Conformational stability and self-association equilibrium in biologics

Benjamin R. Clarkson, Arne Schön, and Ernesto Freire*

Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA

Corresponding author: Freire, E. (ef@jhu.edu)

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Teaser: High structural stability and negligible aggregation are two sought-after properties of protein drugs. However, these properties are linked functions. Understanding their interdependence provides a rigorous way to select the best protein variants and design optimal formulations.

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. Since 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 during the next years and their transformation from drugs administered mainly in hospital settings to drugs self-administered by patients, requires the fulfilment of strict safety and stability conditions. The long term stability of biologics is a function of diverse physical and chemical factors that require 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/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 is an astronomical number of partially denatured (also referred to as partially folded) conformations, the vast majority never becomes populated. In fact, the native/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]. Multi-domain proteins may exhibit partially denatured conformations corresponding to states in which some domains are unfolded while the others remain folded [6]. Figure 1 illustrates two commonly observed situations. The top panel represents the two-state situation in which the native and fully denatured conformations are the only ones that become significantly populated. The bottom panel 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:

$$F_{denatured} = \frac{e^{-\frac{\Delta G}{RT}}}{1 + e^{-\frac{\Delta G}{RT}}} \quad [1]$$

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 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 100mg/mL protein solution with a structural stability of 8 kcal/mol the concentration of denatured protein is 0.1Mg/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. Figure 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].

Thermodynamic linkage between conformational equilibrium and self-association

Different aggregation scenarios can be derived from the conformational equilibrium situations shown in Figure 1. Either the native, partially denatured or denatured states may have a tendency to self-associate and form different types of aggregates. Some self-association processes are functionally and biologically important like those present in oligomeric proteins, and usually involve highly specific native state interactions. Other self-association processes are detrimental and undesirable, especially those occurring in protein engineering and during the development of therapeutic proteins like monoclonal antibodies [19]. These unwanted self-association processes are usually referred to as aggregation processes since they often lead to irreversible aggregates. In protein therapeutics the presence of these aggregates not only reduces the activity of the protein but may also trigger dangerous immune responses. From a physicochemical standpoint, the formation of these aggregates has been described in terms of different variants of the Lumry-Eyring model [16–21]. The basic idea is that the protein exist in equilibrium between different conformations and that some of those conformations have a tendency to self-associate and form aggregates. The initial self-association is usually reversible or partially reversible and, depending on the protein and physical or chemical conditions, it may stay that way or eventually lead to irreversible aggregation. Reversible aggregation affects the conformational equilibrium in well-defined ways. In this review, three different aggregation cases will be considered in order to illustrate their impact in the conformational equilibrium of the protein. The first case is the one originating from native state self-association, the second case is the one in which a partially denatured state self-associates, and the third case is the one that originates from denatured state self-association. The resulting aggregates fall into class 3, class 5 and class 6 aggregates according to the classification proposed by Joubert *et al.* [19].

Case 1: native state aggregation

Figure 3 illustrates the situation in which aggregation originates from self-association of the native state. In this case the equilibrium will be shifted to the left, ΔG of the denatured and partially denatured states will increase and, consequently, the stability of the native state will increase. If this situation occurs, the measured ΔG usually referred to as apparent ΔG , ΔG_{app} , is given by [22]:

$$\Delta G_{app} = \Delta G_o^o + RT \ln(1 + jK_{N,j}[N]^{j-1}) \quad [2],$$

Where ΔG_o^o is equal to the value of the Gibbs energy extrapolated to zero protein concentration (in practice, a concentration low enough at which aggregation is not present), j is the average stoichiometry of the self-associated state and $K_{N,j}$ the self-association constant. Equation 2 indicates that native state self-association will cause the measured Gibbs energy to increase as a function of protein concentration and that the magnitude of the increase is proportional to the self-association constant and the average size of the aggregates. These aggregates are small and fully or partially reversible (class 3 [19]). For the specific case in which the native state forms a dimer ($j = 2$), equation 2 has an exact solution that can be expressed in terms of the total protein concentration as demonstrated before for the HIV-1 protease [23] (see inset in Figure 3). In this case, it is possible to determine the dimerization constant which under the conditions in the figure is 1.6×10^{-8} M. For arbitrary degrees of oligomerization, there are no exact solutions for $K_{N,j}$ but the fraction of aggregated protein can be calculated directly from the data even though j and $K_{N,j}$ cannot be determined:

