Biophysical Chemistry 183 (2013) 57–63

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Tyrosine/tyrosinate fluorescence at 700 MPa: A pressure unfolding study of chicken ovomucoid at pH 12

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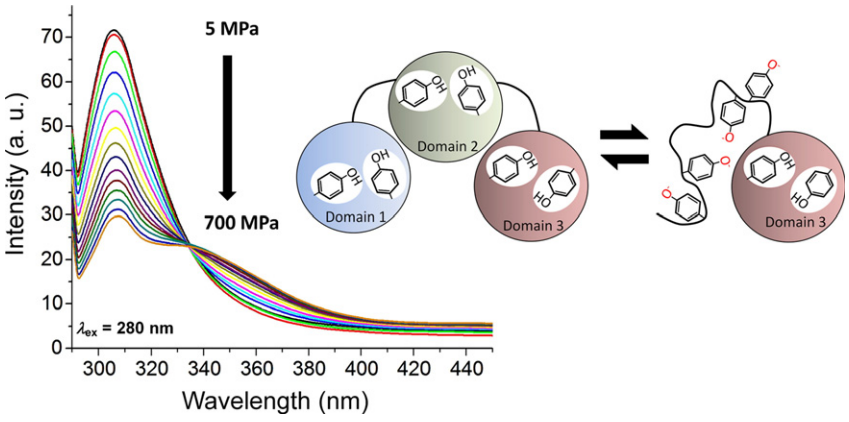
HIGHLIGHTS

- Utility of tyrosine/tyrosinate fluorescence is explored to 700 MPa at pH 12.
- All three domains of ovomucoid are folded at 0.1 MPa at 25 °C at pH 12.
- At 700 MPa at 25 °C at pH 12, only domain 3 is folded, domains 1 and 2 is unfolded.
- The outstanding stability of domain 3 is considered to give the strong allergenicity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 June 2013
Received in revised form 21 July 2013
Accepted 21 July 2013
Available online 29 July 2013

Keywords:
Pressure stability
Tyrosine/tyrosinate fluorescence
Chicken ovomucoid
Unfolding intermediate
Thermodynamic variables

ABSTRACT

The utility of tyrosine/tyrosinate fluorescence for pressure-unfolding studies of Trp-lacking proteins has been explored for the first time, with chicken ovomucoid (OVM) as target. A newly developed fluorescence spectrometer working in the range 0.1–700 MPa is employed for this purpose. At 25 °C at pH 12, all six Tyr residues give tyrosine emission at 306 nm, implying that all five Tyr residues are well buried at pH 12 in the folded OVM, except one giving "half-tyrosinate" emission at 325 nm. Upon increasing pressure, however, a distinct intermediate state, in which domains 1 and 2 are selectively unfolded, appears and increases up to 700 MPa. Extrapolated to 0.1 MPa, this intermediate lies 8.8 ± 2.6 kJ mol^{-1} above the native state, characterized with a partial molar volume smaller by −28.9 ± 7.4 ml mol^{-1}. At 5 °C at 700 MPa, even domain 3 gives a sign of cold denaturation.

1. Introduction

Pressure has emerged as a useful perturbation to trap and characterize the structure and thermodynamic properties of folding intermediates [1]. While many proteins respond sensitively to pressure and often unfold below 400 MPa, some proteins are exceptionally stable and need higher pressures to denature. A class of such proteins is commonly found in proteinase inhibitors [2] and allergenic proteins [3]. Ovomucoid (OVM) from chicken egg white, consisting of 186 amino acid residues (28.6 kDa), is well-known as an allergenic glycoprotein [4] as well as a trypsin inhibitor [2,5]. This protein consists of three sub-domains (domain 1–domain 3), each having three intra-domain disulfide bonds and two Tyr residues, but no Trp residue [6,7]. Investigation of its

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http://dx.doi.org/10.1016/j.bpc.2013.07.008

thermodynamic stability is important from the practical viewpoint, as it consists of 11% of the total protein content and is the most potent allergen in egg white. Unfolding experiments using urea and other denaturant perturbation revealed that the unfolding of OVM proceeds in two distinctive steps with a formation of an intermediate in between [8,9]. More rigorous thermodynamic analyses have been carried out on domains of OVM for turkey [10] and chicken [11,12] mainly by DSC on temperature axis. However, no studies on pressure axis have been carried out on OVM, although pressure-axis experiments are uniquely important, as it can give the structural as well as thermodynamic information on semi-stable high energy conformers of proteins located higher on the folding funnel [13]. Such information is crucial for understanding the inhibitory function, enzymatic degradability, allegenicity and gel formation [14].

