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Short communication

Effects of buffer ionization in protein transition volumes

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ABSTRACT

Protein denaturation events are generally associated with a change in the state of ionization of abnormally titrating groups and, therefore, are coupled with changes in buffer ionization/neutralization equilibria. Consequently, buffer ionization should influence the measured change in volume accompanying protein denaturation. Changes in volume accompanying protein denaturation reflect the differential packing and hydration of polypeptide chains in their native and denatured conformations while also describing the pressure stability of proteins. A characteristic feature of conformational transitions of globular proteins is a near zero change in volume that is comparable in magnitude with the volume of ionization of biologically relevant buffers. Thus, the impact of buffer ionization on the volume of protein denaturation could be very significant with the potential to affect not only its magnitude but also its sign. To investigate this point quantitatively, we performed pressure perturbation calorimetric (PPC) studies of lysozyme and ribonuclease A at pH 3.0 in four buffers differing in their ionization volumes. Our results identify buffer ionization as an important determinant of protein transition volume that needs to be carefully taken into account. We emphasize that the importance of our results is not limited to PPC measurements but is more general and applies to all volumetric investigations, in particular, extending to the derivation of the pressure–temperature phase diagram of protein stability.

The partial molar volume, V°, of a globular protein represents a measure of the fine balance of forces stabilizing/destabilizing the native conformation including the ensemble of intramolecular interactions and the entire range of solute–solvent interactions [1–4]. A change in volume, ΔV, accompanying a conformational transition of a protein collectively reflects all structural and solvation alterations associated with the transition [2–7]. In addition, ΔV is a fundamental thermodynamic property of protein stability determining the pressure-dependent equilibria between its various conformational states [8,9].

Pressure perturbation calorimetry (PPC) has emerged as a versatile and sensitive technique for determining volume changes accompanying temperature-induced order–disorder transitions [10–12]. The reliability of PPC volume measurements and their applicability to protein studies have further increased upon the introduction of a two-state analysis of primary expansibility data [13,14]. The analysis enables one to more rigorously separate the conformational and excess contributions to the measured temperature dependences of protein expansibility thereby significantly alleviating the problem of pre- and post-denaturational baselines. The transition enthalpy required for a two-state approximation of PPC peaks should be determined separately from differential scanning calorimetric (DSC) measurements [13]. There is, however, a heretofore unaccounted contribution that, as demonstrated below, may strongly affect ΔV with the potential of altering not only its magnitude but also its sign.

A change in volume, ΔV, associated with a protein transition contains pH-dependent and pH-independent components. The pH-dependent term reflects changes in volume accompanying an alteration in the state of ionization of abnormally titrating groups (which exhibit distinct pK_a values in the native and unfolded states) and the coupled alteration in the state of buffer ionization. The pH-independent component is the conformational change in volume which reflects the transition-induced alterations in packing and hydration of the polypeptide chain. Importantly, the conformational change in protein volume does not contain any contribution from a change in the state of ionization of titrable groups. The transition volume of a protein can be presented as the sum:

$$\Delta V(T) = \Delta V_{conf}(T) + \Delta V_{ion}(T) + \nu \Delta V_{buff}(T) \quad (1)$$

where $\Delta V_{conf}(T)$ is the pH-independent conformational change in volume; $\Delta V_{ion}(T)$ is the volume change accompanying ionization of abnormally titrating protein groups which change their pK_a upon the transition; $\Delta V_{buff}(T)$ is the volume of buffer ionization; $\nu = \sum_{i=1}^{n} \left[\frac{10^{pH-pKa_{Di}}}{1+10^{pH-pka_{Di}}} - \frac{10^{pH-pKa_{Ni}}}{1+10^{pH-pKa_{Ni}}}\right]$ is the number of protons released to the bulk upon the transition; pK_{aNi} and pK_{aDi} denote the dissociation constants of the *i*-th ionizable group in the native and denatured conformations, respectively.

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Volume changes, ΔV, accompanying protein denaturation may be negative or positive [15,16]. However, the magnitude of ΔV is invariably small, typically, no more than ~100 cm^3mol^{-1} [2–5,15,16]. The volumes of ionization, ΔV_{buff}, of biologically relevant buffers are on the order of 10 cm^3mol^{-1} but may vary in sign and magnitude depending on the buffer [17]. The relative range of ΔV and ΔV_{buff} values suggests that, depending on ν, the last term of Eq. (1) may have a decisive impact on the magnitude and even the sign of ΔV. Thus, unless the term $\nu\Delta V_{buff}(T)$ is properly accounted for, unmasking and estimating the conformational term $\Delta V_{conf}(T)$ would be impossible.

