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Thermal stability of pepsin: A predictive thermodynamic model of a multi-domain protein

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ABSTRACT

Pepsin is generally used in the preparation of $F(ab)_2$ fragments from antibodies. The antibodies that are one of the largest and fastest growing categories of bio- pharmaceutical candidates. Differential scanning calorimetric is principally suitable method to follow the energetics of a multi-domain, fragment to perform a more exhaustive description of the thermodynamics in an associating system. The thermodynamical models of analysis include the construction of a simultaneous fitting of a theoretical expression. The expression depending on the equilibrium unfolding data from multimeric proteins that have a two-state monomer. The aim of the present study is considering the DSC data in connection with pepsin going through reversible thermal denaturation. Afterwards, we calculate the homology modeling identification of pepsin in complex multi-domain families with varied domain architectures. In order to analyze the DSC data, the thermal denaturation of multimer proteins were considered, the "two independent two-state sequential transitions with domains dissociation model" was introduced by using of the effective ΔG concept. The reversible unfolding of the protein description was followed by the two-state transition quantities which is a slower irreversible process of aggregation. The protein unfolding is best described by two non-ideal transitions, suggesting the presence of unfolding intermediates. These evaluations are also applicable for high throughput investigation of protein stability.

1. Introduction

Pepsin is commonly used in the preparation of $F(ab)_2$ fragments from antibodies. Antibodies and antibody-derived molecules are one of the largest and fastest growing classes of bio- pharmaceutical candidates and products. Differential scanning calorimetry (DSC) is the most direct experimental technique to resolve the energies out of conformational transitions of biological macromolecules. The stability of multi-domain proteins is commonly investigated by DSC. One of the great advantages of DSC is that it can detect fine-tuned of interactions between the individual domains of a protein. Followed by measuring the temperature dependence of the partial heat capacity, a basic thermodynamic property, DSC gives immediate access to the thermodynamic mechanism that governs a conformational equilibrium. The way proteins work imposes constraints on their function. Knowing the sources of the protein stability is essential to recognize their structure and function. One of the method for quantifying the stability of a protein is to populate the native and unfolded states by physical and chemical means. Then, the transitions measured by DSC or fluorescence, and absorption spectroscopy were evaluated [1,2]. During the last two decades DSC has significantly contributed to the development of our current understanding of the energetics and thermodynamic properties of protein folding-unfolding transitions [3]. By scanning microcalorimetry, it was shown that thermal transition is connected purely to the denaturation of protein molecules in the crystal and it is not accompanied by the crystal disintegration into separate molecules [4].

At critical temperatures and higher, along with typical loosening of the protein globuleis is observed along with it oligomeric molecules undergo dissociation process into subunits. Subsequently these smaller components may associate with each other and oligomeric structures. This association may result in irreversible denaturation and deviant physicochemical behavior [5]. The denaturation unfolding process is strongly dependent on the heating rate. As it is expected, the unfolding process is kinetically controlled by the presence of an irreversible reaction. CD signal on heating of proteins, constructing ellipticity quantities at wavelength distinct is very applicable for following the denaturation process [6]. A large body of research on the thermodynamics of small monomeric and single domain proteins has indicated that the hydrophobic effect and loss of conformational entropy

Abbreviations: DSC, Differential Scanning Calorimetry; T_m, Thermal midpoint; ΔH^{cal}, Calorimetric enthalpies; ΔH^{VH}, Van't Hoff enthalpy

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are the major determinants of stability in the native state [7]. The unfolding transitions of several small globular proteins are usually highly cooperative. They closely follow a two-state mechanism under equilibrium conditions [8,9]. However, in some cases, stable intermediates have been detected and partially characterized [10,11]. Much of the disagreement between theoretical and experimental results of thermal denaturation of proteins derives from the necessity of using some models to interpret the thermodynamic data for proteins [1]. In these cases that have no easy experimental methods, applying an empirical formula can be a rational. For overcoming to the above difficulties, the present study uses of the effective ΔG concept, the thermodynamics of a multi-domain protein which undergo thermal denaturation is studied as a function of protein concentration. ΔGe_{ff} is a valuable factor that provides an natural increase of the stability of multimeric proteins; one can determine the section of unfolded protein from ΔGe_{ff} of a multimeric protein as easily as doing so from the ΔG° of a monomeric protein [8]. In this regards, we try to discuss and introduce "the two independent two-state transitions with a domain dissociation model".

2. Methods

DSC measurements were carried out on a MicroCall "MC-2" Differential Scanning Calorimeter (Micro All Inc., Northampton, MA) with cell volumes 1.14 mL, at heating rates 1.5 °C min^{-1}. DSC scans were obtained in a temperature range from 283.15 to 373.15 K. During the measurements, the protein concentrations was 30 μM, and pH ranged from 1.0 to 4.0 [12]. The buffers used for acid denaturation of pepsin are at different pH values KCl/HCl (pH 0.8–1.4), Gly/HCl (pH 1.6–3.0), sodium acetate (pH 3.5–5.0) at a concentration of 20 mM [13]. Pepsin, lyophilized powder (≥2500 units mg^{-1} protein) as well as Aldrich. Degassing during the calorimetric measurements was prevented by additional constant pressure of 1 atm over the liquids in the cells. At first, the solvent was placed in both the sample and reference compartments. A DSC curve corresponding to solvent vs. solvent run was used as an instrumental baseline. The calorimetric data was corrected for calorimetric baseline (by subtracting solvent-solvent scan).

