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Applications of pressure perturbation calorimetry to study factors contributing to the volume changes upon protein unfolding☆,☆☆

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ABSTRACT

Background: Pressure perturbation calorimetry (PPC) is a biophysical method that allows direct determination of the volume changes upon conformational transitions in macromolecules.
Scope of this review: This review provides novel details of the use of PPC to analyze unfolding transitions in proteins. The emphasis is made on the data analysis as well as on the validation of different structural factors that define the volume changes upon unfolding. Four case studies are presented that show the application of these concepts to various protein systems.
Major conclusions: The major conclusions are:

1. Knowledge of the thermodynamic parameters for heat induced unfolding facilitates the analysis of the PPC profiles.
2. The changes in the thermal expansion coefficient upon unfolding appear to be temperature dependent.
3. Substitutions on the protein surface have negligible effects on the volume changes upon protein unfolding.
4. Structural plasticity of proteins defines the position dependent effect of amino acid substitutions of the residues buried in the native state.
5. Small proteins have positive volume changes upon unfolding which suggests difference in balance between the cavity/void volume in the native state and the hydration volume changes upon unfolding as compared to the large proteins that have negative volume changes.

General significance: The information provided here gives a better understanding and deeper insight into the role played by various factors in defining the volume changes upon protein unfolding. This article is part of a Special Issue entitled Microcalorimetry in the BioSciences — Principles and Applications, edited by Fadi Bou-Abdallah.

1. Introduction

How a linear sequence of amino acids folds into an intricate and biologically active three-dimensional structure of a protein has captivated the scientific community ever since the myoglobin structure was reported [1,2]. Multiple physico-chemical forces such as hydrogen bond, hydrophobic interaction, van der Waals interaction, disulfide bridges and electrostatic interactions have been widely discussed for their role in defining the protein stability (see e.g. [3–7]. Much of the studies have been exploring the effect of fundamental environmental parameter, temperature (T) on protein stability (defined as the Gibbs energy difference between unfolded and native states $\Delta G = G_U - G_N$). However, much less has been done to explore the dependence of the stability of proteins on the other equally fundamental environmental parameter, hydrostatic pressure (P). This is important to understand the physico-chemical principles that define the stability of proteins in general and in particular for the organisms that live in the deep sea, the so-called barophiles [8,9].

The pressure dependence of the protein stability is defined by the volume changes upon unfolding, ΔV:

$$\Delta V = V_U - V_N = \left(\frac{\partial \Delta G}{\partial P}\right)_T \quad (1)$$

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where V_U and V_N are the volumes of the unfolded and native states, respectively. If ΔV is negative, increase in hydrostatic pressure will, according to Le Châtelier's principle, lead to a decrease in protein stability, while positive values of ΔV will lead to an increase in stability. There are two major factors contributing to the ΔV of protein unfolding. The first factor is the imperfection in the packing of the native proteins, i.e. well documented presence of cavities of voids [10–12]. The second factor is the volume changes of solvent water upon hydrating the protein groups exposed due to the unfolding [13]:

$$\Delta V = V_{voids} + V_{Hydration}. \quad (2)$$

The exact magnitudes of these effects, especially the hydration of newly exposed accessible surface area due denaturation, are still under debate [14–16].

Until recently, the volume changes upon unfolding have been determined indirectly from the pressure dependence of equilibrium constant (see e.g. ref.[16]). Introduction of the commercial instruments to perform pressure perturbation calorimetry (PPC) experiments [17] and subsequent development of data analysis formalism [18] provided the protein folding community the ability to directly determine ΔV of conformational transitions. These measurements combined with other biophysical methods assessing thermodynamic (e.g. DSC) and structural (e.g. circular dichroism and fluorescence spectroscopies, NMR, SAXS) properties of proteins and supported by computational modeling allowed better understanding of the properties of proteins that contribute to the pressure dependence of protein stability [18–40].

In this paper we report several robust approaches to analyze the PPC profiles of proteins and report four novel case studies of protein systems that clarify the importance of various factors in defining the volume changes upon protein unfolding. The article is organized as follows. After the Materials and methods section, we present a brief discussion of the PPC experiment and foundation for a two-state analysis. This is followed by case studies of four different protein systems for which PPC experiments were performed. Each case highlights certain aspects of data analysis and more importantly, probes various structural aspects of proteins that define their volume changes upon unfolding.

