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Thermodynamics of the thermal unfolding of eglin *c* in the presence and absence of guanidinium chloride

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Abstract

The thermal unfolding of eglin *c*, a small proteinase inhibitor of molecular weight 8.1 kDa, is studied by means of high sensitivity scanning calorimetry over a wide pH range in dilute buffer solutions, and in the presence of varying concentrations of guanidinium chloride at pH 7.00 and 10.55. The temperature of half-completion of the unfolding transition, $t_{1/2}$, in dilute buffer varies from 41° C at pH 1.1 to 86° C at pH 7.0 to 10.55, with corresponding enthalpy changes of approximately 40 kcal mol^{-1} and 71 kcal mol^{-1}. This latter enthalpy change, amounting to 8.7 cal g^{-1}, is unusually large for a protein, especially for one of unusually small molecular weight. Addition of 3.3 M guanidinium chloride at pH 10.55 lowered $t_{1/2}$ from 86° C to 40° C and decreased the enthalpy change from approximately 71 kcal mol^{-1} to 25 kcal mol^{-1}.

Keywords: Thermodynamics; Thermal unfolding; Eglin *c*; Guanidinium chloride

1. Introduction

Eglin *c* is a proteinase inhibitor occurring in the leech *Hirudo medicinalis* [1]. It strongly inhibits a number of proteinases, including elastase, cathepsin G, α-chymotrypsin, subtilisin and others, with binding constants in the range of 10^{10}–10^{11} M^{-1}. It contains 70 amino acids and has a molecular weight of 8099 Da. Eglin *c* is a remarkably stable protein considering its small size and lack of an intramolecular disulfide bond, undergoing reversible thermal denaturation at 85.5° C at pH 7.0, with an enthalpy change of 70.4 kcal mol^{-1}.

The crystal structure of eglin *c* has been determined to a resolution of 1.95 Å [2], and a high precision solution structure has been deduced from extensive NMR data [3]. The structure includes a well-defined core, a relatively mobile proteinase binding loop (residues 41–48), and a highly flexible heptapeptide at the amino terminus.

Because of its small size, accurately known structure and unusual stability, eglin *c* is an excellent candidate for detailed thermodynamic study. In this paper we study its thermal unfolding by means of high sensitivity differential scanning calorimetry (DSC) over a wide range of pH in the absence of guanidinium chloride (GuCl), and at pH 7.0 and 10.55 in the presence of GuCl concentrations of 0.5 to 3.3 M.

2. Materials and methods

Eglin *c* was supplied in generous amount by Dr. H.P. Schnebli of Ciba-Geigy Ltd., Basel, Switzer-

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land, and was used without further purification. Analytical grade chemicals were used in the preparation of buffers. GuCl, glycine and bicine were purchased from Sigma Co., St. Louis, MO. The buffers used were glycine (pH 2.0–3.25), sodium phosphate (pH 7.0–8.0), bicine (pH 9.2) and glycine (pH 10.05–10.55), all at 50 mM concentration. The protein was dialysed overnight at 4° C against the appropriate buffer solution. For pH 1.1–1.3 the protein solution was adjusted to the desired pH with dilute HCl.

Calorimetric measurements were performed with a Microcal MC-2 differential scanning calorimeter at a scan rate of 1° C min^{-1}. Instrumental base lines were determined prior to scanning each sample with both cells filled with dialysate which also served in the reference cell during scanning of the protein. The protein concentration for these experiments was in the range of 1.5–6.5 mg ml^{-1}. Samples were degassed at room temperature prior to being scanned.