In this study we used the Swiss-Model template library (SMTL) [14,15] database to analyze the domain organization of proteins. The SCOP [16] and CATH [17] families corresponding to multi-domain proteins have been used to identify single domain homologues. A control dataset of non-redundant single domain proteins obtained from completely different families has also been formed. All the structures have been optimized at 298 K by using the optimizing tool available in FoldX (version 3.0) [18]. Then free energy computations were done.

3. Results and discussion

3.1. The thermodynamic models

We clearly pointed out that this manuscript is the continuation of previously published work, as described in the framework of this model. The previously published paper were also clearly identified (reference [12]) at the manuscript.

The framework used for fitting DSC data offers four models. They all of them use the Levenberg-Marquardt non-linear least-square method [19], there was a conflict with the number of parameters involved:

1) Two-state with zero ΔC_p (parameters: Thermal midpoint (T_m), Calorimetric enthalpies (ΔH^{cal})); 2) Non-two state with zero ΔC_p (parameters: T_m, ΔH^{cal}, Van't Hoff enthalpy (ΔH^{VH})); 3) Two-state with non-zero ΔC_p (parameters: T_m, ΔH^{cal}, ΔC_p, BL0, BL1) and 4) Dissociation with non-zero ΔC_p (parameters: T_m, ΔH^{cal}, ΔC_p, BL0, BL1, n- number of multimers).

With exception of parameter number (4), one or more transitions can be used to fit the models, each transition has a relevant parameter set, for example in the case of two overlying transitions can be used to fit to one or more transitions. In the case of multiple transitions, each transition has its own complete parameter set, e.g., if pattern 1 is used to fit two overlapping transitions there will be two independent parameters sets (T_{m1}, ΔH^{cal1}) and (T_{m2}, ΔH^{cal2}). These parameters specify the thermal midpoint (T_m) and heat change (ΔH^{cal}) for each transition. BL0 and BL1 parameters define the slope and intercept of the low-temperature baseline segment, they are not repeated and appears only once in each model. While all four models use calorimetric heat change, only non-two state with zero ΔC_p model has a van't Hoff heat change (ΔH^{VH}). The ΔH^{cal} is determined only by the area under transition peak, while the van't Hoff heat is determined only by the shape (ΔC_p^{max} at transition midpoint). The transition sharpness is associated with ΔH^{VH} largernes. The relationship between ΔH^{cal} and ΔH^{VH} can sometimes provide insights not accessible from ΔH^{cal} model alone. If a protein is composed of two identical domains, which unfold independently with the same T_m and ΔH^{cal}, then the ratio of H^{cal}/H^{VH}, will be 2, while it would be 1 if the protein had a single domain. If, on the other hand, the protein dimerized and dimer underwent only a single coupled transition then, the ratio will be 0.5, etc.

In the model 1, it is possible to define that overlapping transitions are either independent or sequential in nature, for example, if two architectural domains are interacting strongly, it is possible to assume that their transitions will be coupled in a sequential manner. However, the independent model might better describe two transitions that are thoroughly uncoupled from one another. In practice, this option is often not critical because the sequential and independent models lead essentially identical effects whenever the T_m's of two transitions are separated by a couple of degrees or more. The mathematical derivations for each pattern has been introduced in the previous article [19]. Generally is the aim, the objective is using the simplest model which produces a good framework for the data. Therefore, if data is described by two-state model using two transitions, it would be preferred over a two-state model using three transitions or a non-two state model having two transitions. In this study, a noble two independent two-state transitions with domains dissociation model is introduced, as the fifth fitting model.

3.2. The two independent two-state transitions with domains dissociation model

First, for investigating of thermal multi-domains protein stability, the modified Gibss-Helmholts equation is determined. The fundamental assumption in this model is the equilibrium reversibility of thermal denaturation process in multimeric protein between the folded and unfolded states. As the inherent difficulty in the treatment and analysis of their equilibrium behavior in experimental scope, the use of the Gibss-Helmholts empirical modified function in terms of the number of domains can be a cross cut to thermodynamic purposes.

Eq. (1) shows the equilibrium between n-identical domain protein and its unfolded monomer without any intermediate state is shown in:

$$F_n \leftrightarrow nU \tag{1}$$

The reaction equilibrium constant is as follows:

$$K_D = \frac{[U]^n}{[F_n]} \tag{2}$$

The definition of unfolded protein fraction, f_D is expressed as follow:

$$f_D = \frac{[U]}{P_t} \tag{3}$$

Where, P_t is the total protein concentration in domain units. As fallow K_{unf} and ΔG^o can be expressed as functions of f_D: