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Conformational stability and self-association equilibrium in biologics

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Biologics exist in equilibrium between native, partially denatured, and denatured conformational states. The population of any of these states is dictated by their Gibbs energy and can be altered by changes in physical and solution conditions. Some conformations have a tendency to self-associate and aggregate, an undesirable phenomenon in protein therapeutics. Conformational equilibrium and self-association are linked thermodynamic functions. Given that any associative reaction is concentration dependent, conformational stability studies performed at different protein concentrations can provide early clues to future aggregation problems. This analysis can be applied to the selection of protein variants or the identification of better formulation solutions. In this review, we discuss three different aggregation situations and their manifestation in the observed conformational equilibrium of a protein.

Introduction

The expected growth of biologics over the next few years and their transformation from drugs administered mainly in hospital settings to drugs self-administered by patients, requires the fulfillment of strict safety and stability conditions. The long-term stability of biologics is a function of diverse physical and chemical factors that need to be addressed in an effective and rapid way. Among them, it is necessary to identify conditions that maximize the structural stability of the native state and prevent aggregation or other undesirable processes [1–4]. Maximizing structural stability and minimizing aggregation tendencies involve decisions in at least two different development stages: the selection of the protein variants with the top stability and/or aggregation profiles; and, the identification of the solution conditions (formulations) that maximize stability and minimize aggregation. These decisions will be facilitated by a better understanding of the existing linkage between conformational and self-association processes.

Conformational equilibrium in proteins

Proteins exist in equilibrium between the native, partially denatured, and fully denatured conformations. Even though there are an astronomical number of partially denatured (also referred to as partially folded) conformations, most never become populated. In fact, the native and/or denatured state equilibrium of most single-domain proteins can be accounted for by the two-state model, in which the population of partially denatured conformations is negligible [5]. Multidomain proteins can exhibit partially denatured conformations corresponding to states in which some domains are unfolded whereas the others remain folded [6]. Fig. 1 illustrates two commonly observed situations. Fig. 1a represents the two-state situation in which the native and fully denatured conformations are the only ones that become significantly populated. Fig. 1b represents the situation in which a partially denatured conformation becomes populated in addition to the native and fully denatured states. The partially denatured state usually originates from the unfolding of the least-stable domain. In all cases, the stability of a protein or a structural domain is determined by the Gibbs energy of stability, ΔG, characteristic of each structure. The population or fraction of denatured protein or domain is given by Eq. (1):

$$F_{denatured} = \frac{e^{-\Delta G/RT}}{1 + e^{-\Delta G/RT}}. \quad (1)$$

A large and positive ΔG minimizes the fraction of denatured protein or domain. Table 1 shows a look-up table with characteristic ΔG values and the corresponding populations of denatured protein. The percent of denatured protein is simply the fraction

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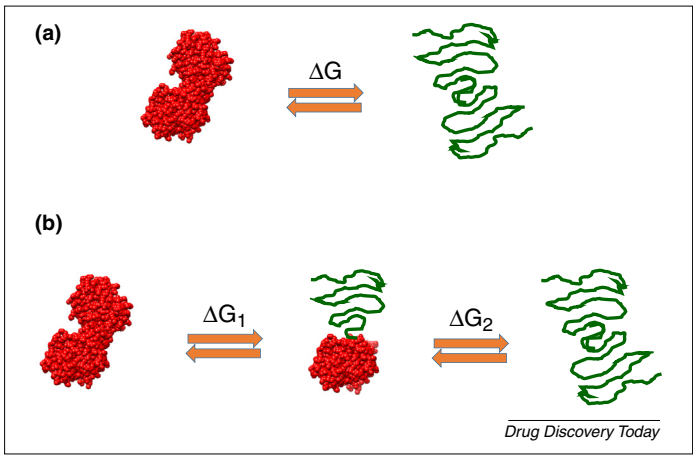

FIGURE 1

Typical protein denaturation situations encountered in the laboratory. **(a)** The classical two-state situation in which protein denaturation occurs without the presence of partially denatured conformation. This situation is mostly observed for single-domain proteins or multidomain proteins with highly cooperative interdomain interactions. **(b)** The situation in which denaturation occurs with the presence of a significantly populated partially denatured state. This situation often occurs with multidomain proteins.