To study the impact of buffer ionization on the volume of a protein transition, we performed PPC investigations of the heat-induced native-to-unfolded transitions of lysozyme and ribonuclease A in pyrophosphate, citric, glycine, and HEPES buffers all adjusted to pH 3.0. The volumes of ionization, ΔV_{buff}, of these buffers at 25 °C are −16.0, −10.7, −6.0, and 9.4 cm^3mol^{-1}, respectively [17]. This is a range sufficiently wide to facilitate the investigations reported here.

Fig. 1 shows representative PPC profiles of ribonuclease A in a citric buffer (panel a) and lysozyme in a HEPES buffer (panel b). We calculate a change in volume, ΔV_M, accompanying protein denaturation at the transition temperature, T_M, from such PPC profiles by fitting experimental data with an analytical function which was derived based on the two-state approximation (see Experimental Section). This approach is analogous to that recently described by Makhatadze et al. [13,14]. The transition enthalpy, ΔH_M, needed for the two-state approximation was measured separately by DSC.

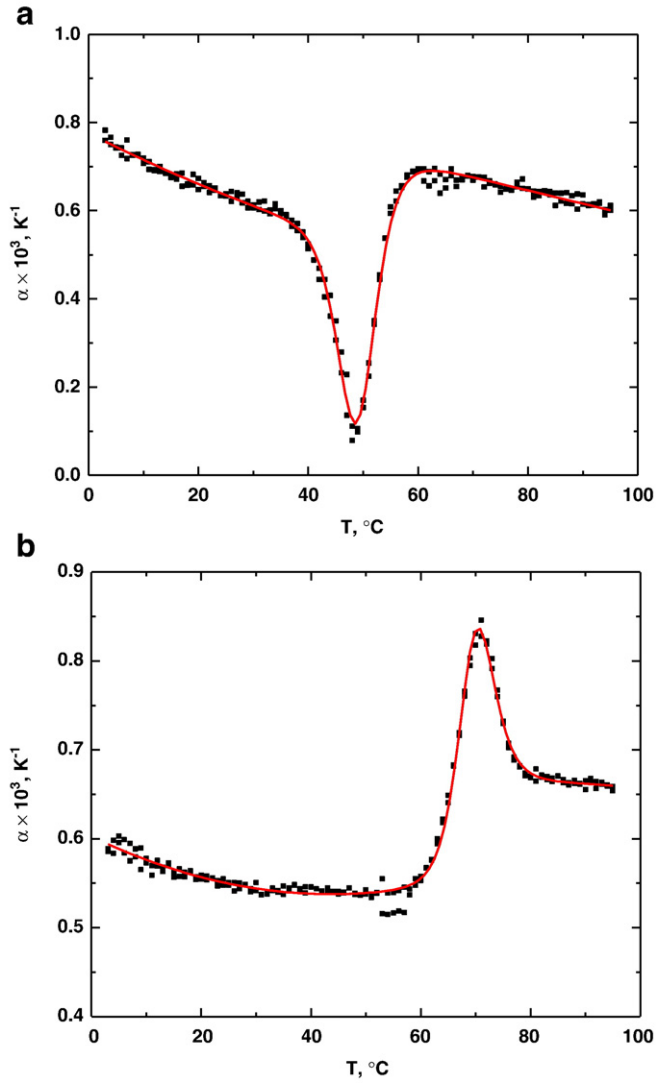

Fig. 1. Representative PPC profiles of ribonuclease A in citric buffer (panel a) and lysozyme in HEPES buffer (panel b). Experimental data were fitted with Eq. (3).

Table 1
Thermodynamic parameters of temperature-induced unfolding transitions of lysozyme and ribonuclease A.

Buffer	ΔV_{buff}	T_M[a]	ΔH_M	ΔV_M
	cm^3mol^{-1}	°C	kcal mol^{-1}	cm^3mol^{-1}
Pyrophosphate	−16.0[b]	73.9 ± 0.2[c]	102.5 ± 2.3[c]	1.3 ± 0.7[c]
		58.6 ± 1.6[d]	95.3 ± 2.7[d]	−43.6 ± 0.9[d]
Citrate	−10.7[b]	72.1 ± 0.2[c]	107.9 ± 2.5[c]	−4.9 ± 0.3[c]
		50.0 ± 0.6[d]	89.2 ± 2.9[d]	−46.3 ± 0.5[d]
Glycine	−6.0[b]	74.6 ± 0.2[c]	108.2 ± 2.7[c]	3.3 ± 0.2[c]
		49.4 ± 0.9[d]	82.9 ± 3.3[d]	−26.1 ± 2.2[d]
HEPES	9.4[b]	71.1 ± 0.2[c]	104.7 ± 2.5[c]	18.2 ± 1.3[c]
		45.5 ± 0.5[d]	85.1 ± 2.1[d]	−10.8 ± 1.2[d]
Hypothetical buffer	0			9.1 ± 3.2[c]
				−23.4 ± 4.2[d]

[a] The tabulated transition temperatures, T_M, are the averages of PPC and DSC measurements.
[b] Reported in ref. [17] at 25 °C.
[c] Lysozyme.
[d] Ribonuclease A.

Our measured parameters of the temperature-induced denaturation transitions of lysozyme and ribonuclease A are listed in Table 1. The transition temperatures, T_M, of lysozyme in the four buffers studied in this work are grouped around 72.9 ± 1.6 °C, an observation suggesting no significant interactions between the protein and the components of the buffers. On the other hand, ribonuclease A exhibits a significant deviation in T_M. In the citric, glycine, and HEPES buffers, the values of T_M are close to each other with the average of 48.3 ± 2.4 °C, while, in pyrophosphate, the T_M is 10 °C higher (58.6 ± 1.6 °C). This observation is consistent with the binding of pyrophosphate to ribonuclease A with an equilibrium constant, K_b, of ~7000 M^{-1}. The latter can be calculated from $\Delta T_M = (RT_MT_C/\Delta H_M)\ \ln(1 + K_b a_L)$ [18]. The binding of pyrophosphate to ribonuclease A is not unexpected given the chemical and structural similarities of pyrophosphate and ribonucleotides, natural substrates of the protein.

Fig. 2 presents the PPC-determined changes in volume at T_M, ΔV_M, accompanying the heat-induced unfolding of lysozyme and ribonuclease A in pyrophosphate, citrate, glycine, and HEPES plotted against the volume of buffer ionization, ΔV_{buff}. Note that the values of ΔV_{buff} plotted on the *X*-axis in Fig. 2 have been reported at 25 °C [17], while our ΔV_M values plotted on the *Y*-axis were determined at higher temperatures. However, this disparity should not be critical for our analyses presented below. This notion stems from results of our recent study in which we measured the volumes of ionization of all titrable amino acid side chains and terminal carboxyl and amino groups between 18 and 55 °C [19]. In all instances, the temperature dependences of ionization volumes were weak with the difference between the values at 18 and 55 °C not exceeding ~25% [19]. For example, the volume of ionization of the aspartic acid side chain is 11.2 ± 0.7, 12.1 ± 1.4, 12.1 ± 0.3, and 12.8 ± 0.5 cm^3mol^{-1} at 18, 25, 40, and 55 °C, respectively [19]. By extension, it is plausible to assume that the reported volumes of ionization of pyrophosphate, citrate, glycine, and HEPES at 25 °C do not differ by more than ~25% from those at the transition temperatures, T_M, of the two proteins studied in this work.

The apparent transition volume of ribonuclease A in pyrophosphate determined by PPC should be contributed by the change in volume accompanying the dissociation of pyrophosphate from the protein. Therefore, we analyzed our data with and without the experimental point corresponding to ribonuclease A in pyrophosphate. The two modes of analysis produced very similar results, which is consistent with an insignificant change in volume of accompanying pyrophosphate unbinding.

