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 (\(-10^\circ\)C~50\(^\circ\)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 \(\Delta G = G_U - G_N\) were evaluated from changes in the wavelength of maximum fluorescence intensity (\(\lambda_{\text{max}}\)) as a function of pressure and temperature. The dependence of \(\Delta G\) on temperature exhibits concave curvatures against temperature, showing positive heat capacity changes (\(\Delta C_p = C_p^U - C_p^N = 1.8\sim1.9\ \text{kJ mol}^{-1}\ \text{deg}^{-1}\)) at all pressures studied (250~400 MPa), while the temperature \(T_S\) for maximal \(\Delta G\) increased from about 10\(^\circ\)C at 250 MPa to about 40\(^\circ\)C at 550 MPa. The dependence of \(\Delta G\) on pressure gave negative volume changes (\(\Delta V = V_U - V_N\)) upon unfolding at all temperatures studied (\(-86\sim-17\ \text{ml mol}^{-1}\) for \(-10^\circ\text{C}~50^\circ\text{C}\), which increase significantly with increasing temperature, giving a positive expansivity change (\(\Delta \alpha = 0.07\ \text{ml mol}^{-1}\ \text{deg}^{-1}\)). 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 (\(\Delta G\)) is presented on the \(p-T\) plane.

Key words: hen lysozyme, high pressure fluorescence, high pressure NMR, thermodynamic stability on pressure-temperature axes, free energy landscape

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 \(-10^\circ\text{C} and 50^\circ\text{C}\) 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
similar structure has been reported in solution\textsuperscript{17}. The folded structure consists of two domains, $\alpha$ and $\beta$, the $\alpha$ domain having a large hydrophobic core with a large water-containing cavity\textsuperscript{17}. There are in total six tryptophan residues in the molecule, two of which (Trp 62 and 63) are found in the $\beta$ domain and the rest are found in the $\alpha$ 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. (\texttimes{} 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\% $^1$H$_2$O/10\% $^1$H$_2$O, pH 2), which has one of the smallest $\Delta V$ values for the dissociation of the carboxyl group ($\Delta V=-5.1$ ml mol\textsuperscript{-1})\textsuperscript{14}. Even so, the $\Delta V$ 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\textsuperscript{20}. At each pressure, intensity corrections are made for the pressure-induced compaction of the solvent water (e.g., by $\sim$9\% at 300 MPa, 298K)\textsuperscript{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\textsuperscript{21}. $^1$H chemical shifts were referenced to the methyl signal of 1,4-dioxane added internally ($\delta=3.70$ 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

Tryptophan fluorescence spectra of hen lysozyme (35 $\mu$M or 0.5 mg ml\textsuperscript{-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 $\sim$100 $\mu$l 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

$$
N \leftrightarrow U
$$

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 $\lambda_{\text{max}}$ at pressure $p$ is determined by the following relation

$$
\lambda_{\text{max}} = f_N \lambda_{\text{max},N} + f_U \lambda_{\text{max},U}
$$

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

$$
K = \frac{f_U}{f_N} = \frac{f_U}{1-f_U} = \frac{\lambda_{\text{max},N} - \lambda_{\text{max},U}}{\lambda_{\text{max},U} - \lambda_{\text{max},N}}
$$

(3),

![Figure 1](image-url)
from which the Gibbs free energy difference \( \Delta G \) between N and U will be determined experimentally as a function of \( p \) and \( T \) by using the relation

\[
\Delta G = G_U - G_N = -RT \ln K
\]

(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 \( \Delta G \) (eq. 5). Eq. 5 is expressed as a Taylor expansion of \( \Delta G \) at a reference point \( (p_0, T_0) \) to the second order to \( p \) and \( T \)

\[
\Delta G = \Delta G^0 + \Delta V^0 (p-p_0) + \frac{\Delta \chi}{2} (p-p_0)^2 - \Delta S_p (T-T_0)
\]

\[
- \frac{\Delta C_p}{2T_0} (T-T_0)^2 + \Delta \alpha (p-p_0) (T-T_0)
\]

(5)

where \( \Delta G^0 \) is the Gibbs free energy difference of conformer U relative to N at a reference point \( (p_0, T_0) \). \( \Delta V^0 \) is the change in partial molar volume of conformer U relative to N \( (\Delta V^0 = V^U - V^N) \) also at a reference point \( (p_0, T_0) \) and \( \Delta \chi \) is the isothermal compressibility change. \( \Delta S_p \) is the entropy change at a reference point \( (p_0, T_0) \). \( \Delta C_p \) 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 \( T = T_m \), eq. 5 conforms to

\[
\Delta G = \Delta G^0 + \Delta V^0 (p-p_0) + \frac{\Delta \chi}{2} (p-p_0)^2
\]

\[
+ \Delta \alpha (p-p_0) (T_m - T_0)
\]

(6)

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

\[
\Delta V = V_U - V_N = \Delta V^0 + \Delta \alpha (T-T_0)
\]

(7)

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

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

(8).

At the midpoint of transition between N and U, where \( \Delta G = 0 \) and \( f_0 = f_{\text{eq}} \), we obtain the relations

\[
p_m = \Delta G^0/\Delta V
\]

(9)

\[
\Delta G = \Delta H_m \left( 1 - \frac{T}{T_m} \right) + \Delta C_p \left( T_m - T \right) \ln \left( \frac{T}{T_m} \right)
\]

(10),

\[
\Delta S_m = \frac{\Delta H_m}{T_m}
\]

(11)

where \( T_m \), \( p_m \), \( \Delta H_m \), \( \Delta S_m \) and \( \Delta C_p \) 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**

\( ^1H \) NMR spectra at 0.1–400 MPa

\( ^1H \) 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 \(-5^\circ C\), we could not attain full unfolding even at 400 MPa. In Figure 2A, the \( ^1H \) NMR spectrum (800 MHz) of hen lysozyme recorded at various pressures from 100 MPa to 400 MPa at \(-5^\circ C\). The spectrum at 0.1 MPa (bottom) was obtained at \(25^\circ C\) at 600 MHz. The spectrum at 0.1 MPa (top) was also obtained at \(25^\circ C\), but in the presence of 8 M urea. Chemical shifts are referenced to dioxane \( \delta = 3.70 \) ppm. All the protein solutions were prepared in 50 mM maleate buffer, 90% \( ^1H_2O/10% \) \( ^1H_2O \) (pH 2). (B) The fraction of unfolding against pressure as estimated from the fractional decrease of the combined intensity of high-field shifted methyl \( ^1H \) NMR signals of Leu17, Thr51, Ile56, Ile88 and Ile98 (\( \delta = 0.3–0.8 \) ppm) (open circles) and from the red shift of the wavelength of maximum fluorescence emission \( (\lambda_{\text{max}}) \) (closed circles). The solid curve is drawn by best-fitting the fluorescence data to eq. 8.

