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The pressure-temperature phase diagram of hen lysozyme at low pH

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The equilibrium unfolding of hen lysozyme at pH 2 was studied as a function of pressure (0.1~700 MPa) and temperature (–10°C~50°C) using Trp fluorescence as monitor supplemented by variable pressure ^{1}H NMR spectroscopy (0.1~400 MPa). The unfolding profiles monitored by the two methods allowed the two-state equilibrium analysis between the folded (N) and unfolded (U) conformers. The free energy differences ΔG ($=G_U-G_N$) were evaluated from changes in the wavelength of maximum fluorescence intensity (λ_{max}) as a function of pressure and temperature. The dependence of ΔG on temperature exhibits concave curvatures against temperature, showing positive heat capacity changes ($\Delta C_p=C_{pU}-C_{pN}=1.8$–$1.9$ kJ mol^{-1} deg^{-1}) at all pressures studied (250~400 MPa), while the temperature T_S for maximal ΔG increased from about 10°C at 250 MPa to about 40°C at 550 MPa. The dependence of ΔG on pressure gave negative volume changes ($\Delta V=V_U-V_N$) upon unfolding at all temperatures studied (–86~–17 ml mol^{-1} for –10°C~50°C), which increase significantly with increasing temperature, giving a positive expansivity change ($\Delta\alpha$~1.07 ml mol^{-1} deg^{-1}). A phase-diagram between N and U (for $\Delta G=0$) is drawn of hen lysozyme at pH 2 on the pressure-temperature plane. Finally, a three-dimensional free energy landscape (ΔG) is presented on the *p-T* plane.

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Knowledge on thermodynamic stability of a globular protein forms a basis for understanding its function, folding as well as misfolding into amyloid fibrils[1]. In addition to the characterization of thermodynamic stability on the temperature axis[2,3], the characterization of the same on the pressure axis is increasingly important in basic protein science[4–6] as well as in the applied protein science[7]. Hen lysozyme, consisting of 129 amino acids with four disulfide bonds, is one of the most well characterized globular proteins both in structure and function. Hen lysozyme and its mutants have been widely used as a model system for studying enzyme function and protein folding[8,9] as well as for studying amyloid fibril formation in recent years[10–15]. In the present study, pressure-induced equilibrium unfolding of hen lysozyme is carried out at pH 2 using a high pressure fluorescence spectrometer which operates in the pressure range of 3~700 MPa along with high pressure ^{1}H NMR spectroscopy which works in the pressure range of 3~400 MPa. Temperature was varied widely between –10°C and 50°C so that a rather complete free energy landscape of hen lysozyme may be obtained on the pressure-temperature plane.

Figure 1 shows the three-dimensional structure of hen lysozyme (wild-type) in crystal (PDB ID: 135L)[16], and a

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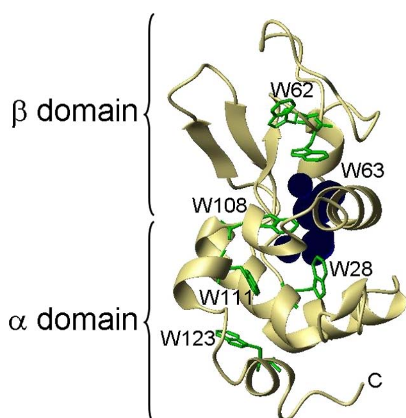

Figure 1 The structure of hen lysozyme in crystal (PDB ID; 135 L), which consists of two domains, α and β. The six tryptophan residues are colored green and the water-accessible internal cavity is shown by navy-spheres.

similar structure has been reported in solution[17]. The folded structure consists of two domains, α and β, the α domain having a large hydrophobic core with a large water-containing cavity[17]. There are in total six tryptophan residues in the molecule, two of which (Trp 62 and 63) are found in the β domain and the rest are found in the α domain, of which Trp 28, 108 and 111 are close to the large cavity (Fig. 1). The fluorescence from the six Trp residues will be used for the thermodynamic analysis of equilibrium unfolding, while the side chain signals of ^{1}H NMR spectrum will be used to monitor the cooperative transition.

Materials and methods

Materials

Hen egg white lysozyme was obtained from Seikagaku Co. (6×crystallized and lyophilized, Lot E40314) and was used without further purification. The protein solutions for NMR measurements were concentrated to 1.7 mM in 50 mM maleate buffer (90% ^1H_2O/10% ^2H_2O, pH 2), which has one of the smallest ΔV values for the dissociation of the carboxyl group (ΔV=−5.1 ml mol^{-1})[18]. Even so, the ΔV value predicts that the pH of the buffer may decrease as much as 0.6 unit at 700 MPa. Therefore, the effective pH of the solution in the entire range of pressure (3~700 MPa) should vary between 2.0 and 1.4.