The difficulty of studying the conformational stability of OVM on pressure axis is two-fold: (1) The workable pressure range on a commercial fluorescence spectrometer is usually limited up to 400 MPa and (2) neither can we utilize the sensitive Trp fluorescence to report the conformational state. Thus in the pressure-axis study, we have recently constructed a high pressure fluorescence spectrometer that can operate in the pressure range from 0.1 up to 700 MPa in our laboratory and have proven its utility in getting the thermodynamic stability diagram on p–T plane of hen lysozyme at pH 2, another allergenic protein from egg white [15]. In the present work, the high pressure fluorescence measurement was performed by utilizing the improved high-pressure fluorescence spectrometer that can operate in the pressure range up to 700 MPa. Then, to detect unfolding transitions of OVM, we utilize the fluorescence emission from six Tyr residues, two each per domain, in the pressure range between 5 MPa and 700 MPa at 25 °C and 5 °C. A highly alkaline solution condition, pH 12.0, is chosen to ensure that a Tyr residue of OVM will give distinct tyrosinate fluorescence when exposed to the solvent by denaturation of the protein. A clear transition of the tyrosine fluorescence from tyrosine (R-OH, λ_{max} ~ 306 nm) to tyrosinate (R-O^-, λ_{max} ~ 350 nm) was found with increasing pressure, allowing two-state thermodynamic analysis of the transition of OVM from the native to a selectively unfolded intermediate, in which two domains out of the three are selectively disordered, while the last domain remains largely intact at the extreme condition of pH 12.0 and 700 MPa at 25 °C.

2. Materials and methods

2.1. Protein sample preparation

Purified OVM from hen egg white was purchased from Sigma Chem. Co. Since we found on this sample an extra band on SDS-PAGE at ~14.4 kDa beside that of OVM (likely to be hen lysozyme), we performed a cation-exchange chromatography to remove the contamination. After lyophilization, the sample was dissolved in 50 mM KCl-NaOH buffer (pH 12.0) at a concentration of 1.60 mg ml^{-1}, the concentration of OVM determined from the absorbance at 278 nm using a molar extinction coefficient of 11,726 M^{-1} cm^{-1} (0.410 A_{278} = 1 mg ml^{-1}) [16].

2.2. Properties of tyrosine and tyrosinate fluorescence

When the pH of the solution is below its pK_a (~10.4), Tyr gives its intrinsic fluorescence emission at λ_{max} ~ 306 nm by excitation at 280 nm (Fig. 1, black) [17]. The λ_{max} of the intrinsic fluorescence of tyrosine (pH ≪ 10.4) is insensitive to the polarity of the solvent, namely its λ_{max} does not change whether a Tyr residue is buried in the interior of the protein or exposed to the solvent as in the event of unfolding. When its phenolic ring is deprotonated above pH > pK_a, tyrosine turns into tyrosinate (Fig. 1, inset), the ionized form of tyrosine gives emission at λ_{max} ~ 340 nm by excitation at 280 nm (Fig. 1, green) [18,19]. When the pH of the solution is close to its pK_a, the fluorescence maximum arises in between that of tyrosine and that of tyrosinate, e.g. at 315 nm

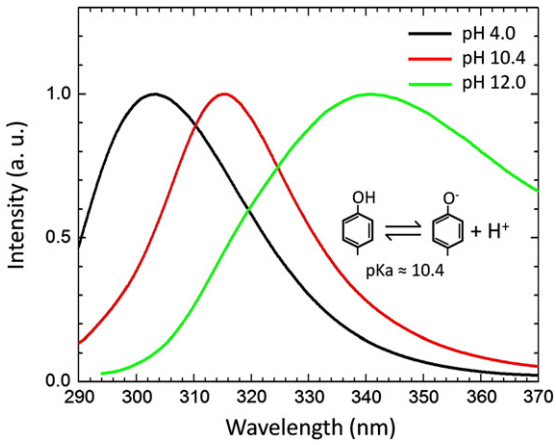

Fig. 1. The fluorescence spectra of amino acid tyrosine at three pH values, at 0.1 MPa at 25 °C. All the excitations were made at 280 nm and the emission intensity is normalized to unity at its maximum. The experiments were performed on L-tyrosine (1.0 mg/ml) in 50 mM acetate buffer (pH 4.0), in 50 mM Glycine-NaOH buffer (pH 10.4, close to pK_a of tyrosine [17]) and in 50 mM KCl-NaOH buffer (pH 12.0). The emission with λ_{max} at 306 nm is assignable to tyrosine, the emission with λ_{max} at ~350 nm to tyrosinate, and the emission with λ_{max} at ~315 nm to a tyrosine/tyrosinate mixture.

by excitation at 280 nm (Fig. 1, red). This would arise, if tyrosine and tyrosinate exchanges very rapidly. Otherwise, it will result when phenolic OH of tyrosine is polarized due to hydrogen bonding or salt-bridges. In this report, we investigate the possibility of utilizing this basic fluorescence property of a tyrosine residue in solution as a probe for unfolding of a Trp-lacking protein, chicken ovomucoid, with pressure.