2. Materials and methods

2.1. TrpZip peptide

TRPZIP4 with sequence GEWTWDDATKTWTWTE-NH_2 was synthesized using standard Fmoc chemistry as described previously [41]. The peptide has a molecular weight of 2013 Da and extinction coefficient ($\varepsilon_{280,0.1\%}$) of 11.41 optical units. The partial specific volume, V_{pr}, was calculated to be 0.70 cm^3/g based on the amino acid composition as described previously [42]. Both DSC and PPC experimental procedures were performed using TRPZIP4 concentrations between 0.5 and 3.5 mg/ml. Peptide purification was done using a C18 reverse phase HPLC column (Discovery Bio Wide pore C18, 10 μm, Supelco Sigma-Aldrich, Bellefonte, PA) and a 0 to 100% acetonitrile gradient in the presence of 0.05% trifluoroacetic acid (TFA). Pooled fractions were lyophilized and then washed with Milli-Q (Millipore, Billerica, MA) water. This procedure was repeated at least three times to remove residual TFA which could potentially interfere with spectroscopic and calorimetric measurements. MALDI-TOF (Bruker Ultraflex III, Bruker Daltonics, Billerica, MA) was carried out to determine the molecular weight and purity of the samples. The peptide concentrations were determined using an UV–Vis spectrophotometer (Hitachi U-2900). Prior to the calorimetric experiments, all samples were thoroughly dialyzed using Spectra/Por 1000 Da molecular weight cutoff membrane (18 × 11.5 mm) into 20 mM sodium phosphate buffer pH 7.0. Three dialysis changes of 4 h each were performed.

2.2. BPTI, ubiquitin and acylphosphatase variants

Bovine pancreatic trypsin inhibitor (BPTI) was purchased from United States Biochemical Corp. (Cleveland, OH, USA, cat. No. 11,388) and used without further purification.

The constructs for ubiquitin and protocol for expression and purification has been described by us previously [43,44].

The constructs for ACP-wt and ACP-GA2 have been described previously [45]. QuickChange site directed Mutagenesis Kit (Stratagene) was used to introduce mutations Ala at position corresponding to Ile13, Phe22, Leu35, Val39, Val47, Leu51 and Val58 in the overall background of ACP-GA2. The presence of the desired mutation in the gene of interest in the cloning vector, pGia, was confirmed by direct DNA sequencing (Eurofins MWG Operon). Expression plasmids were transformed into *Escherichia coli* BL21(DE3) competent cells (Novagen, Madison, WI) using the heat shock method. The cells were grown in 1 L LB medium containing 100 μg/ml ampicillin at 37 °C to an optical density of 0.8 at 600 nm and induced by adding 1 mM (final concentration) of IPTG. The induction of protein expression was carried out for 6 h. Cells were harvested at 7500 × *g* and the pellet was suspended in 15 ml of Ni-NTA denaturing lysis buffer B (Novagen) (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8) and stored at −20 °C until further use. The frozen pellet was thawed and passed through a French pressure cell and the lysed cells were centrifuged at 39,000 × *g* for 45 min at 4 °C to remove cell debris. The supernatant was diluted with Ni-NTA denaturing lysis buffer B (Novagen) in 1:1 ratio and applied to Ni-NTA resin (Novagen) column twice. Stationary phase was washed with denaturing wash buffer pH 6.25 (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl). Additional wash with pH 5.9 wash buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl) was done until optical density at 280 nm was less than 0.01 o.u. The protein was eluted using pH 4.45 elution buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl). The eluted protein was thoroughly dialyzed against 1 L 5% acetic acid and applied onto a G-50 Sephadex gel filtration column (2.5 × 150 cm) equilibrated in 5% acetic acid. Protein containing fractions were pooled, lyophilized and stored at −20 °C. Purity of the protein was evaluated by SDS-PAGE. In addition the molecular mass of the proteins were determined by MALDI-TOF mass spectrophotometer (Bruker Ultraflex III, Bruker Daltonics, Billerica, MA) and found to be within 1–4 Da of the theoretically expected.

All proteins were thoroughly dialyzed using 3500 Da molecular weight cutoff dialysis tubing prior to calorimetric experiments. All discussed PPC and DSC experiments were performed in 20 mM glycine buffer in pH range from 1.75 to 3.55.

2.3. Differential scanning calorimetry (DSC)

DSC experiments were performed from 5 to 115 °C at a heating rate of 90 ° per hour on a VP-DSC (GE-Microcal) following a protocol discussed elsewhere. [46] The acquired raw data was converted to heat capacity profiles using Origin lab software (Origin, Northampton, MA). The heat capacity profiles were analyzed according to a two-state model as described previously (ref [46] using in-house written script for nonlinear regression software, NLREG.

2.4. Circular dichroism spectroscopy (CD)

CD measurements were performed using a JASCO J-710 spectropolarimeter and a 1 mm rectangular quartz cuvette. The temperature was controlled at 20 °C using a Peltier effect cell holder. Prior to the protein scans (200 to 260 nm), scans of the buffer alone were performed to determine the baseline signal. The ellipticity (θ) was converted to mean