^{1}H NMR measurements at variable pressure

^{1}H one-dimensional variable-pressure NMR measurements of hen lysozyme were performed also at various temperatures in the pressure range between 3 and 400 MPa at a ^{1}H frequency of 800.16 MHz on a Bruker DRX-800 spectrometer[20]. At each pressure, intensity corrections are made for the pressure-induced compaction of the solvent water (e.g., by ~9% at 300 MPa, 298K)[19]. Data were processed with XWIN-NMR (Bruker BioSpin). A specially prepared pressure-resistive quartz cell (inner diameter <1 mm), which endures pressure up to 400 MPa and gives a reasonably good spectral resolution, was chosen for this particular study. The detailed procedure for preparing the pressure-resistive quartz cell for NMR is described in the literature[21]. ^{1}H chemical shifts were referenced to the methyl signal of 1,4-dioxane added internally (δ=3.70 ppm for ^{1}H). For comparison, ^{1}H NMR experiments at 0.1 MPa were also performed at 25°C, pH 2, in Shigemi tube (5 mm outer diameter) with/without 8 M urea at a ^{1}H frequency of 600.13 MHz using a 3-9-19 pulsed field gradient for water suppression on a Bruker AVANCE-600 spectrometer.

Tryptophan fluorescence measurements at variable pressure

Trp fluorescence spectra of hen lysozyme (35 μM or 0.5 mg ml^{-1} in 50 mM maleate buffer, pH 2) were recorded on a fluorescence spectrophotometer (FP-6500, JASCO) with a high pressure chamber (Syn Corporation, Kyoto) within which a quartz cell containing ~100 μl of the sample solution is placed, connected to a high pressure pump system (Techno Corporation, Hiroshima) using water as pressure mediator. Measurements were enabled in a wide pressure range between 3 MPa and 700 MPa, 3 MPa being used instead of 0.1 MPa to avoid any effect from air bubbles. The temperature of the high pressure chamber was controlled by circulating water-ethylene glycol 1-to-1 mixture to the high-pressure sample-holding chamber. The excitation was made at 295 nm with a bandwidth of 3 nm, and the emission from 310 nm to 450 nm was collected with a bandwidth of 10 nm. The data were processed with Microcal Origin 6.0 (Microcal Software, Inc.).

Analysis of high pressure fluorescence data

We assume that the protein exists in the two-state equilibrium between the folded conformer N and the unfolded conformer U, namely

$$N \leftrightarrow U \quad (1)$$

with equilibrium constant K dependent on pressure p and temperature T. Furthermore, we assume that at a fixed temperature T; the wavelength of maximum fluorescence intensity λ_{max} at pressure p is determined by the following relation

$$\lambda_{max}=f_N\,\lambda_{maxN}+f_U\,\lambda_{maxU} \quad (2)$$

where f_N represents the fraction of N, f_U represents the fraction of U, λ_{maxN} represents the wavelength of maximum fluorescence intensity for N and λ_{maxU} represents the wavelength of maximum fluorescence intensity for U. Then the equilibrium constant K at any pressure p will be determined experimentally by

$$K=\frac{f_U}{f_N}=\frac{f_U}{1-f_U}=\frac{\lambda_{max}-\lambda_{max\,N}}{\lambda_{max\,U}-\lambda_{max}} \quad (3),$$

from which the Gibbs free energy difference ΔG between N and U will be determined experimentally as a function of p and T by using the relation

$$\Delta G = G_U - G_N = -RT \ln K \quad (4).$$

In order to proceed further to determine thermodynamic parameters associated with the folding-unfolding transition as quantities independent of pressure and temperature, we must recourse to the theoretical expression of ΔG (eq. 5). Eq. 5 is expressed as a Taylor expansion of ΔG at a reference point (p_0 and T_0) to the second order to p and T[20]

$$\Delta G = \Delta G^0 + \Delta V^0(p-p_0) + \frac{\Delta\kappa}{2}(p-p_0)^2 - \Delta S_0(T-T_0) - \frac{\Delta C_p}{2T_0}(T-T_0)^2 + \Delta\alpha(p-p_0)(T-T_0) \quad (5)$$

where ΔG^0 is the Gibbs free energy difference of conformer U relative to N at a reference point (p_0 and T_0), ΔV^0 is the change in partial molar volume of conformer U relative to N ($\Delta V^0 = V^0_{\mathrm{U}} - V^0_{\mathrm{N}}$) also at a reference point (p_0 and T_0) and $\Delta\kappa$ is the isothermal compressibility change. ΔS_0 is the entropy change at a reference point (p_0 and T_0), ΔC_p is the heat capacity change and $\Delta\alpha$ is the change in expansivity. In an experiment in which pressure is varied at a constant temperature, say $T = T_x$, eq. 5 conforms to

$$\Delta G = \Delta G^0_x + \Delta V^0(p-p_0) + \frac{\Delta\kappa}{2}(p-p_0)^2 + \Delta\alpha(p-p_0)(T_x - T_0) \quad (6)$$

where ΔG^0_x is the free energy difference extrapolated to a reference pressure (0.1 MPa) at T_x, and the slope against p gives the volume change at T_x. In the further analysis, we neglect the compressibility term $\Delta\kappa(p-p_0)^2/2$, as is often found permissible for many globular proteins[22], whence we obtain the expression for the volume difference as a temperature-dependent quantity,

$$\Delta V = V_U - V_N = \Delta V_0 + \Delta\alpha(T - T_0) \quad (7)$$

where the subscript x is eliminated from T. Then the change in the wavelength of maximum fluorescence intensity ($\lambda_{\max}$) can be expressed by combining eq. 2, 3, 4 and 6.