2.3. High pressure fluorescence spectrometer operating at 0.1–700 MPa

To enable the study of the conformational stability of an exceptionally stable protein as a function of pressure, we have recently developed a high-pressure fluorescence spectrometer system that operates in the pressure range up to 700 MPa, incorporating a newly designed high-pressure optical vessel and inner optical cell (Syn-Corporation Co. Ltd., Japan) with a FP-6500 spectrofluorometer (JASCO Inc., Japan). The stable detection of the emission spectrum is assured in this construction due to the tightly fixed position of the optical cell against any convection in the pressure medium (here water) produced by applying pressure, as well as by minimizing any leak in the high pressure line connected to the sample holder. The successful construction of such a system allowed us to perform the thermodynamic analysis of the conformational transition of hen lysozyme at pH 2 up to 700 MPa using Trp fluorescence [15]. Essentially the same set-up is used for the present study, but using tyrosine/tyrosinate fluorescence. Details of the design of the sample holder connected with a high pressure pump will be published elsewhere.

2.4. High pressure tyrosine/tyrosinate fluorescence measurements

We studied the unfolding transition of OVM at pH 12.0 in the pressure range from 5 MPa to 700 MPa on a high pressure fluorescence spectrometer system as described briefly below. Tyrosine and tyrosinate fluorescence spectra of OVM were collected from 5 MPa to 700 MPa at 25 °C and at 5 °C using FP-6500 spectrofluorometer (JASCO Inc., Japan) combined with a high pressure chamber (Syn-Corporation Co. Ltd., Japan). We applied 5 MPa instead of 0.1 MPa to avoid any effect from air bubbles in inner optical cell. Temperature of the pressure resistive chamber was maintained by circulating water–ethylene glycol 1-to-1 mixture. The excitation wavelength from a xenon arc lamp was set at 280 nm for excitation of dominant tyrosine emission and at 295 nm for isolated-tyrosinate emission with a slit-width of 5 nm [20,21], while the emission in the range of 290–450 nm was collected with a slit-