The DSC data were fit by a non-linear least

Table 1
Thermodynamic parameters for the thermally-induced unfolding of eglin *c* in dilute buffer solution

pH	Conc. (mg ml^{-1})	$t_{1/2}$ (° C)	ΔH_{cal} at $t_{1/2}$ (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	ΔCp at $t_{1/2}$ (kcal K^{-1} mol^{-1})	St. dev. (% of C_{max})
1.10	3.16	41.12	39.0	1.01	0.86	1.1
1.30	1.43	41.27	36.7	1.20	0.97	0.9
1.30	2.14	42.08	35.9	1.31	0.66	0.8
1.65	2.58	46.09	39.3	1.30	0.21	0.8
1.80	2.47	47.28	41.9	1.25	0.11	0.9
2.00	2.18	46.90	41.3	1.23	0.58	1.2
2.15	2.70	51.13	46.9	1.20	0.51	0.9
2.25	1.56	51.14	46.7	1.21	0.51	1.0
2.50	1.80	51.46	44.2	1.33	0.41	1.1
2.50	2.16	54.52	42.8	1.44	0.24	1.0
2.50	2.56	55.40	48.2	1.27	0.30	1.0
2.50	3.37	54.91	46.9	1.28	0.29	0.9
2.50	3.78	51.76	49.3	1.13	0.39	0.7
2.50	5.51	55.00	53.5	1.06	0.49	0.6
2.50	7.08	51.59	48.5	1.13	0.56	0.8
2.75	2.65	57.56	51.0	1.22	0.35	0.9
2.85	2.78	59.46	52.3	1.18	1.02	1.1
2.85	2.91	60.44	51.8	1.22	0.64	1.0
2.93	2.30	63.93	60.1	1.05	0.44	0.6
3.00	2.32	63.30	57.4	1.10	0.84	0.6
3.25	2.37	68.98	56.1	1.20	0.22	1.1
7.00	2.21	85.83	74.8	1.07	0.18	0.7
7.00	2.45	85.99	67.5	1.21	0.20	0.9
7.00	2.91	85.16	64.7	1.31	0.43	1.1
7.00	4.42	85.43	74.0	1.09	0.21	0.6
7.01	5.01	85.29	71.1	1.15	0.17	1.8
7.05	2.54	85.49	65.5	1.17	0.36	0.7
8.00	2.20	86.52	72.3	1.21	0.51	0.4
9.20	3.00	86.30	72.1	1.08	0.70	0.7
10.05	2.61	86.55	72.5	1.07	0.53	0.9
10.50	1.48	87.05	73.6	1.12	0.05	1.1
10.50	2.84	85.45	67.5	1.18	1.08	1.3
10.50	3.23	86.58	72.3	1.12	0.55	0.8
10.50	5.77	86.37	71.6	1.12	0.55	0.6
10.55	2.81	86.02	68.5	1.19	0.80	1.1
10.55	6.56	85.68	72.7	1.09	0.77	0.7
			Mean	1.18	0.493	0.90
			S.E.	±0.02	±0.045	±0.04

squares analysis to a modified two-state model with the adjustable parameters $t_{1/2}$, the temperature of half-completion of the transition, ΔH_{cal}, the molar enthalpy change, and the ratio $\Delta H_{vH}/\Delta H_{cal}$, where ΔH_{vH} is the van 't Hoff enthalpy [4,5]. For a strictly two state process $\Delta H_{vH} = \Delta H_{cal}$. In cases where $\Delta H_{vH} > \Delta H_{cal}$, as was true in all our experiments with eglin c, it can be concluded that there is some degree of intermolecular cooperation resulting from oligomerization in either the native state, the unfolded state or both. The fact that the ratio $\Delta H_{vh}/\Delta H_{cal}$ averaged 1.18 indicates that oligomerization amounted to only a small degree of dimerization. The standard deviation of the calculated points from the observed points, expressed as a percentage of the maximal excess heat capacity, gives a measure of the adequacy of the fit in each case. The chemical baseline was computed by changing the linearly least squared pre-transition baseline to the post-transition baseline in proportion to the extent of the reaction. Since these two baselines in general have different slopes, the apparent value of the change in heat capacity accompanying the transition, ΔCp, which is equal to the vertical distance between the extrapolated baselines, varies with temperature. This temperature variation, which in some cases is very pronounced, and its effect on the variation of ΔH_{cal} with temperature, are included in the curve fitting conputation, with the assumption that the ratio $\Delta H_{vH}/\Delta H_{cal}$ is temperature independent.

