High pressure spectrofluorimetry – a tool to determine protein-ligand binding volume

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High pressure spectrofluorimetry – a tool to determine protein-ligand binding volume

G Skvarnavičius[1], Z Toleikis[1], M Grigaliūnas[1], J Smirnovienė[1], P Norvaišas[1], P Cimmperman[1,2], D Matulis[1], and V Petrauskas[1]

[1]Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Vilnius University, Vilnius, Lithuania
[2]Baltic Institute of Advanced Technology, Vilnius, Lithuania

E-mail: vytautas.petrauskas@bti.vu.lt

Abstract. The change in protein volume observed upon protein-ligand interaction (termed as the binding volume) is an important but largely neglected thermodynamic parameter from the perspective of both fundamental science and potential applications in the development of specific protein ligands. The binding volume is the pressure derivative of the Gibbs energy, thus elevated pressure is required to determine the volumetric properties of proteins. Here we describe the use of high-pressure spectrofluorimetry to determine both unfolding and ligand binding-induced volume changes of a protein. The degree of protein unfolding at elevated pressures was monitored by an intrinsic tryptophan fluorescence. Different approaches of experimental fluorescence spectra analysis are described and the impact on the quality of thermodynamic parameters is discussed.

1. Introduction

Despite the laborious nature of pressure techniques, numerous studies were devoted to determine the thermodynamic properties of pressure-induced protein unfolding [1–13]. Relatively high pressures are required to determine the volumetric properties of proteins, and that is probably the most serious obstacle for the pressure to become a standard descriptor of the thermodynamic state of a protein.

Of particular interest is the change in volume related to protein-ligand interaction, hereafter termed as protein-ligand binding (or reaction) volume. A majority of high pressure studies were devoted to reveal the thermodynamics of protein unfolding/refolding reaction and the dissociation of multimeric proteins under pressure. However, relatively little attention has been paid to the volume changes resulting from the interaction between a protein and small molecule [4, 12, 14–19].

High pressure spectrofluorimetry has been extensively used during the past several decades [20] and helped to reveal various aspects of protein folding and stability. Here we describe how high pressure fluorescence could be used to determine protein-ligand binding volume. We continue the development and validation of the method on several isoforms of human carbonic anhydrase (CA) – a protein involved in cancer progression and therapy. We describe two different approaches of experimental fluorescence spectra analysis and discuss their impact on the reliability of calculated thermodynamic parameters.

2. Materials and Methods

Three proteins, namely CA I, CA II, and CA XIII, which are isoforms of human carbonic anhydrase (CA), were used to illustrate the determination of protein-ligand binding volume by fluorescent pressure shift assay (FPSA) technique. The FPSA was previously described using other protein-ligand systems [12, 19, 21]. The protein unfolding experiments were performed using an ISS PC1 photon counting spectrofluorimeter equipped with a high-pressure cell connected to a hydrostatic pump. The pressure of 380 MPa was reached by gradual increments of 20 MPa while maintaining the constant temperature of 25 ℃ by circulating water bath. The system was allowed to equilibrate for approximately two minutes after each pressure increment prior to recording the fluorescence spectrum. The intrinsic fluorescence of tryptophan residues at atmospheric and elevated pressures was observed by exciting the tryptophan at 295 nm and recording the emission spectra from 320 to 400 nm. A quartz cuvette was filled with the aqueous protein solution containing 3 µM CA, 0–1.5 M guanidine hydrochloride (GndHCl), 1% dimethyl sulfoxide (DMSO), 10 mM Bis-Tris buffer, *p*H 7.0 and covered with sealing film to separate the protein solution from water in the pressure cell. Various concentrations of acetazolamide (AZM) ligand was used to determine the stability of CA against pressure-induced denaturation.

3. Model

The pressure-induced unfolding profiles of a protein, $f(p)$, can be described by an equation

$$f(p) = f_N + \frac{f_U - f_N}{1 + \exp(\Delta G(p)/RT)}, \tag{1}$$

where R is the universal molar gas constant and T – absolute temperature. Here we use an assumption that there are only two protein states at equilibrium – native (N) and unfolded (U) – and the transition between states is stepwise (i.e., without any intermediate states). If protein unfolding is monitored by fluorescence intensity at a fixed wavelength, λ, the parameters f_N and f_U denote the experimental fluorescence yields for the native and unfolded protein states, respectively (solid square curves in Figure 1 (c) and (d)). Equation (1) is also valid, if protein unfolding profile is obtained from the center of spectral mass (CSM) [4], which is defined as

$$\lambda_{CSM} = \frac{\sum_i f_i \lambda_i}{\sum_i f_i}, \tag{2}$$

where f_i is the fluorescence intensity at a wavelength λ_i and the summation is over entire spectrum range. The Gibbs energy of a protein, ΔG, as a function of pressure, p, at a constant temperature is [2, 10]

$$\Delta G = \Delta G_0 + \Delta V_0 (p - p_0) + \frac{\Delta\beta}{2} (p - p_0)^2 . \tag{3}$$

ΔG_0, ΔV_0 and $\Delta\beta$ denote changes of the standard state thermodynamic parameters of protein unfolding – the Gibbs energy, volume and compressibility factor, respectively. The pressure value at the midpoint of protein unfolding curve (i.e., when both native and unfolded protein concentrations are equal) is referred to as the melting pressure, p_m. The addition of ligand to the protein solution usually changes the value of its melting pressure. The relationship between the concentration of added ligand, L_t, total protein concentration, M_t, and the midpoint of unfolding transition is [21]

$$L_t = (\exp(-\Delta G_U/RT) - 1) \left(\frac{M_t}{2\exp(-\Delta G_U/RT)} + \frac{1}{\exp(-\Delta G_b/RT)} \right), \tag{4}$$

where indexes U and b stand for the changes in Gibbs energy related to protein unfolding and protein-ligand binding, respectively:

$$\Delta G_x = \Delta G_{0\_x} + \Delta V_{0\_x}(p_m - p_0) + \frac{\Delta\beta_x}{2}(p_m - p_0)^2; \quad x = U, b. \tag{5}$$

The relationship (4) is often referred to as a dosing curve. The main parameter of interest – protein-ligand binding volume, ΔV_{0_b} – can be obtained by fitting experimental dosing curve with Equation (4).

4. Results and Discussion

To determine the protein-ligand binding volume a series of pressure-induced protein unfolding experiments at various concentrations of added ligand have to be performed. Typical fluorescence spectra of CA II and CA I proteins at various pressures are shown in the Figure 1 (a) and (b), respectively. Native state of the protein dominates at low pressure, which is manifested by the peak position of tryptophan fluorescence at emission wavelength approximately equal to 332 nm [22]. The increase of pressure promotes the unfolded states of a protein and shifts the maximum of emission spectra, λ_{max}, towards 350–353 nm. This wavelength range corresponds to the fluorescence peak of tryptophan residues in a highly polar environment.