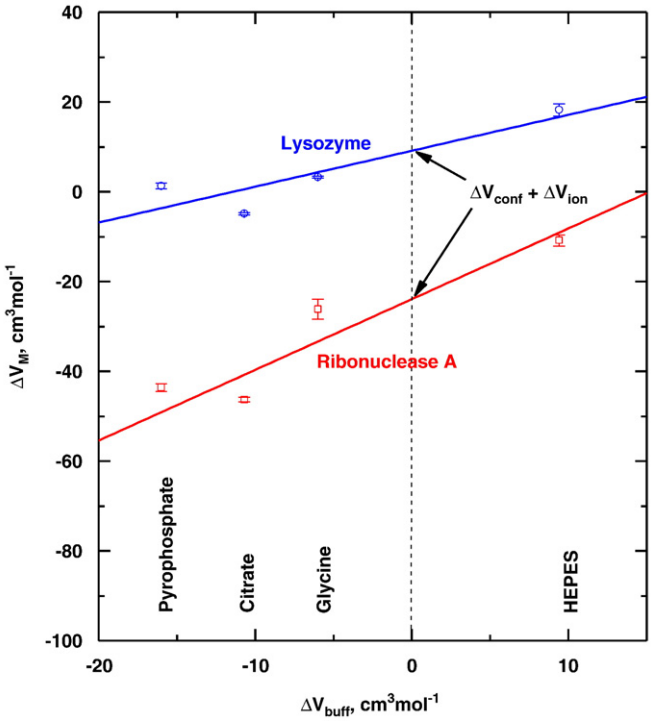

Fig. 2. Dependences of the transition volumes, ΔV_M, on the volume of buffer ionization, ΔV_{buff}, for lysozyme and ribonuclease A.

As expected on the basis of Eq. (1), the transition volumes, ΔV_M, of the two proteins exhibit a good linear correlation with the volume of buffer ionization, ΔV_{buff} (see Fig. 2). Interpolation of the linear dependences in Fig. 2 to the point corresponding to a hypothetical buffer with $\Delta V_{buff} = 0$ yields the values of $(\Delta V_{conf} + \Delta V_{ion})$ for lysozyme and ribonuclease A which are presented in the last rows of Table 1.

The slope of the dependence of ΔV_M on ΔV_{buff} equals the number of released protons, ν. The values of ν calculated in this way are 0.8 and 1.6 for lysozyme and ribonuclease A, respectively. An analogous approach, based on correlating the binding enthalpy with the enthalpy of buffer ionization, has been employed to determine the number of protons released or absorbed upon ligand–protein, peptide–lipid, and protein–protein association events [20–22].

In an alternative procedure, the number of released protons can be determined from the pH-dependence of the transition temperature, T_M [23]:

$$\nu = \frac{\Delta H_M}{2.303 R T_M^2} \frac{dT_M}{dpH} \quad (2)$$

From our measured pH-dependences of T_M (data not shown), we calculate the values of ν at pH 3.0 of 0.9 and 2.1 for lysozyme and ribonuclease A, respectively. These numbers are in good agreement with the values of ν calculated as the slopes of the dependences of ΔV_M on ΔV_{buff}. The agreement lends credence to the experimental protocols used in our PPC measurements and the subsequent analysis of the resulting data.

The span of our observed buffer-dependent changes in ΔV_M for lysozyme is 23 cm^{3}mol^{-1}, while that for ribonuclease A is 36 cm^{3}mol^{-1}. Understandably, these changes will be even larger for greater numbers of released protons, ν. For example, for α-chymotrypsinogen A, the value of ν at pH 3.0 is ~4 as can be evaluated from the pH-dependence of the transition temperature, T_M, and the transition enthalpy, ΔH_M, reported in ref. [24]. Given the magnitude of the relative contribution of the $\nu\Delta V_{buff}(T)$ term to the net value of the transition volume, ΔV_M, the choice of the buffer should have a major impact on the stability of a protein with the potential ability to alter the nature of its pressure dependence from pressure-induced denaturation to pressure-induced stabilization and *vice versa*.

Our data graphically presented in Fig. 2 in conjunction with Eq. (1) suggest that different experimental approaches and evaluation strategies should be selected depending on the aims of the specific investigation. If the study is aimed at determining the conformational contribution, ΔV_{conf}, either the measurements should be conducted within a pH-range where $\nu \approx 0$ (where T_M is pH-independent) or the buffer should be chosen in a way that the ionization term, ΔV_{ion}, in Eq. (1) compensates the $\nu\Delta V_{buff}$ term. The latter provision can be approximately met by selecting a buffer that has the same titrating groups as those involved in protein ionization (e. g., a glycine or a diglycine buffer). A similar reasoning has been put forward regarding the components of the transition enthalpy in DSC measurements [23]. On the other hand, if the goal of the study is to evaluate the ionization term, ΔV_{ion}, in addition to the conformational term, ΔV_{conf}, buffer-dependent studies (similar to those described here) should be carried out with the measured values of ΔV_M being extra- or interpolated to the point corresponding to a hypothetical buffer with $\Delta V_{buff} = 0$ as shown in Fig. 2.