3. Results and discussion

A typical DSC curve obtained with eglin c is shown in Fig. 1. The protein concentration was 2.45 mg ml^{-1} in 50 mM sodium phosphate buffer at pH 7.00. The solid curve shows the experimental data, the open circles are the values of the excess specific heat, C_{ex}, calculated by our curve fitting program, and the dashed curve is the chemical baseline calculated to progress from the pre-transition baseline to the post-transition baseline in proportion to the extent of the reaction. The good fit of the calculated to the observed data (standard deviation = 0.9% of the value of C_{max}, the maximal value of C_{ex}) was obtained with the ratio of van 't Hoff to calorimetric enthalpies equal to 1.21. The van 't Hoff enthalpy,

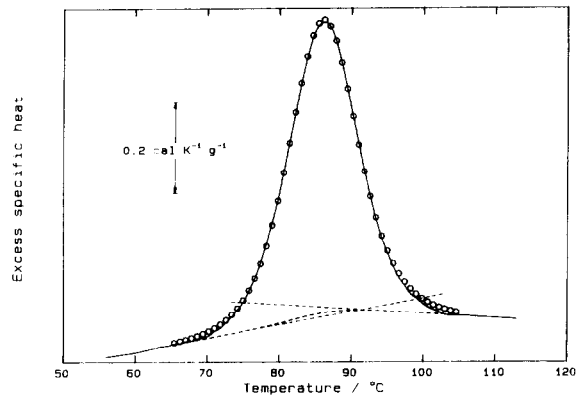

Fig. 1. A DSC curve observed with eglin c at a concentration of 2.45 mg ml^{-1} in 50 mM phosphate buffer at pH 7.00. The solid curve represents the observed data, and the open circles the points calculated with the evaluated parameters $t_{1/2} = 86.0°$ C, $\Delta H_{cal} =$ 67.5 kcal mol^{-1}, $\Delta H_{vH} = 81.7$ kcal mol^{-1}, and $\Delta Cp = 0.20$ kcal K^{-1} mol^{-1}, all at $t_{1/2}$.

ΔH_{vH}, controls the sharpness of the unfolding transition according to the van 't Hoff equation

$$\frac{d\ln K}{dT} = \frac{\Delta H_{vH}}{RT^2} \tag{1}$$

where K is the equilibrium constant for the two-state process: eglin c (native) ⇄ eglin c (unfolded).

The data obtained by curve fitting for 36 DSC experiments with eglin c in the pH range 1.10 to 10.55 are listed in Table 1, but with no experiments in the range pH 3.25 to 7.00 because of precipitation of the unfolded protein in the neighborhood of its isoelectric pH 5.4. The transition temperature varies from 41° C at pH 1.1 to 86° C at pH 10.55. Fig. 2 gives a plot of $t_{1/2}$ vs. pH, with mean values being shown at those pH values where more than one scan was run. The slope of the plot at pH 2.5 is 15.0° C/pH, which in the equation

$$\Delta\nu = \frac{1000\,\Delta H_{cal}}{2.303RT_{1/2}^2} \cdot \frac{dT_{1/2}}{dpH} \tag{2}$$

gives $\Delta\nu = 1.5$. Thus the molecule takes up approximately 1.5 protons on unfolding at low pH. It is evident that at higher pH, in the range between pH 7.0 and 10.55, there is no significant change in protonation during unfolding. The proton take-up at low pH is presumably due to the exposure to the