$$F_{aggregated} = \frac{\frac{\Delta \Delta G}{(e^{\frac{\Delta \Delta G}{RT}} - 1)}}{(1 + e^{\frac{\Delta G_o^o}{RT}} + \frac{\Delta \Delta G}{(e^{\frac{\Delta \Delta G}{RT}} - 1)})} \quad [3] \quad (3)$$

Where $\Delta \Delta G = \Delta G_{app} - \Delta G_o^o$. In general, if experiments are performed at different protein concentrations, an increase in the measured ΔG with protein concentration is indicative of native state self association. This effect is observed both for oligomeric proteins or proteins that exhibit unwanted aggregation (e.g. antibodies that exhibit native state aggregation).

Case 2: partially denatured state aggregation

In some situations, partially denatured conformations have a higher tendency to self-associate than the native or denatured states. The situation is illustrated in Figure 4. In this case, the first transition, corresponding to the transition from monomeric native to self-associated partially denatured states is shifted down upon increasing protein concentration whereas the second transition, corresponding to the transition from the self-associated partially denatured to monomeric fully denatured state is shifted up upon increasing protein concentration. Experimentally, this situation has been observed with recombinant human growth hormone (rHGH) at low pH [24]. rHGH is a monomeric protein, upon denaturation at low pH it forms a partially denatured dimer which fully dissociates upon complete denaturation. It must be noted that if the dimeric partially denatured state were the end point of the denaturation transition, it will result in only the destabilization of the native state. In the DSC experiments, as shown in Figure 5, the T_m of the first transition decreases with rHGH concentration whereas the

T_m of the second transition increases with rHGH concentration. A similar behavior will be observed in chemical denaturation experiments. In this case the first transition occurs at lower denaturant concentrations and the second transition at higher denaturant concentrations. It is worth mentioning, that several monoclonal antibodies exhibit two chemical denaturation transitions. If this is the case, an assessment of native state aggregation requires that the two transitions exhibit higher ΔG values upon increasing protein concentration.

Case 3: denatured state aggregation

Figure 6 illustrates the situation in which aggregation originates from self-association of the denatured state. In its early stages the process is fully or partially reversible (class 6 [19]). In this case the equilibrium will be shifted to the right, ΔG will decrease and the stability of the native state will decrease. If this situation occurs, the measured ΔG is given by [22]:

$$\Delta G_{app} = \Delta G_o^o - RT \ln(1 + jK_{N,j}[N]^{j-1}) \quad (2)$$

Previously, this behavior has been reported for cetuximab in urea denaturation experiments [22]. ΔG decreased from about 9 kcal/mol at 24 Mg/mL to 7 kcal/mol at 180 Mg/mL consistent with denatured state aggregation. In those experiments, urea was able to resolve only one transition; however, subsequent experiments performed with guanidinium hydrochloride (GndnHCl), a more potent chemical denaturant, were able to resolve two transitions. However, only the first transition exhibited a consistent decrease in ΔG upon increasing antibody concentration suggesting that the aggregating conformation is the partially denatured state emerging from the first transition. For several antibodies, CH2 domains have been identified as the low stability domains, also responsible for the first transition observed in DSC experiments [25]. Also, partially denatured conformations in which the CH2 domains are unfolded can be prone to aggregate [26]. The second transition, involving the most stable domains, appeared to be independent of concentration further emphasizing that, in this case, the least stable domain is responsible for the observed aggregation. Figure 7 illustrates this situation. Even though the partially denatured state initiates the self-association process, full denaturation does not lead to dissociation of the aggregate. This situation is different than the one observed with rHGH in which full denaturation leads to dissociation of the oligomeric intermediates. For rHGH, this dissociation is reflected in a ΔG increase for the second transition upon increasing protein concentration. For cetuximab, the Gibbs energy of the second transition remains unchanged, strongly suggesting that the high stability domain does not affect the self-association situation resulting from the denaturation of the low stability domain. In this case, measures to prevent aggregation should be primarily aimed towards the least stable domain.