$$\lambda_{\max} = \frac{\lambda_{\max} + \lambda_{\max}\exp[-\{\Delta G^0 + \Delta V^0(p-p_0) + \Delta\alpha(p-p_0)(T-T_0)\}/RT]}{1 + \exp[-\{\Delta G^0 + \Delta V^0(p-p_0) + \Delta\alpha(p-p_0)(T-T_0)\}/RT]} \quad (8).$$

At the midpoint of transition between N and U, where $\Delta G = 0$ and $f_N = f_U$, we obtain the relations

$$p_m = \Delta G^0/\Delta V \quad (9)$$

$$\Delta G = \Delta H_m\left(1 - \frac{T}{T_m}\right) + \Delta C_p\left\{T - T_m - T\ln\left(\frac{T}{T_m}\right)\right\} \quad (10),$$

$$\Delta S_m = \frac{\Delta H_m}{T_m} \quad (11)$$

where T_m, p_m, ΔH_m, ΔS_m and ΔC_p represent the temperature at the midpoint (T_m) and pressure at the midpoint (p_m) of transition between N and U, the unfolding enthalpy change at T_m, the unfolding entropy change at T_m and the unfolding heat capacity change, respectively.

Experimental results

^{1}H NMR spectra at 0.1~400 MPa

^{1}H one-dimensional NMR measurements were carried out on hen lysozyme (pH 2) at varying pressures up to 400 MPa, the highest pressure available in the current high pressure NMR system at 800 MHz at various temperatures. Except at subzero temperatures, e.g. at −5°C, we could not attain full unfolding even at 400 MPa. In Figure 2A, the ^{1}H NMR

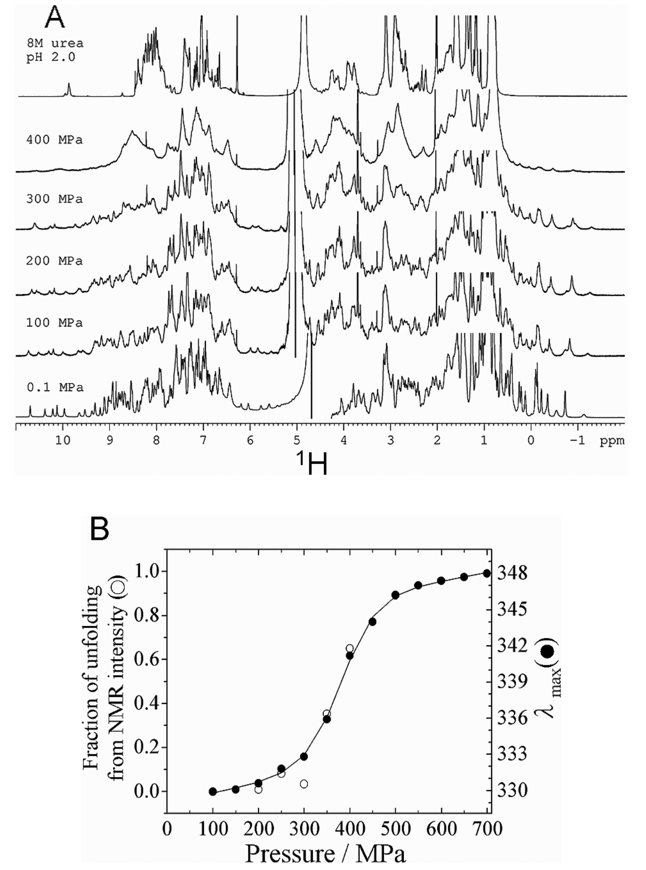

Figure 2 (A) ^{1}H NMR spectra (800 MHz) of hen lysozyme recorded at various pressures from 100 MPa to 400 MPa at −5°C. The spectrum at 0.1 MPa (bottom) was obtained at 25°C at 600 MHz. The spectrum at 0.1 MPa (top) was also obtained at 25°C, but in the presence of 8 M urea. Chemical shifts are referenced to dioxane δ= 3.70 ppm. All the protein solutions were prepared in 50 mM maleate buffer, 90% ^1H_2O/10% ^2H_2O (pH 2). (B) The fraction of unfolding against pressure as estimated from the fractional decrease of the combined intensity of high-field shifted methyl ^{1}H NMR signals of Leu17, Thr51, Ile56, Ile88 and Ile98 (δ=−0.3~0.8 ppm)[26] (open circles) and from the red shift of the wavelength of maximum fluorescence emission ($\lambda_{\max}$) (closed circles). The solid curve is drawn by best-fitting the fluorescence data to eq. 8.

spectra recorded at −5°C above 100 MPa only to avoid freezing are shown. The spectrum at 0.1 MPa was recorded at 25°C and is shown in Figure 2A (bottom). The spectrum for the "fully unfolded" structure was recorded also at 25°C at 0.1 MPa in the presence of 8 M urea and is also shown in Figure 2A (top).

Figure 2A shows that the spectrum at 100 MPa is essentially the same as that at 0.1 MPa (25°C) in chemical shifts except for significant line broadening. This result indicates that hen lysozyme at −5°C and at 100 MPa retains almost the same "folded" structure as that at 0.1 MPa at 25°C, which is expected to be close to that in Figure 1. The broader signals at −5°C and at 100 MPa would be largely attributable to the lower spectral resolution of the particular pressure-resistive cell used, although the possibility of extra broadening due to partial hydration of the protein matrix[23,24] may not be denied.

As the pressure is increased above 100 MPa, the characteristic spectral features of the folded structure are gradually lost and replaced by a featureless spectrum typical for an unfolded conformer with disordered and hydrated polypeptide chain[25], similar to that in 8 M urea (Fig. 2A (top)) except for line broadening. In particular, the ε-proton signals of all the six Trp residues are observed and well dispersed at 9.4, 10.1, 10.2, 9.5, 10.4 and 10.7 ppm, which are assignable to Trp 28, 62, 63, 108, 111 and 123, respectively[23,26], showing the characteristic folded structure of hen lysozyme at low pressure. At 400 MPa, however, all these signals are lost and replaced by new signals at ~10 ppm typical for ε-protons of solvent-exposed Trp residues[25], giving evidence that all the six Trp residues are exposed to the solvent. Also the extremely high-field shifted methyl proton signals (<0 ppm), representing another characteristic signature of the folded hen lysozyme (Fig. 1), are nearly completely lost at 400 MPa, which must have merged into the biggest resonance peak at ~0.9 ppm[25]. All the spectral changes are reversible with pressure. These observations clearly indicate that the folded structure is almost fully lost as the pressure is increased to 400 MPa.

We take the combined intensity (I) of the high-field shifted methyl proton signals at −0.8~0.3 ppm in Figure 2A (assignable to Leu17, Thr51, Ile56, Ile88 and Ile98)[26] at pressure p relative to its initial intensity (I_0) at 100 MPa, after correcting the intensity due to the compression of the solvent water (See Materials and Methods), as representing the folded fraction (f_N) of hen lysozyme at −5°C. In Figure 2B, we plot the fraction of the unfolded conformer U ($f_U=1-f_N$) as a function of pressure (open circle). In the same figure, we also plot the change in the wavelength of maximum fluorescence intensity (λ_{max}) measured under the same condition (pH 2, at −5°C, except for the concentration) up to 700 MPa (closed circle). The good coincidence of the two plots certifies that the change in λ_{max} of Trp fluorescence represents the fraction of unfolding as correctly as predicted by the ^{1}H NMR signal intensity. Furthermore, the solid curve in Figure 2B represents the best-fit of eq. 8, based on the two-state equilibrium of eq. 1, to the change in the wavelength of maximum fluorescence intensity (λ_{max}). The coincidence is excellent, which prompts us to use the wavelength of maximum fluorescence intensity (λ_{max}) in conjunction with eq. 8 to obtain thermodynamic parameters associated to the folding-unfolding transition of hen lysozyme at pH 2. Thus in the following section, we use the fluorescence data carried out up to 700 MPa, which realized complete unfolding at all temperatures studied.

Trp fluorescence spectra at 0.1~700 MPa

As hen lysozyme is highly resistive to pressure even at pH 2 and full unfolding is hardly attainable at 400 MPa, the highest pressure available in our current high pressure NMR system[20,21] at normal temperatures, we now turn to high-pressure fluorescence spectroscopy[27] with which we can reach 700 MPa.

Figure 3 (A–G left) compiles the fluorescence spectral data from six Trp residues of hen lysozyme (35 μM in 50 mM maleate buffer, pH 2), measured as a function of pressure from 3 MPa to 700 MPa at different temperatures (−5°C, −10°C, 5°C, 15°C, 25°C, 40°C and 50°C). In all cases, the fluorescence spectrum changes with pressure both in intensity and wavelength, which are fully reversible with pressure with respect to the maximum wavelength of emission (λ_{max}) but less reversible (~80%) with respect to the intensity of emission. The lack of full reversibility is often encountered in high-pressure fluorescence experiments owing to some technical reasons. On the other hand, the shift in λ_{max} of Trp fluorescence is considered to represent correctly the change in the microenvironment of the tryptophan ring[22]: The blue shift (λ_{max} ~330 nm) indicates that the Trp ring is in the non-polar environment or buried in the hydrophobic core, while the red shift (λ_{max} ~350–355 nm) indicates that the Trp ring is in the polar environment or exposed to the solvent water[27]. Although at 700 MPa below ~5°C water is expected to go into ice VI, the smooth transitions in Figure 3A, B and C suggest that the solution went into the super-cooled state.

Figure 3 (A–G right) plots the maximum emission wavelength (λ_{max}) against pressure at all temperatures studied. At all temperatures, λ_{max} stayed initially within 330~335 nm, showing that Trp residues are almost fully buried in the folded conformation. Finally at 700 MPa, λ_{max} shifted to 349~350 nm at all temperatures, showing that all the six Trp residues become exposed to the solvent in accordance with the high pressure NMR result (Fig. 2). Therefore, we conclude that the protein is totally unfolded at 700 MPa. In Figure 3 right, we plot λ_{max} as a function of pressure at all temperatures studied, which presumably represents transitions of hen lysozyme from the folded (N) to the unfolded (U) conformer.

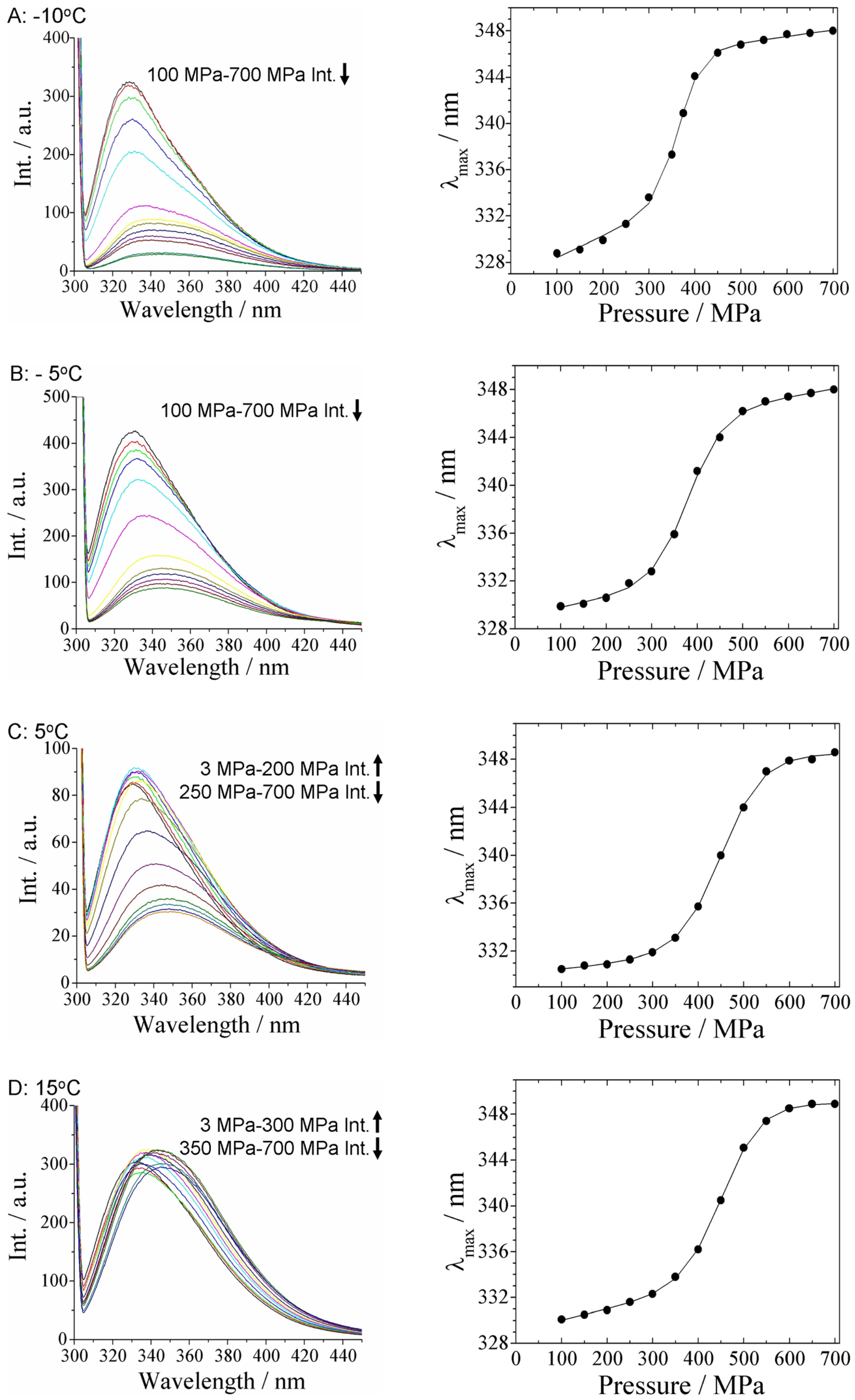

Figure 3 (A–D)