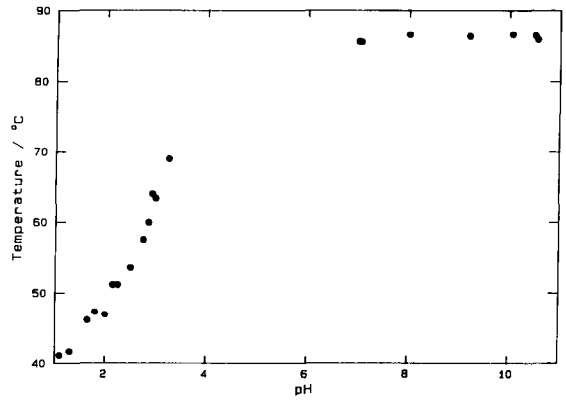

Fig. 2. The variation with pH of $t_{1/2}$ for the thermal unfolding of eglin c in dilute buffer. The slope at pH 2.5 indicates that 1.5 protons per molecule are taken up by the protein during unfolding at this pH. No proton uptake occurs in the range pH 7.00 to 10.55.

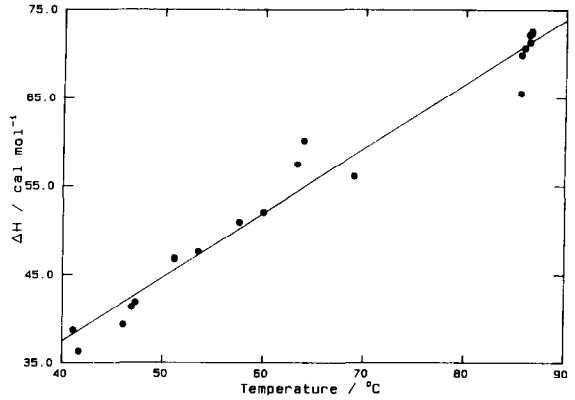

Fig. 3. The dependence of the enthalpy of unfolding of eglin c on $t_{1/2}$ in dilute buffer. The slope gives 0.730 kcal K^{-1} mol^{-1} for the heat capacity change in the unfolding process.

solvent during unfolding of buried carboxyl groups with abnormally low pK_a's.

The values for ΔH_{cal} listed in Table 1 are plotted as a function of $t_{1/2}$ in Fig. 3. These data are fitted with a standard deviation of ± 2.8 kcal mol^{-1} by the equation

$$\Delta H_{cal} = 8.164 + 0.730\ t \text{ kcal mol}^{-1} \quad (3)$$

The value of $\Delta Cp = 0.730$ kcal K^{-1} mol^{-1} is presumably a more significant value than the mean value, 0.493 kcal K^{-1} mol^{-1}, obtained from the individual scans (column 6, Table 1). That fact that ΔCp obtained in this way is apparently independent of temperature over the temperature range 40–90° C is inconsistent with the temperature variation, noted above, of ΔCp as evaluated in individual experiments. In the experiment shown in Fig. 1, for example, the apparent ΔCp actually becomes negative above 93° C. We have no explanation for this inconsistency, which has been observed in numerous other cases of protein thermal unfolding.

The denaturational enthalpy of unfolding of eglin c averages 70.7 kcal mol^{-1} at pH 7 to 10.55, or 8.73

Table 2
Thermodynamic parameters for the thermally-induced unfolding of eglin c in the presence of GuCl

pH	Protein conc. (mg ml^{-1})	GuCl conc. (M)	$t_{1/2}$ (° C)	ΔH_{cal} at $t_{1/2}$ (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	ΔCp at $t_{1/2}$ (kcal K^{-1} mol^{-1})	St. dev. (% C_{max})
7.00	2.64	0.503	77.87	65.5	1.10	0.97	1.0
7.00	2.62	0.945	73.40	65.8	1.03	0.33	0.5
7.00	1.74	1.316	70.14	62.5	1.01	0.52	0.5
7.00	2.60	1.451	67.01	54.4	1.19	0.76	1.7
7.00	2.45	1.493	67.65	56.2	1.11	0.44	0.6
7.00	2.65	1.737	63.69	53.2	1.06	0.56	0.5
7.00	2.37	2.461	56.41	40.3	1.27	0.46	1.0
7.00	1.75	3.121	48.39	35.6	1.15	0.52	1.8
10.55	2.03	0.953	73.54	59.9	1.09	0.54	0.9
10.55	1.54	1.432	69.10	49.0	1.26	0.29	1.0
10.55	2.42	1.939	61.39	45.9	1.11	0.88	0.4
10.55	2.23	2.336	56.00	40.3	1.21	0.63	0.9
10.55	2.48	2.793	48.44	32.1	1.25	0.82	1.0
10.55	2.32	3.311	41.31	24.1	1.40	0.63	1.3
				Mean	1.16	0.597	1.16
				S.E.	±0.03	±0.058	±0.33

cal g^{-1}. This is an unusually large enthalpy, especially considering that eglin c includes a strongly disordered N-terminal heptapeptide (10% of its amino acid residues) and a somewhat disordered proteinase binding loop (residues 41–48). We are unable to account for this large enthalpy of unfolding in terms of the known structure of the protein.

Application of the Gibbs–Helmholtz equation to evaluate the pH variation of the change in the standard free energy of unfolding, $\Delta\Delta G_u^\circ = \Delta G_u^\circ(\mathrm{pH}) - \Delta G_u^\circ(\mathrm{pH}\ 7.0)$, from the value zero at $t_{1/2}$ at pH 7.00, 85.5° C,

$$\Delta\Delta G_u^\circ = \Delta H(T)\left(1 - \frac{T_{1/2}}{T}\right)i - \Delta Cp\left(T - T_{1/2} + T_{1/2}\ln\frac{T_{1/2}}{T}\right) \qquad (4)$$

where $T = T_{1/2}$ at the lower pH, gives values which fit the equation

$$\Delta\Delta G_u^\circ = -14.86 + 0.1725(T - 273.15) \qquad (5)$$

with a standard deviation of ± 0.24 kcal mol^{-1}. Thus lowering the pH from 7.0 to 1.0, where $t_{1/2} = 40°$ C, destabilizes the protein by 8 kcal mol^{-1}.

The mean value of the ratio $\Delta H_{vH}/\Delta H_{cal} = 1.18$ usually indicates a small degree of oligomerization. The experiments at pH 2.50, 7.00, and 10.55, where sizeable changes in protein concentration were made, indicated no significant dependence of $t_{1/2}$ on concentration, which leads to the conclusion that no significant changes in oligomerization of the protein accompany the unfolding process at these values of the pH.

A number of DSC experiments were run at pH 7.00 and pH 10.55 in the presence of varying concentrations of GuCl. The results of these experiments are summarized in Table 2, and the transitions observed at pH 7.00 and various GuCl concentrations are illustrated in Fig. 4. As is to be expected, increasing concentrations of GuCl at constant pH lead to decreasing transition temperatures and decreasing enthalpies of denaturation. Since, as shown in Table 2, the ratio $\Delta H_{vH}/\Delta H_{cal}$ is not affected by the addition of GuCl, the transition broadening seen in Fig. 4 with increasing GuCl concentration does not involve a decrease in cooperativity. Fig. 5 shows the decrease in $t_{1/2}$ expressed as a function of $-\log$

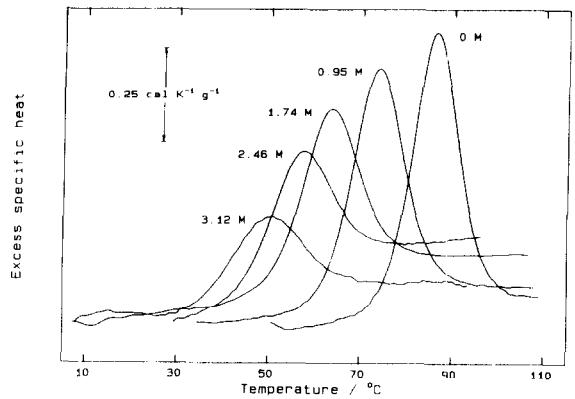

Fig. 4. DSC curves observed with eglin c at pH 7.00 and various concentrations of GuCl.