In conclusion, this work identifies buffer ionization as an important determinant of the protein transition volume that needs to be properly taken into account. While our experimental results are backed by fundamental physico-chemical considerations, to the best of our knowledge, this work represents the first quantitative study of the effect of buffer ionization on the volume of protein denaturation. We emphasize that the importance of our results is not limited to PPC measurements but is more general and applies to all volumetric investigations, in particular, extending to the derivation of the pressure–temperature phase diagram of protein stability.

Experimental section

The proteins lysozyme and ribonuclease A of the highest commercially available purity were purchased from Sigma-Aldrich Canada, Ltd. and used without further purification. The buffer ingredients sodium chloride, glycine, sodium citrate, citric acid, HEPES, and sodium pyrophosphate were also purchased from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario, Canada).

The following buffers all adjusted to pH 3.0 were used: (i) 10 mM glycine–HCl buffer; (ii) 10 mM sodium citrate–citric acid buffer; (iii) 10 mM HEPES–HCl buffer; and (iv) 10 mM sodium pyrophosphate–HCl buffer. Solutions were prepared using doubly distilled water. All measurements were performed in the presence of 10 mM NaCl.

The proteins were dissolved in and exhaustively dialyzed against each buffer under study using Spectra/Por dialysis tubing with a 3500 Da molecular weight cutoff (VWR Canada, Ontario, Canada). The concentrations of the proteins were determined spectrophotometrically after the dialysis using the following molar extinction coefficients: $\varepsilon_{282} = 37{,}600\ M^{-1}\,cm^{-1}$ for lysozyme [25] and at $\varepsilon_{278} = 9800\ M^{-1}\,cm^{-1}$ for ribonuclease A [26]. For all of the PPC and DSC measurements reported here, the concentrations of the proteins were within the range of 2 to 5 mg/mL.

The PPC measurements were conducted between 3 and 95 °C on a MicroCal VP-DSC instrument with a PPC accessory (MicroCal, LLC, Northampton, MA). We analyzed the PPC profiles to determine the values for the denaturation temperature, T_M, and the volume change, ΔV_M, for each transition. Experimental expansibility data, $\alpha(T)$, were fitted with an analytical function that has been derived based on the two-state approximation analogous to that described by Makhatadze et al. [13]:

$$\alpha(T) = \frac{1}{1 + K(T)} \left(\alpha_N(T) + K(T)\alpha_D(T) + \frac{K(T)}{1 + K(T)} \times \frac{\Delta H_M (\Delta V_M / V^\circ)}{RT^2} \right) \quad (3)$$

where $\alpha_N(T)$ and $\alpha_D(T)$ denote the pre- and post-denaturational baselines, respectively, which were both approximated by parabolic functions; ΔH_M is the change in enthalpy accompanying protein denaturation at T_M; ΔV_M is the requisite transition volume at T_M; $V°$ is the partial molar volume of the proteins [27]; and the equilibrium constant $K(T)$ is equal to [D]/[N], with [N] and [D] corresponding to the concentrations of the native and denatured protein states, respectively. Using basic thermodynamic relationships, $K(T)$ can be expressed as $K(T) = \left(\frac{T}{T_M}\right)\Delta C_p / R \exp\left[\frac{(T_M\Delta C_p - \Delta H_M)(T^{-1} - T_M^{-1})}{R}\right]$, where ΔC_P is the heat capacity change accompanying protein denaturation.

Transition enthalpies, ΔH_M, and heat capacity changes, ΔC_P, required for the two-state analysis were measured using a Calorimetry Sciences Corporation model 6100 NanoDSCII differential scanning calorimeter (Calorimetry Sciences Corporation, Provo, UT). Protein solutions were loaded into the calorimeter cell and each sample was heated/cooled repeatedly from 4 to 95 °C, at a heating rate of 1 °C/min. For each sample, the first DSC scan was used to obtain values for T_M, ΔH_M, and ΔC_P using previously described protocols [28]. Subsequent scans were used to assess the reversibility of each denaturation process.

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