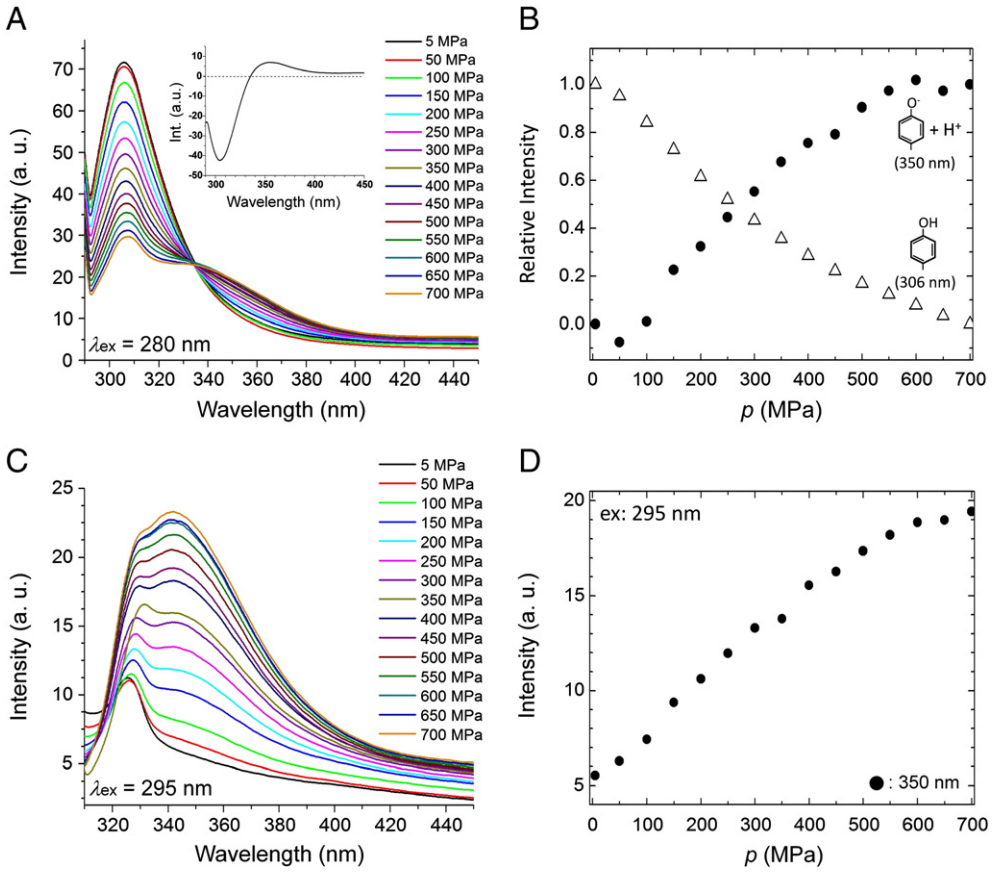

Fig. 2. Pressure-dependent changes of the fluorescence emission spectra of OVM (1.6 mg ml^{-1}) at pH 12.0 at 25 °C between 5 and 700 MPa. (A) Spectral changes by excitation at 280 nm (tyrosine absorption). An isoemissive point is observed at 335 nm. The inset shows the difference spectrum obtained by subtracting the spectrum at 700 MPa from that at 5 MPa. (B) Plot of emission intensities at 306 nm (tyrosine) and at 350 nm (tyrosinate) (from panel A), after correcting the measured intensity by taking the solvent contraction into account (for example, ~9% by 300 MPa at 25 °C) [22]. Open triangle: 306 nm; filled circle: 350 nm. (C) Spectral changes by excitation at 295 nm (tyrosinate absorption). (D) Plot of emission intensity at 350 nm (tyrosinate) (from panel C).

width of 10 nm at a scanning rate of 500 nm min^{-1}. The shutter was kept open only when the emission was recorded. At each pressure, corrections were made for fluorescence intensities for the increase in the effective protein concentration by the pressure-induced compaction of the solvent water (for example, ~9% by 300 MPa at 25 °C) [22]. The data were processed with Microcal Origin 6.0 (Microcal Software, Inc.).

2.5. Thermodynamic analysis of the fluorescence data

Under physiological conditions, say at 0.1–5 MPa, the OVM is nearly fully folded in its native conformer N, giving tyrosine emission intensity i_N. All fluorescence intensities at pressure i_p were normalized to i_N. When the protein exists in the two-state equilibrium between the folded conformer N and the intermediate conformer I (N ⇌ I) with an equilibrium constant K at any pressure p is determined experimentally (cf. Fig. 4) by

$$K = \frac{[\mathrm{I}]}{[\mathrm{N}]} = \frac{i_p - i_N}{i_I - i_p} \tag{1}$$

where i_N and i_I are the fluorescence intensities of the folded and intermediate conformers, respectively. From the experimental variation of K against pressure, we obtain the Gibbs energy difference between N and I by fitting to the following relation,

$$\Delta G^p{}_{\mathrm{NI}} = G_I - G_N = -RT\ln K = \Delta G^0{}_{\mathrm{NI}} + \Delta V^0{}_{\mathrm{NI}}(p-p_0) + \frac{\Delta\kappa}{2}(p-p_0)^2, \tag{2}$$

in which, in often case, the differential compressibility $\Delta\kappa$ between N and I may be negligible. Here, R is the gas constant, T is the absolute temperature, K is the equilibrium constant, and the values $\Delta G^p{}_{\mathrm{NI}}$ and $\Delta G^0{}_{\mathrm{NI}}$ are the Gibbs free energy differences at pressures p and p_0 (=0.1 MPa (1 bar)), respectively. The value $\Delta V^0{}_{\mathrm{NI}}$ is the partial molar volume difference at ambient pressure between the two conformers. By combining Eqs. (1) and (2), the fluorescence intensity is expressed as a function of pressure by

$$i_p = \frac{i_N + i_I \exp\left\{-\dfrac{\Delta G^0_{\mathrm{NI}} + \Delta V^0_{\mathrm{NI}}(p-p_0)}{RT}\right\}}{1 + \exp\left\{-\dfrac{\Delta G^0_{\mathrm{NI}} + \Delta V^0_{\mathrm{NI}}(p-p_0)}{RT}\right\}}. \tag{3}$$

Fluorescence intensity values experimentally determined at various pressures are best fit to Eq. (3) with four parameters, i_N, i_I, $\Delta G^0{}_{\mathrm{NI}}$ and $\Delta V^0{}_{\mathrm{NI}}$.

3. Experimental results and analysis

3.1. Fluorescence of OVM by excitation at 280 nm

Fig. 2A shows an overlay of the fluorescence spectra from six Tyr residues of OVM in the solution condition of pH 12.0 measured at pressures from 5 MPa to 700 MPa at 25 °C by excitation of tyrosine absorption at 280 nm. At 5 MPa, the fluorescence emission is dominated by the emission with λ_{max} at 306 nm (Fig. 2A) assignable to tyrosine

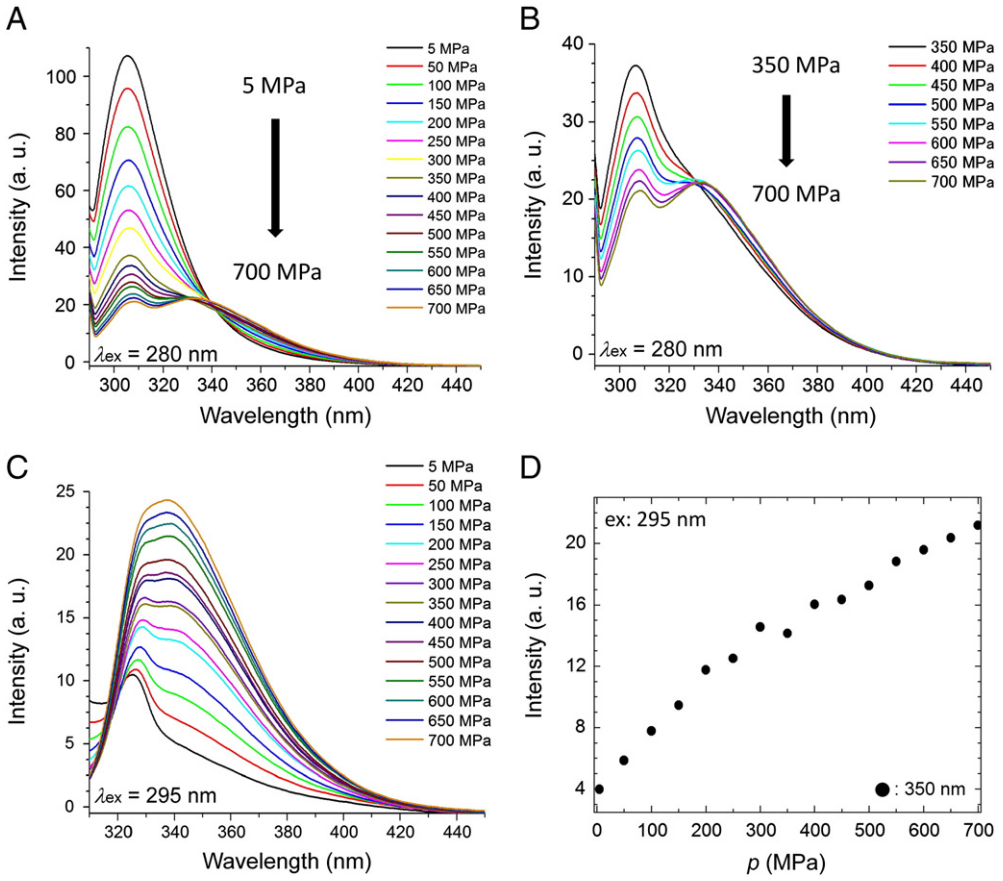

Fig. 3. Pressure-dependent changes of the fluorescence emission spectra of OVM (1.6 mg ml^{-1}) at pH 12.0 at 5 °C between 5 and 700 MPa. (A) Spectral changes by excitation at 280 nm (tyrosine absorption) between 5 MPa and 700 MPa. An isoemissive point is observed at 335 nm. (B) The spectral changes by excitation at 280 nm (tyrosine absorption) between 350 and 700 MPa. A new isoemissive point is found at 330 nm. (C) Spectral changes by excitation at 295 nm (tyrosinate absorption). (D) Plot of the emission intensity at 350 nm (for tyrosinate), obtained by excitation at 295 nm at various pressures from 5 to 700 MPa at 5 °C.

(R-OH) (cf. Fig. 1). The observation indicates that even at 25 °C in the solution of pH 12.0 the *effective* pK_a values of all six Tyr of OVM are larger than 12. Such a large pK_a value was observed previously in other proteinase inhibitor [23]. This finding indicates, in turn, that all the three domains of OVM are folded and that the phenolic OH groups of the Tyr residues are largely protected from the solvent, even at pH 12.0 at 25 °C. With increasing pressure, the decrease of the emission at 306 nm and a concomitant increase in the emission around 350 nm (assignable to tyrosinate (R-O$^-$), see inset) were observed, with a clear isoemissive point at 335 nm (Fig. 2A). The result strongly suggests that a two-state transition takes place between the tyrosine (R-OH) and tyrosinate (R-O$^-$) forms of Tyr residues of OVM in the wide pressure range from 5 to 700 MPa (Fig. 2B). Quantitatively, the fraction (47/75 ≃ 2/3) of the original emission at 306 nm at 5 MPa is decreased at 700 MPa, while a significant fraction (28/75 ≃ 1/3) of the original emission still remains even after the transition is nearly complete at 700 MPa. The results indicate that 4 Tyr residues out of six corresponding to two domains have transformed into tyrosinate (R-O$^-$), while 2 Tyr residues corresponding to one domain remain as tyrosine (R-OH) even at 700 MPa, strongly suggesting that the folding state of one domain of OVM is strongly resistive against denaturation, even to the extreme condition of pH 12.0 at 700 MPa. The situation at 5 °C is similar to that at 25 °C, in which the original emission at 306 nm decreases to near 1/3 (~36/110) and instead the emission at λ_{max} ~ 350 nm

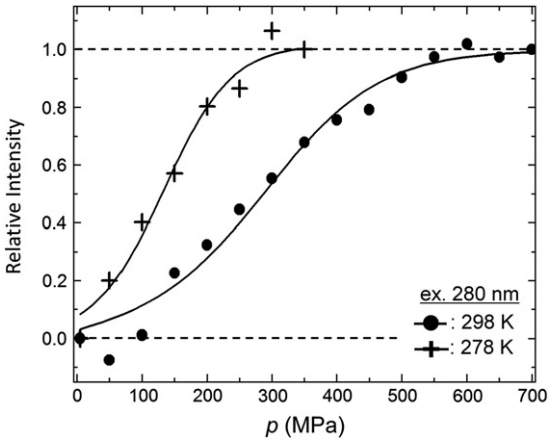

Fig. 4. Plots of the emission intensity at 350 nm of OVM as a function of pressure at 25 °C and at 5 °C. Both emissions were obtained by excitation at 280 nm. The solid curves are the best-fit of Eq. (3) to fluorescence intensities, giving the Gibbs free energy difference (ΔG^0_{NI}) and the partial molar volume difference (ΔV^0_{NI}) between I and N at ambient pressure, which are listed in Table 1.

Table 1
Thermodynamic parameters for the unfolding of chicken ovomucoid determined from tyrosine/tyrosinate fluorescence experiments at pH 12.0.

Temperature (°C)	ΔG^0_{NI} (kJ mol^{-1})[a]	ΔV^0_{NI} (ml mol^{-1})[b]
λ_{ex} = 280 nm		
25 °C	8.8 ± 2.6	−28.9 ± 7.4
5 °C	5.9 ± 0.6	−43.7 ± 5.1

[a] Gibbs free energy change at 0.1 MPa calculated with Eq. (3) by assuming $\Delta\kappa = 0$.
[b] Partial molar volume change at 0.1 MPa calculated with Eq. (3) by assuming $\Delta\kappa = 0$.

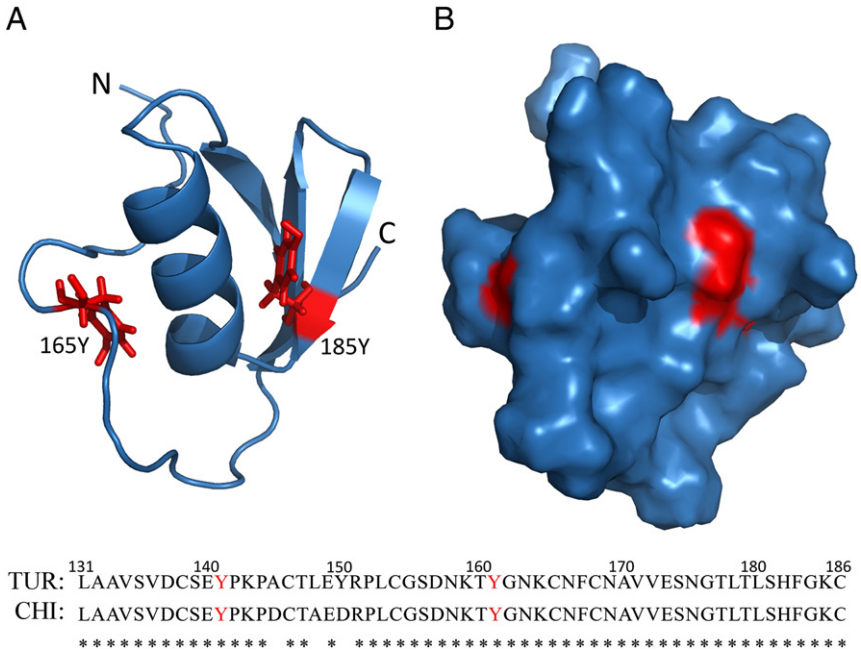

Fig. 5. NMR solution structure of turkey ovomucoid third domain (PDB ID: 1TUR) [31]; expressed in Ribbon model (A) and in Surface model (B). The two Tyr residues (165Y and 185Y), conserved in chicken ovomucoid, are marked by red in both figures. Bottom: The amino acid sequence of turkey ovomucoid third domain and that of chicken ovomucoid third domain (sequence similarity ~ 94%).

increases with an isoemissive point at 338 nm (Fig. 3A), but this transition finishes already at ~350 MPa. When pressure is further increased above 350 MPa, the intensity of emission at 306 nm shows a further decrease and the emission at λ_{max} ~ 350 nm increases with a new isoemissive point at 330 nm (Fig. 3B), suggesting that a Tyr residue belonging to the most stable domain begins to transform from tyrosine (R-OH, λ_{max} ~ 306 nm) to tyrosinate (R-O^-, λ_{max} ~ 350 nm), suggesting the onset of unfolding of the most stable domain at 5 °C above 350 MPa.