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**Figure 2** (A) \( ^1H \) NMR spectra (800 MHz) of hen lysozyme recorded at various pressures from 100 MPa to 400 MPa at \(-5^\circ C\). The spectrum at 0.1 MPa (bottom) was obtained at \(25^\circ C\) at 600 MHz. The spectrum at 0.1 MPa (top) was also obtained at \(25^\circ C\), but in the presence of 8 M urea. Chemical shifts are referenced to dioxane \( \delta = 3.70 \) ppm. All the protein solutions were prepared in 50 mM maleate buffer, 90% \( ^1H_2O/10% \) \( ^1H_2O \) (pH 2). (B) The fraction of unfolding against pressure as estimated from the fractional decrease of the combined intensity of high-field shifted methyl \( ^1H \) NMR signals of Leu17, Thr51, Ile56, Ile88 and Ile98 (\( \delta = 0.3–0.8 \) ppm) (open circles) and from the red shift of the wavelength of maximum fluorescence emission \( (\lambda_{\text{max}}) \) (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-resistant cell used, although the possibility of extra broadening due to partial hydration of the protein matrix 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, similar to that in 8 M urea (Fig. 2A (top)) except for line broadening. In particular, the ω-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, 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 ω-protons of solvent-exposed Trp residues, 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. 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) at pressure p relative to its initial intensity (Ip) at 100 MPa, after correcting the intensity due to the compression of the solvent water (See Materials and Methods), as representing the folded fraction (fC) of hen lysozyme at −5°C. In Figure 2B, we plot the fraction of the unfolded conformer U (fU = 1 – fC) as a function of pressure (open circle). In the same figure, we also plot the change in the wavelength of maximum fluorescence intensity (λmax) 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 verifies that the change in λmax of Trp fluorescence represents the fraction of unfolding as correctly as predicted by the 1H 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 (λmax). The coincidence is excellent, which prompts us to use the wavelength of maximum fluorescence intensity (λmax) in conjunction with eq. 8 to obtain thermodynamic parameters associated with 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 system20,21 at normal temperatures, we now turn to high-pressure fluorescence spectroscopy20 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 (λmax) 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 λmax of Trp fluorescence is considered to represent correctly the change in the microenvironment of the tryptophan ring22. The blue shift (λmax ~330 nm) indicates that the Trp ring is in the non-polar environment or buried in the hydrophobic core, while the red shift (λmax ~350–355 nm) indicates that the Trp ring is in the polar environment or exposed to the solvent water22. 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 (λmax) against pressure at all temperatures studied. At all temperatures, λmax stayed initially within 330–335 nm, showing that Trp residues are almost fully buried in the folded conformation. Finally at 700 MPa, λmax 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 λmax 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)
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 ($\lambda_{\text{max}}$) in Figure 3 (right) with eq. 8, which gives $\Delta V$ (eq. 7) and $\Delta G^0$ 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 ($\Delta G^0$) shows an increasing trend by lowering temperature, but the values may not be as reliable as $\Delta V$ in the present case, because 0.1 MPa is far from $p_m$ and no reliable data points are available to 0.1 MPa.

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 ($\lambda_{\text{max}}$) as a function of pressure at various temperatures. The solid curves are the best-fit of eq. 8 to $\lambda_{\text{max}}$, giving $\Delta G^0$ and $\Delta V$ (eq. 7) values at different temperatures, which are listed in Table 1.
Table 1 Thermodynamic parameters for unfolding of hen lysozyme determined from Trp fluorescence experiments

| T (°C) | ΔG° (kJ mol⁻¹) | ΔV (kJ mol⁻¹) | Pm (MPa) |
|--------|----------------|---------------|-----------|
| -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     |

1 Gibbs free energy change at 0.1 MPa calculated with eq. 8.
2 Partial molar volume change (ΔV=ΔV°+Δα(T−T°)) calculated with eq. 8.
3 The denatured temperature

Figure 4 gives the plot of ΔV=V_c−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 Δα=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 Δα 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 ΔCp, T_m (the temperature for ΔS=0), T_n, 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 ΔCp value at each pressure, covering both the cold denaturation and heat denaturation ranges. In general, a positive ΔCp 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_n, the temperature of maximum stability (the temperature for ΔS=0) increases with increasing pressure.

Phase diagram and free energy landscape

Figure 6 shows the phase diagram (for ΔG=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, including metmyoglobin, chymotrypsinogen, ribonuclease A, and Staphylococcal nuclease. In these proteins, except for chymotrypsinogen which clearly gives a region of pressure-induced folding at elevated temperature, an ellipsoid type pattern like that in Figure 6 is generally 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 Δκ within the pressure range studied. This will provide the basis for studying hen lysozyme at low pH by varying temperature and/or pressure.

Figure 4 Plots of the change in partial molar volume ΔV on unfolding against temperature. Best-fit to eq. 7 gives a change in expansivity Δα 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.
Concluding remark

Since hen lysozyme is highly resistive to pressure, thermodynamic unfolding studies on the pressure axis have been carried out in the presence of denaturants. 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 the 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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