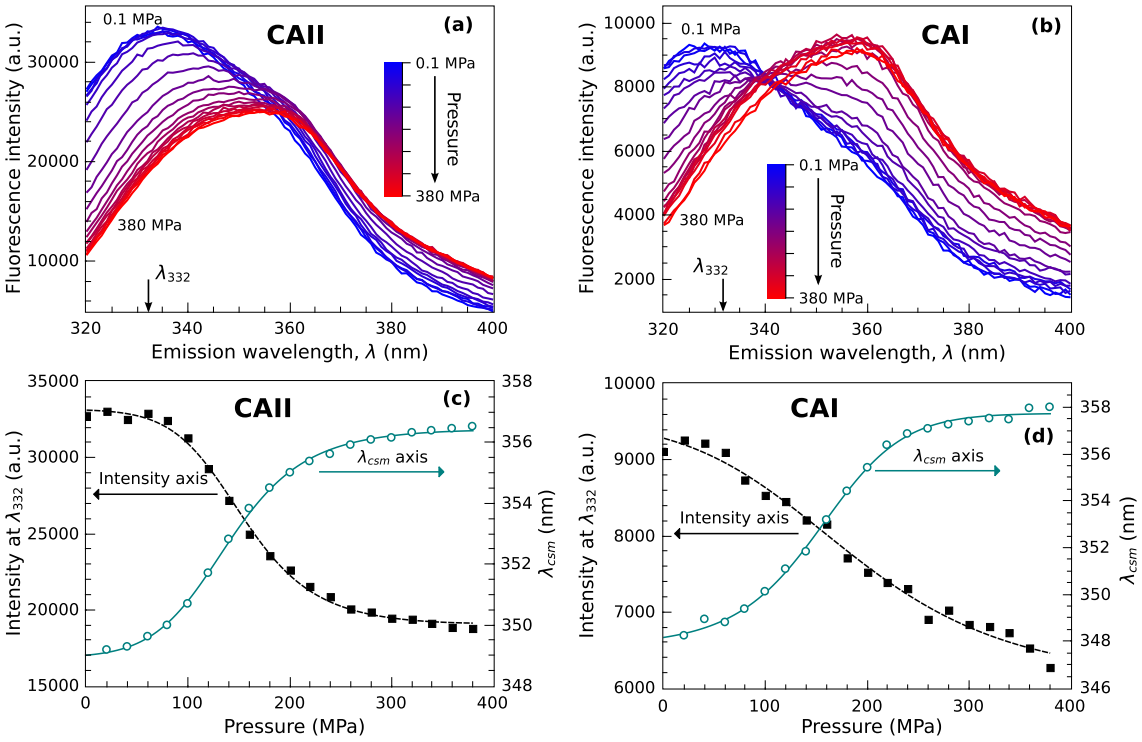

Figure 1. Intrinsic tryptophan fluorescence spectra at various pressures and pressure-induced unfolding profiles of CA II and CA I proteins. Lines in (c) and (d) are fits to Equation (1), λ_{CSM} data points are calculated from the corresponding spectra using Equation (2).

Several types of unfolding profiles could be obtained from the spectral information shown in Figure 1 (a) and (b), including the pressure-induced shift in either λ_{CSM} (Equation (2)) or λ_{max}, or by following the change in fluorescence intensity at a particular wavelength, I_λ.

Both CSM and λ_{max} unfolding profiles require an entire tryptophan fluorescence spectrum and thus are more time consuming than I_λ, which could be obtained from the fluorescence yield measurements at a single wavelength. I_λ unfolding profile is shown by black solid squares, while calculated values of λ_{CSM} versus pressure are represented by open circles in Figure 1 (c) and (d). Our results show that for some proteins (e.g., CA II) both methods provide reliable unfolding profiles while in some cases (e.g., CA I) these profiles could not be used interchangeably to precisely determine the thermodynamic parameters of unfolding (compare fluorescence intensity curves of CA II and CA I). It was possible to fit the unfolding transition of CA I using I_λ profile, but the thermodynamic parameters were obtained with lower accuracy than in the case of CA II. For example, the relative errors of fitted values of protein unfolding volume ΔV_{0_U} were 27% (I_λ profile) and 22% (λ_{CSM} profile) for CA II protein, while that values for CA I protein were 91% and 36%, respectively. In case of pressure-induced unfolding of CA XIII protein, the transition to unfolded state was almost hindered in the I_λ profiles (not shown here). In contrary to I_λ profile, the shift in λ_{CSM} of CA XIII denaturation demonstrated unambiguous unfolding transition.

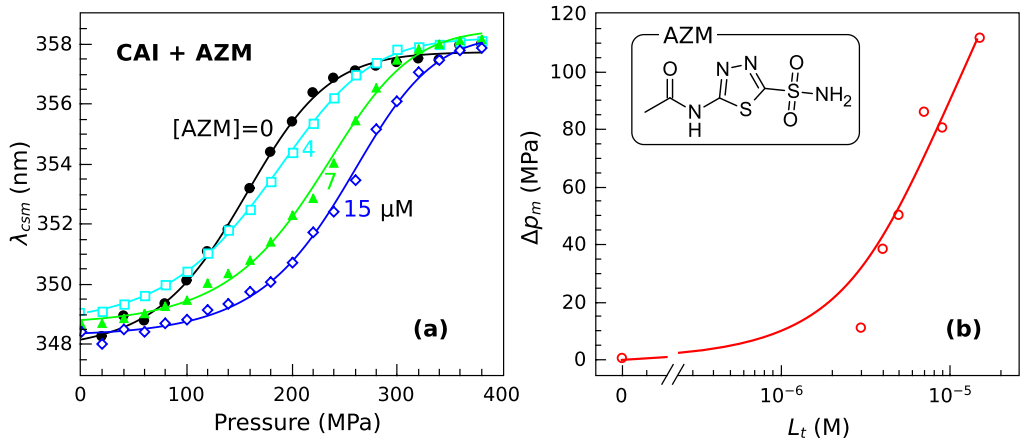

Figure 2. (a) Pressure-induced unfolding profiles of CA I at various concentrations of added ligand AZM (lines are fits to Equation (1)). All protein solutions contained 1.2 M of GndHCl. (b) The shift of protein melting pressure, Δp_m, versus total concentration of added ligand (line is the fit to Equation (4)).

The next step in the determination of protein ligand binding volume is the analysis of protein-ligand dosing curve, which is obtained by plotting the shift of p_m values versus total concentration of added ligand. Higher hydrostatic pressure is necessary to reach the protein melting point upon addition of stabilizing ligand. λ_{CSM} unfolding profiles of CA I titration by acetazolamide (AZM) are shown in Figure 2 (a). The titration of CA I protein solution with AZM yields the dosing curve shown in Figure 2 (b). The binding volume can be determined by fitting data with model Equation (4). In the shown example of CA I – AZM system (Figure 2 (b)) the fitted value of ΔV_{0_b} is equal to (-32 ± 4) ml/mol.

Many proteins can withstand relatively high pressures without being unfolded. An addition of stabilizing ligands requires even higher hydrostatic pressures to unfold a protein. The unfolding pressure values are often higher than 400 MPa, which is the limit of our experimental equipment. Partial protein unfolding or unfolding profiles without a post-transition baseline result in unreliable fitting parameters. Thus, chaotropic agent guanidine hydrochloride was used to assist the pressure-induced protein unfolding. Many previous reports on chemical denaturation

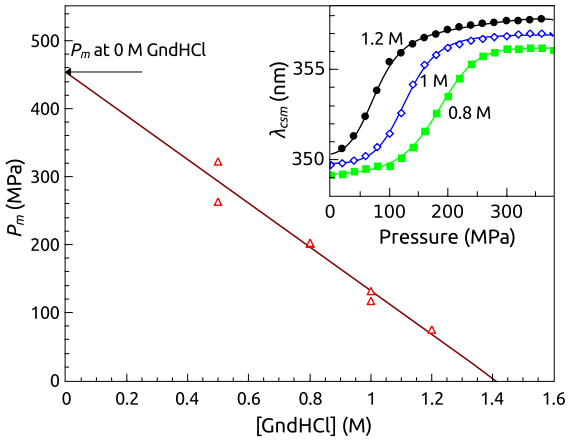

Figure 3. Dependence of CA XIII melting pressure on the concentration of GndHCl. Inset: pressure-induced unfolding profiles of CA XIII at 0.8 M, 1.0 M and 1.2 M of added GndHCl.

of proteins (see, e.g., [23, 24]) show that dependence of the Gibbs energy of unfolding on the concentration of denaturant appears to be linear. The same law is applicable for the melting pressure:

$$p_m([\text{GndHCl}]) = p_m(0) + m \times [\text{GndHCl}], \tag{6}$$

where m is the proportionality constant and $p_m(0)$ is the melting pressure at zero GndHCl concentration, which could be obtained by extrapolation of the linear fit as it is shown in Figure 3.

In conclusion, the protein-ligand binding volume can be determined using fluorescent pressure shift assay technique. The unfolding profiles obtained from the shifts of CSM ensure the quality of these profiles and subsequently the accuracy of fitted thermodynamic parameters of protein unfolding. For some proteins I_λ unfolding profiles with an accuracy similar to that of CSM could be used; in such cases considerably less time is required to obtain the protein unfolding curve.

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