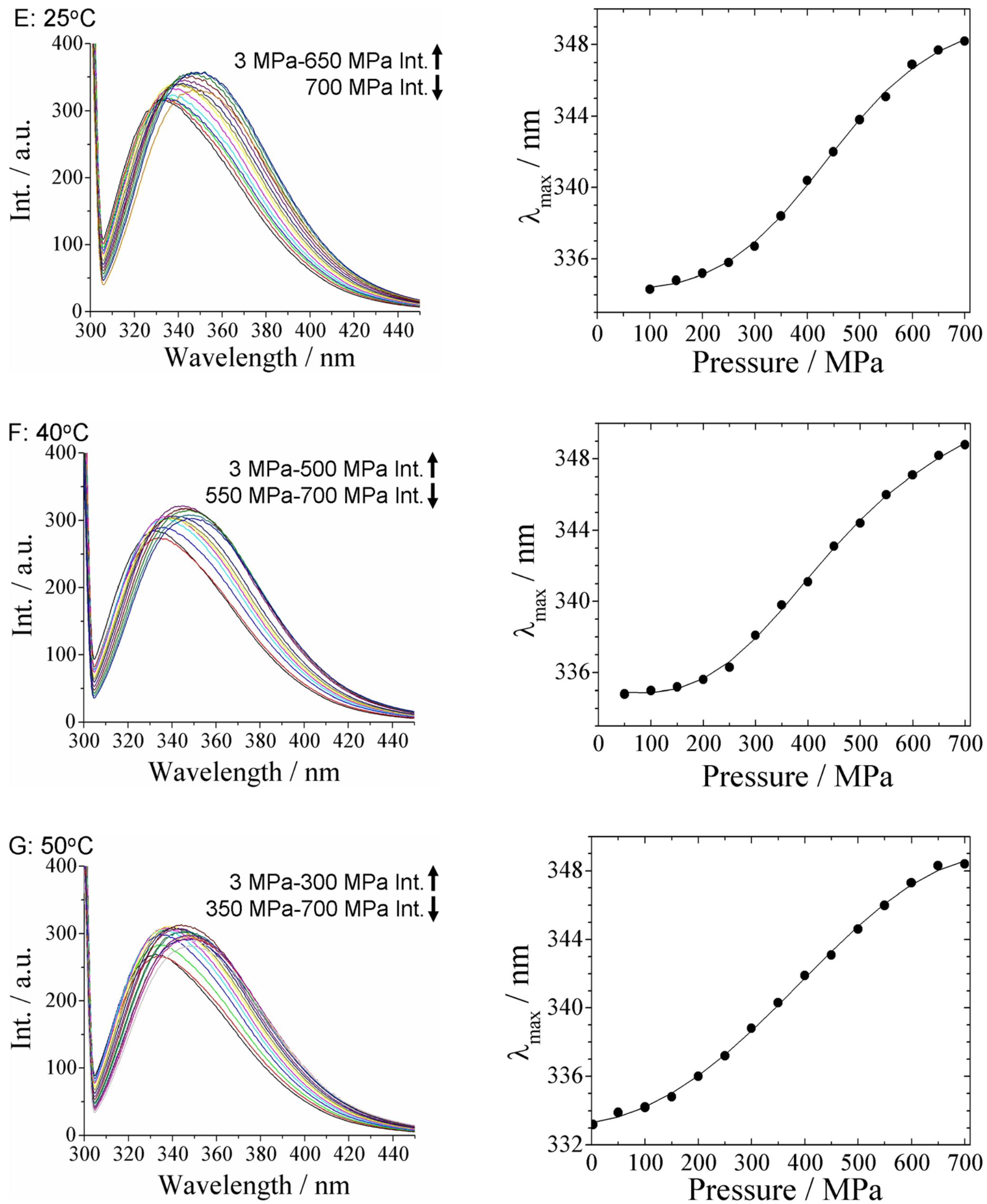

Figure 3 (E–G)

Figure 3 Trp fluorescence changes of hen lysozyme at pH 2 as a function of pressure at various temperatures. (A–G) (Left) Overlay of fluorescence spectra of hen lysozyme recorded as a function of pressure at various temperatures. The upward and downward arrows indicate whether the fluorescence intensity is increased or decreased with increasing pressure in the pressure range indicated. (A–G) (Right) Plots of the wavelength of maximum fluorescence intensity (λ_{max}) as a function of pressure at various temperatures. The solid curves are the best-fit of eq. 8 to λ_{max}, giving ΔG^0 and ΔV (eq. 7) values at different temperatures, which are listed in Table 1.

Thermodynamic analysis and discussion

Volume and expansivity changes

We assume the two-state transition between N and U (eq. 1) and best-fit the observed changes in the wavelength of maximum fluorescence intensity (λ_{max}) in Figure 3 (right) with eq. 8, which gives ΔV (eq. 7) and ΔG^0 values listed in Table 1, together with pressures at the midpoint of transition p_m (eq. 9). In Table 1, we note that p_m ranges from 250 MPa (50°C) to 453 MPa (5°C), revealing a relatively high stability of hen lysozyme against pressure denaturation even at low pH. The stability extrapolated to 0.1 MPa (ΔG^0) shows an increasing trend by lowering temperature, but the values may not be as reliable as ΔV in the present case, because 0.1 MPa is far from p_m and no reliable data points are available to 0.1 MPa.