TABLE 1

The amount of denatured protein is determined by ΔG.[a]

ΔG (kcal/mol)	% Denatured protein
0	50
1.3	10
2.7	1
4.1	0.1
5.5	0.01
6.8	0.001
8.2	0.0001
9.6	0.00001
10.4	0.000001

[a] Knowledge of ΔG enables us to calculate the population of denatured protein or domains. Given that denatured conformations are prone to aggregation, ΔG should have a crucial role in the selection of protein variants and formulation optimization.

denatured multiplied by 100. Also, the actual concentration of denatured protein is simply the total protein concentration multiplied by the fraction denatured. For example, in a 100 mg/ml protein solution with a structural stability of 8 kcal/mol, the concentration of denatured protein is 0.1 μg/ml. In protein engineering or formulation development, a common goal is to optimize the structural stability of the protein, which is equivalent to maximizing ΔG.

There are two techniques capable of measuring Gibbs energies and, therefore, able to provide a window into the conformational equilibrium of a protein: differential scanning calorimetry (DSC) and isothermal chemical denaturation (ICD). DSC induces protein denaturation by increasing the temperature of the solution [7–9], whereas ICD induces protein denaturation by the addition of a chemical denaturant, most commonly urea or guanidine hydrochloride [10–15]. Both techniques are able to determine ΔG not at high temperature or high denaturant concentration but at room temperature or zero denaturant concentration. In both cases, the necessary requirement for ΔG determination is that the experimental measurement is performed under reversible conditions. This condition is tested by scanning the same sample twice or by diluting the chemical denaturant concentration and recovering the original state. Fig. 2 illustrates the type of data obtained by DSC and ICD for a protein characterized by the presence of two cooperative domains. In both cases, analysis of the data permits determination of ΔG_1 and ΔG_2, and their subsequent analysis [7–15].

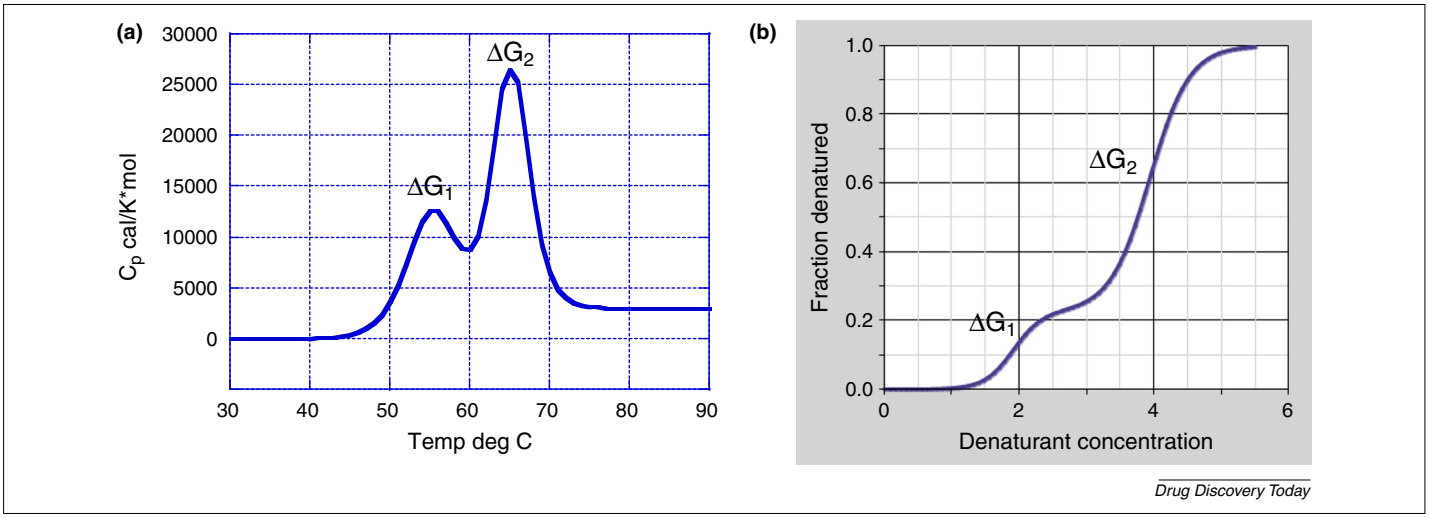

FIGURE 2

Differential scanning calorimetry (DSC) **(a)** and isothermal chemical denaturation (ICD) **(b)** allow measurement of the folding–unfolding reaction of a protein and determination of the Gibbs energy of stability (ΔG) for each step. Folding–unfolding thermodynamics can only be determined if the measurement is done under equilibrium conditions.

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