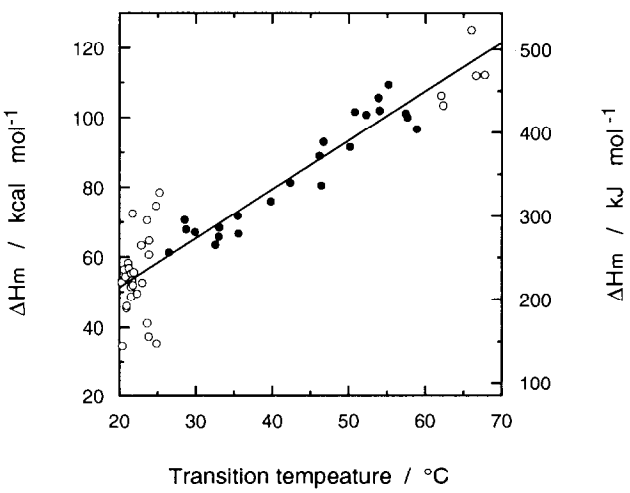

Fig. 4. ΔH_m as a function of the transition temperature for the thermal unfolding of RNase HI. (●) From the pH range 2–3.5; (O) from less than pH 2 and above 3.5. The line represents a least-squares analysis of the values from pH 2 to 3.5.

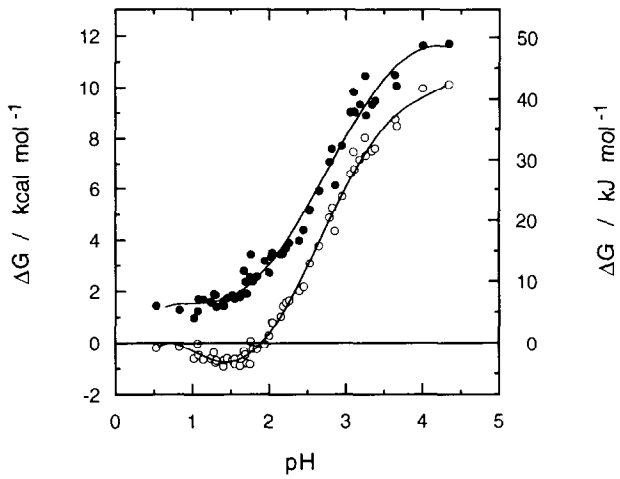

Fig. 5. ΔG as a function of pH for the thermal unfolding of RNase HI. (●) At 10°C; (○) at 25°C. The lines represent least-squares analyses of the values using a fifth-order polynomial expression.

ments; Oobatake, unpublished result). However, this modification slightly increases the ΔG (10°C) value by 0.13 kcal mol^{-1}. Using a non-linear least-squares analysis, with a fifth-degree polynomial expression over the pH range 0.5–4.3, ΔG is given by

$$\Delta G\ (10°C, \text{kcal mol}^{-1}) = 0.114pH^5 - 1.583pH^4 + 7.599pH^3 - 14.495pH^2 + 12.023pH - 2.122 \quad (5)$$

$$\Delta G\ (25°C, \text{kcal mol}^{-1}) = 0.236pH^5 - 3.060pH^4 + 14.065pH^3 - 26.605pH^2 + 20.608pH - 5.541 \quad (6)$$

At pH 4, ΔG (25°C) = 10 kcal mol^{-1} and ΔG (10°C) = 12 kcal mol^{-1}. An important point is that below pH 2, this protein denatures at 25°C with ΔG (25°C) = –1 kcal mol^{-1}, but is stable at 10°C with ΔG (10°C) = 2 kcal mol^{-1}. Using Eqs. (2)–(6), the calculated thermodynamic values at pH 3.0 were as follows: t_m = 50.2°C, $\Delta H_m(t_m)$ = 93.8 kcal mol^{-1} (392.4 kJ mol^{-1}), ΔG (25°C) = 6.08 kcal mol^{-1} (25.4 kJ mol^{-1}), and ΔG (10°C) = 8.14 kcal mol^{-1} (34.1 kJ mol^{-1}). These values are in good agreement with the experimental values at pH 2.95 (Table 1), t_m = 50.1°C and ΔH_m = 91.6 kcal mol^{-1}.

4. Conclusions

Thermal denaturation reveals the nature of the forces that stabilize the folded conformation of globular proteins in solution. There are three types of folded conformations at different pHs. The first one is a tightly packed conformation, which shows a highly cooperative thermal transition at a pH above 2.18. The second one is a loosely packed conformation, which has weak stability and shows cold denaturation at temperatures below

10°C between pH 2.03 and 1.55, as well as cooperative heat denaturation. The third one is as weak as the second one, but has a broad thermal transition and shows no cold denaturation.

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