Table 1 Thermodynamic parameters for unfolding of hen lysozyme determined from Trp fluorescence experiments

T (°C)	ΔG^0 (kJ mol^{-1})^a	ΔV (kJ mol^{-1})^b	p_m (MPa)^c
−10	31.5±3.8	−85.9±3.8	366.7
−5	22.1±2.6	−58.2±6.7	379.7
5	23.1±1.5	−51.0±3.5	452.9
15	25.7±1.1	−56.2±2.4	357.3
25	9.5±3.4	−22.4±9.3	424.1
40	7.6±2.8	−23.4±8.2	324.8
50	4.2±3.1	−16.8±11.8	250.0

[a] Gibbs free energy change at 0.1 MPa calcurated with eq. 8.
[b] Partial molar volume change ($\Delta V = \Delta V^0 + \Delta\alpha(T - T^0)$) calculated with eq. 8.
[c] The denatured temperature

Figure 4 gives the plot of $\Delta V = V_U - V_N$, as obtained from the fit in Figure 3 (right) against temperature, which are all negative within the temperature range studied (−10°C~50°C), but with a significant temperature dependence. From the slope, we obtain as the expansivity change upon unfolding $\Delta\alpha = 1.07$ ml mol^{-1} deg^{-1} in eq. 7. This value is comparable to those in staphylococcal nuclease (1.33 ml mol^{-1} deg^{-1})[28] as well as in metmyoglobin (1.8 ml mol^{-1} deg^{-1})[4] and in ribonuclease A (1.32 ml mol^{-1} deg^{-1})[29]. The positive value of $\Delta\alpha$ is taken to indicate the increased thermal volume due to the increased exposure of the polypeptide chain upon unfolding[22].

Stability and heat capacity changes

In Figure 5, we plotted the experimentally determined values of ΔG (eq. 4) against temperature at constant pressures, which depict concave features at all pressures studied. Data are limited to above 200 MPa, as no significant fraction unfolds in the lower pressure range to give sufficiently reliable ΔG values below 200 MPa. The plots were best-fitted with eq. 10, giving parameters of ΔC_p, T_S (the temperature for $\Delta S = 0$), T_m, ΔH_m and ΔS_m (for both heat and cold denaturations) as summarized in Table 2. The Gibbs free energy changes (ΔG) are fitted reasonably well with a single positive ΔC_p value at each pressure, covering both the cold denaturation and heat denaturation ranges. In general, a positive ΔC_p upon unfolding is accepted as due to the exposure of nonpolar amino acid groups into the solvent water[2]. In accordance with this, ΔH_m for heat denaturation increases with increasing T_m. Interestingly, while the stability is found to decrease with pressure, T_S, the temperature of maximum stability (the temperature for $\Delta S = 0$) increases with increasing pressure.

Phase diagram and free energy landscape

Figure 6 shows the phase diagram (for $\Delta G = 0$) of hen lysozyme for the first time in aqueous environment on the pressure-temperature plane. So far, the thermodynamic stability on the temperature-pressure plane in aqueous environment has been reported for a limited number of proteins,

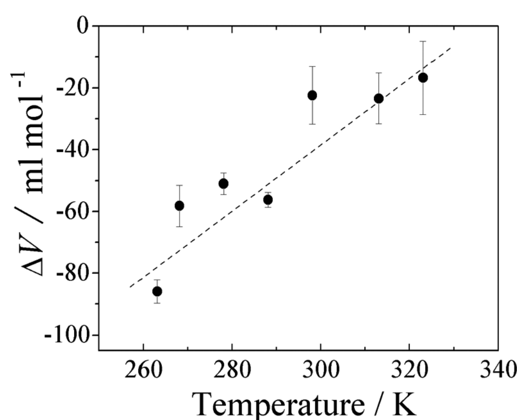

Figure 4 Plot of the change in partial molar volume ΔV on unfolding against temperature. Best-fit to eq. 7 gives a change in expansivity $\Delta\alpha$ on unfolding (260~320 K, pH 2) to be 1.07 ml mol^{-1} deg^{-1}.

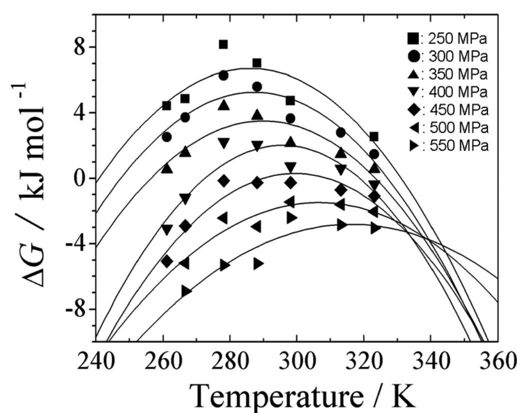

Figure 5 Plots of the change in free energy ΔG on unfolding for hen lysozyme (pH 2) at various pressures. The solid lines show best-fit of eq. 10 to the experimental points, with melting temperature (T_m), enthalpy change at T_m (ΔH_m) and heat capacity change on unfolding (ΔC_p) as fitting parameters listed in Table 2.

including metmyoglobin[4] chymotrypsinogen[30], ribonuclease A[29] and *Staphylococcal* nuclease[28,31–32]. In these proteins, except for chymotrypsinogen which clearly gives a region of pressure-induced folding at elevated temperature[30], an ellipsoid type pattern like that in Figure 6 with no regions of pressure-induced folding has been commonly observed, although the individual pattern is characteristic of each protein[33].

In Figure 7, we draw the energy landscape of hen lysozyme at low pH on pressure and temperature axes based on eq. 5 on the approximation of null isothermal compressibility change $\Delta\kappa$ within the pressure range studied. This will provide the basis for studying hen lysozyme at low pH by varying temperature and/or pressure.

Table 2 Thermodynamic parameters for heat and cold denaturation of hen lysozyme

P (MPa)		ΔH_m (kJ mol^{-1})^a	ΔS_m (J mol^{-1} K^{-1})^b	ΔC_p (kJ mol^{-1} K^{-1})^c	T_m (K)^d	T_s (K)^e
250	Heat	95.1±14.2	287.0±14.5	1.9±0.8	331.4±3.0	286
	Cold	−77.4±10.6	−319.4±14.3		242.3±9.6	
300	Heat	83.9±7.5	256.3±8.6	1.9±0.6	327.4±4.2	287
	Cold	−70.2±8.3	−282.8±8.5		248.2±2.0	
350	Heat	66.3±5.6	204.7±6.0	1.8±0.5	323.9±2.2	290
	Cold	−56.4±13.0	−219.6±13.8		256.8±4.5	
400	Heat	54.2±14.0	170.0±14.6	1.8±0.6	318.8±4.1	296
	Cold	−48.5±6.0	−178.0±6.3		272.5±1.8	

^{a} Unfolding entharpy at T_m
^{b} Unfolding entropy at T_m
^{c} Unfolding heat capacity at T_m
^{d} Denaturation temperature
^{e} Temperature for maximum stability $((\partial \Delta G/\partial T)=\Delta S=0)$

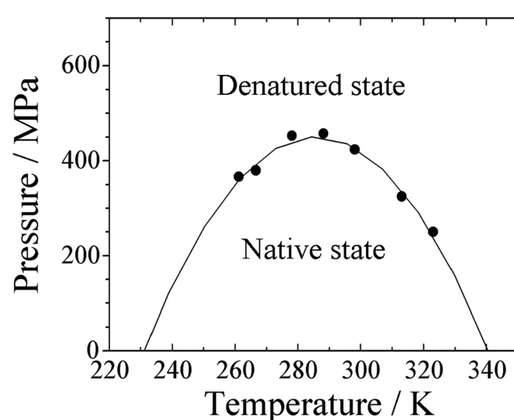

Figure 6 The phase diagram of hen lysozyme at pH 2 as obtained by fluorescence measurements. Plot of p_m (pressure at the midpoint of transition between N and U or half-denaturation) obtained from eq. 9 against T_m. The solid curve represents the best-fit of eq. 5 for $\Delta G=0$ to the experimental points.

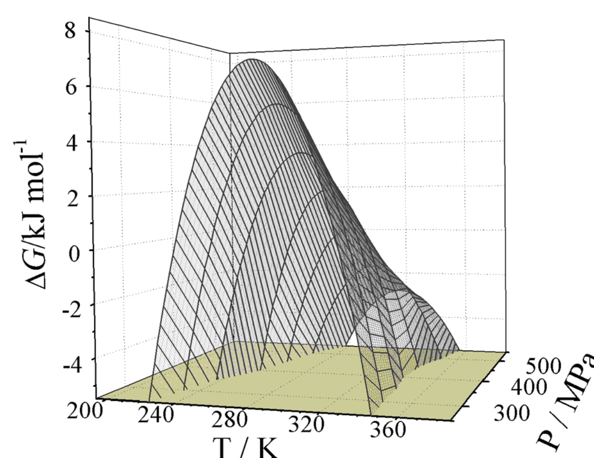

Figure 7 Three-dimensional free-energy landscape of hen lysozyme at pH 2 in the range of pressure from 250 MPa to 550 MPa and temperature from 200 K to 380 K, drawn as overlay of eq. 5 at various pressures.

Concluding remark

Since hen lysozyme ishighly resistive to pressure, thermodynamic unfolding studies on pressure axis have been carried out in the presence of denaturants[34]. In the present study, the study on the thermodynamic stability was carried out on the pressure and temperature axes on hen lysozyme in an aqueous environment at low pH. This was made possible by the use of a high pressure fluorescence spectrometer developed in our laboratory that operates up to 700 MPa, the details of which will be published elsewhere. The knowledge and the method presented here will serve as a basis for studying dynamics and folding of hen lysozyme as a whole, but will also provide information as to the condition for the formation of amyloid fibrils or insoluble aggregates in a wider perspective. In general, the extension of thermodynamic characterization of globular proteins to pressure axis will increase our understanding of proteins as well as our ability for manipulating conformational states of proteins for practical purposes.

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