(GuCl concentration). Estimates of the slope of these data substituted into an equation analogous to Eq. (2) lead to estimates of the number of molecules of GuCl added per molecule of eglin c during the unfolding transition: 8 molecules of GuCl at 3 M GuCl, 5 molecules at 1.7 M GuCl, and 2.5 molecules at 1 M GuCl.

As in the absence of GuCl, ΔH_{cal} is quite accurately a linear function of $t_{1/2}$ as illustrated in Fig. 6, and expressed by the equation

$$\Delta H_{cal} = -23.203 + 1.155\ t \qquad (5)$$

with a standard deviation of ± 3.15 kcal mol^{-1}. It is interesting that $\Delta Cp = 1.155$ kcal K^{-1} mol^{-1} is

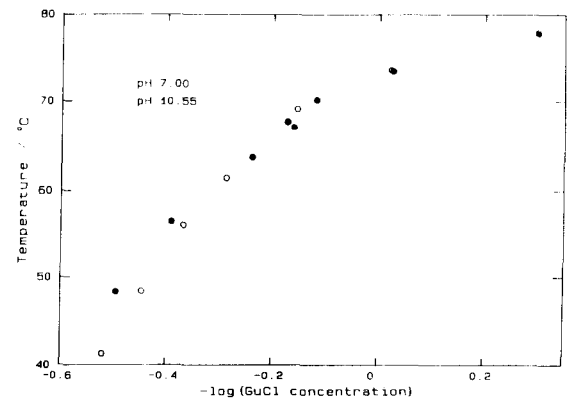

Fig. 5. The variation with GuCl concentration of $t_{1/2}$ for the thermal unfolding of eglin c at pH 7.00 and 10.55. The slope at 1.7 M GuCl ($-\log[\mathrm{GuCl}] = -0.230$) indicates that 5 moles of GuCl are taken up per mole of protein during unfolding.

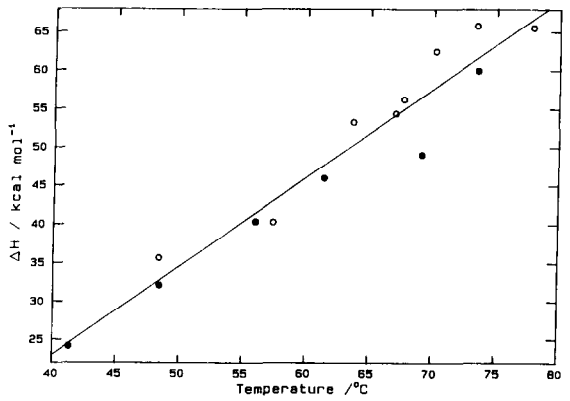

Fig. 6. The variation of the enthalpy of unfolding of eglin *c* at pH 7.00 (○) and pH 10.55 (●) with the temperature of half-completion of the unfolding. The slope of the least squared line gives 1.155 kcal K^{-1} mol^{-1} for ΔCp, a value 60% higher than observed in the absence of GuCl.

almost 60% larger than ΔCp in the absence of GuCl. This may constitute an example of a quite general phenomenon (Y. Liu and J.M. Sturtevant, unpublished data, 1994), that the apparent value of ΔCp for the unfolding of a protein, and perhaps for other macromolecular processes, may vary with the means employed to change the temperature at which the process takes place.

Eq. (4) leads to values for $\Delta\Delta G_u^\circ$ relative to the same reference point as used for the data in the absence of GuCl which fit the Eq. (6)

$$\Delta\Delta G_u^\circ = -13.313 + 0.1474(T - 273.15) \qquad (6)$$

where T is the value of $T_{1/2}$ at a specified concentration of GuCl at pH 7, with a standard deviation of ± 0.23 kcal mol^{-1}. This gives $\Delta\Delta G_u^\circ$ = -7.4 kcal mol^{-1} at 40° C, indicating that a given decrease in $T_{1/2}$ produced by the addition of GuCl represents a smaller destabilization than the same decrease produced by lowering the pH in the absence of GuCl.

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