3.2. Fluorescence of OVM by excitation at 295 nm

To confirm the production of tyrosinates (R-O^-) in ground state in OVM at high pressure, next we excite directly the tyrosinate absorption at 295 nm to observe its fluorescence at pH 12.0 both at 25 °C (Fig. 2C) and 5 °C (Fig. 3C). Initially, we observe emission with λ_{max} around 325 nm at 5 MPa. By increasing pressure, the fluorescence emission at λ_{max} ~ 350 nm, assignable to tyrosinates (R-O^-), increased dramatically to the final value at 700 MPa (Figs. 2C and 3C). Thus in Fig. 2C, we have at least two fluorescent species, one giving the emission with λ_{max} around 325 nm existent already at 5 MPa and the other giving a broad emission with λ_{max} at ~350 nm that increases with pressure. First, the species giving the emission with λ_{max} at ~350 nm can be identified with fully exposed tyrosinates (R-O^-), and the results of Fig. 2D indicate that the fraction of tyrosinates (R-O^-) increases dramatically with the increase in pressure. The results confirm those from Figs. 2A and 3A that a majority of tyrosine (R-OH) residues turn into tyrosinates (R-O^-) with increasing pressure. Figs. 2D and 3D show plots of the emission intensities at 350 nm at various pressures at 25 °C and 5 °C, obtained from Figs. 2C and 3C, respectively. Second, the relatively weak emission with λ_{max} around 325 nm needs attention. This emission appears already at 5 MPa when OVM is in state N

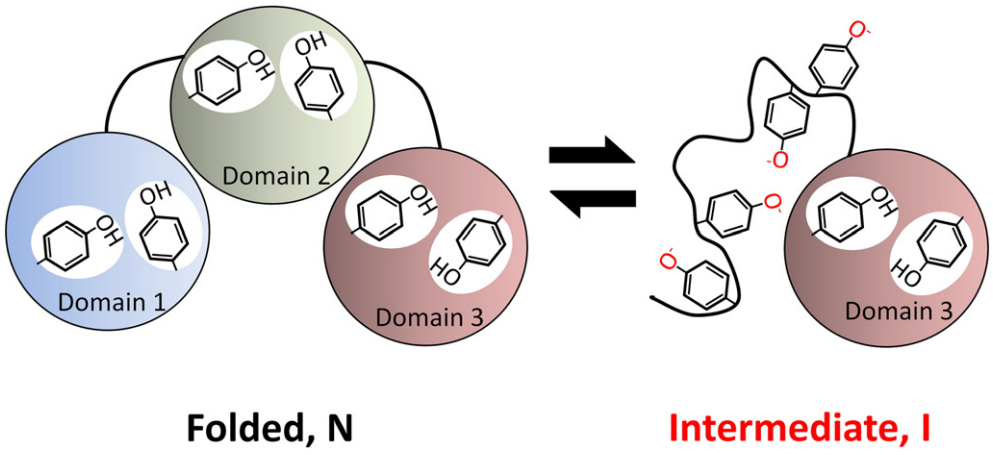

Fig. 6. The conformational fluctuation of OVM between the folded conformer N and the intermediate conformer I deduced from the present experiments at pH 12.0 at 25 °C. All the tyrosine side-chains of OVM are buried at 0.1 MPa at 25 °C at pH 12.0, but those in domains 1 and 2 become exposed to the solvent in the form of tyrosinate at 700 MPa, while those in domain 3 remain buried.

and stays as shoulder even at 700 MPa when OVM is in state I in which only the most stable domain is folded. The most reasonable interpretation seems, therefore, that this emission belongs to a Tyr residue in the most stable domain.

3.3. Thermodynamic analysis

The two-state transitions from tyrosine (R-OH) to tyrosinate ($R\text{-}O^-$) involving approximately four Tyr residues commonly occur between 5 MPa and 700 MPa at 25 °C and between 5 MPa and ~350 MPa at 5 °C. A reasonable interpretation of these observations would be that a major transition of OVM takes place from the initial state N in which all the three domains are folded to the intermediate state I in which two domains of OVM corresponding to 4 Tyr become unfolded and one domain corresponding to 2 Tyr remains largely intact. In any pressures in between, there is an equilibrium between N and I states, $N \leftrightarrows I$, allowing thermodynamic analysis based on Eq. (3) (Fig. 4). The stability and volume parameters obtained at 25 °C and 5 °C are summarized in Table 1.

4. Discussion

Fluorescence remains as a useful technique for protein conformational studies, because of its high sensitivity and because it is a relatively handy spectroscopy that can be used with pressure perturbation, particularly at relatively high pressure, e.g. 400 MPa or higher. For most proteins, tryptophan fluorescence has been a convenient natural probe to study protein conformation and/or solution environment [24]. On the other hand, there is a class of proteins which lack a tryptophan residue, for which tyrosine residues could play a substitute. This was explored in the past, mostly in the 70s and 80s [19,25–29]. Thus in the present work, we have explored, for the first time, the utility of tyrosine/tyrosinate fluorescence in studying the conformational fluctuation of a Trp-lacking protein in solution using pressure perturbation up to 700 MPa. With chicken ovomucoid at pH 12.0 as target, we found a distinct intermediate I at 700 MPa, in which two domains are unfolded, leaving one domain intact. At any pressure in between, an equilibrium $N \leftrightarrows I$ is reached, the conformer I located 8.8 kJ mol^{-1} above N with the volume smaller by -28.9 ml mol^{-1} at 0.1 MPa at 25 °C.

So far, we have not specifically identified the conformer I, under our specific experimental conditions, as to which one of the three domains of chicken ovomucoid is the domain that remains folded even at 700 MPa at 25 °C or at 350 MPa at 5 °C. However, a previous experiment carried out by Das et al., though under milder different experimental conditions, appears to give a reasonable answer with regard to its assignment [30]. They carried out mainly urea titration experiments on isolated domains or domain combinations of chicken ovomucoid at pH 7.0, and found that domain 3 has by far the highest stability ($[urea]_{1/2} \approx 6.4$ M) compared to two other domains ($[urea]_{1/2} \approx 2.7$ M) [30]. Based on this result, it appears rather certain that the single domain remaining intact at an extreme condition of pH 12.0 and 700 MPa be assigned to domain 3, giving the conformational equilibrium of OVM as depicted in Fig. 6. In this regard, we plan to carry on an independent assignment of domain 3 using high pressure NMR in later studies. The outstanding stability of domain 3 is considered responsible for the strong allergenicity of this protein.

Another concern is the state of a Tyr residue in domain 3, giving the emission at $\lambda_{max} \sim 325$ nm in Figs. 2C and 3C. The emission wavelength is rather close to that in Fig. 1, green line, suggesting that the Tyr residue is in half way to tyrosinate, possibly by partial exposure of its side chain to the solvent. On the other hand, in the literature, emission peaks in this intermediate range of wavelength have been detected at ambient pressure in various Trp-lacking proteins [25–29], in which the emissions are ascribed to Tyr residues whose phenolic groups form ionic bonds with positively charged side chains. Thus another possibility for the 325 nm emission is that the Tyr residue responsible for this emission forms an ionic bonding with a positive charged side chain, thus contributing the stability of domain 3. Although the tertiary structure of chicken OVM domain 3 is not known, the solution structure of turkey OVM domain 3, having ~94% sequence homology with the conserved two Tyr residues, has been reported [31] (Fig. 5A). Here we find that the two Tyr residues form part of the folded structure and are located by partially exposing their side chains to the solvent (Fig. 5B). Granted chicken domain 3 has nearly identical structure as that of turkey domain 3, either one of the two Tyr residues could form partial tyrosinate by forming an ionic bonding with a positive charged side chain or by partly exposing its side chain to the alkaline water environment. The state of the Tyr residues of chicken OVM domain 3 could be a target of study by high pressure NMR or crystallography to further expand the utility of tyrosine/tyrosinate fluorescence in future studies.

In the present work, we have restricted our solution condition to a highly alkaline pH, pH 12.0, to study conformational fluctuations of a Trp-lacking protein by utilizing the strong tyrosinate fluorescence at high pH. The experiment has been successful in monitoring differential stabilities of domains and to find a locally unfolded intermediate state of OVM in a clear-cut manner (Fig. 6). This work can be extended to the study of conformational fluctuations in Trp-lacking proteins in the neutral pH region by using tyrosine/tyrosinate fluorescence in combination with various proton acceptors to facilitate tyrosinate production [19,26]. Such studies are under way in our laboratory and will be reported in future publications.

Acknowledgments

This work was supported by the Academic Frontier Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan (07F010), and by a Japan Science and Technology Agency program for the Niigata Industrial Creation Organization, Niigata, Japan. We thank Prof. H. Tachibana for his valuable comments for improving the manuscript.

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