Concluding remarks

The analysis presented here indicates that different modes of partially or fully reversible self-association, that may eventually lead to irreversible aggregation, affect the concentration dependence of the conformational equilibrium of a protein in very specific ways. Since the conformational equilibrium and its concentration dependence can be measured by DSC or ICD, this analysis can provide valuable diagnosis and critical information for the identification of better protein variants or better formulations. Preventing aggregation processes that are linked to the conformational equilibrium of a protein requires: 1) minimizing the concentration of aggregating species; and, 2) Minimizing the self-association constant of the aggregating species. This task can be accelerated by a quantitative knowledge of the conformation/self-association equilibrium.

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References

- 1 Ericsson, U.B. *et al.* (2006) Thermofluor-based high-throughput stability optimization of proteins for structural studies. *Anal. Biochem.* 357, 289–298
- 2 Vedadi, M. *et al.* (2006) Chemical screening methods to identify ligands that promote protein stability, protein crystallization and structure determination. *Proc. Natl. Acad. Sci. U. S. A.* 103, 15835–15840
- 3 Capelle, M.A.H. *et al.* (2007) High throughput screening of protein formulation stability: practical considerations. *Eur. J. Pharm. Biopharm.* 65, 131–148
- 4 Senisterra, G.A. and Finerty, P.J. Jr (2009) High throughput methods of assessing protein stability and aggregation. *Mol. Biosyst.* 5, 217–223
- 5 Privalov, P.L. (1979) Stability of proteins: small globular proteins. *Adv. Protein Chem.* 33, 167–239
- 6 Privalov, P.L. (1982) Stability of proteins: proteins which do not present a single cooperative system. *Adv. Protein Chem.* 35, 1–104
- 7 Brandts, J.F. *et al.* (1970) Thermodynamics of protein denaturation. Effect of pressure on the denaturation of ribonuclease A. *Biochemistry* 9, 1038–1047
- 8 Privalov, P.L. and Khechinashvili, N.N. (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *J. Mol. Biol.* 86, 665–684
- 9 Privalov, G. *et al.* (1995) Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution. *Anal. Biochem.* 232, 79–85
- 10 Greene, R.F., Jr and Pace, C.N. (1974) Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, alpha-chymotrypsin, and beta-lactoglobulin. *J. Biol. Chem.* 249, 5388–5393
- 11 Pace, C.N. *et al.* (1992) Urea denaturation of barnase: pH dependence and characterization of the unfolded state. *Biochemistry* 31, 2728–2734
- 12 Santoro, M.M. and Bolen, D.W. (1988) Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl.alpha.chymotrypsin using different denaturants. *Biochemistry* 27, 8063–8068
- 13 Bolen, D.W. and Santoro, M.M. (1988) Unfolding free energy changes determined by the linear extrapolation method. 2. Incorporation of dGn-u values in a thermodynamic cycle. *Biochemistry* 27, 8069–8074

- 14 Myers, J.K. *et al.* (1995) Denaturant m values and heat capacity changes: relation to changes in accessible surface area of protein unfolding. *Prot. Sci.* 4, 2138–2148
- 15 Freire, E. *et al.* (2013) Chemical denaturation as a tool in the formulation optimization of biologics. *Drug Discov. Today* 18, 1007–1013
- 19 Joubert, M.K. *et al.* (2011) Classification and characterization of therapeutic antibody aggregates. *J. Biol. Chem.* 286, 25118–25133
- 16 Lumry, R. and Eyring, H. (1954) Conformation changes in proteins. *J. Phys. Chem.* 58, 110–120
- 17 Sanchez-Ruiz, J.M. (1992) Theoretical analysis of Lumry-Eyring models in differential scanning calorimetry. *Biophys. J.* 61, 921–935
- 18 Li, Y. and Roberts, C.J. (2010) Protein aggregation pathways, kinetics, and thermodynamics. In *Aggregation of Therapeutic Proteins* (Roberts, W.W.a.C.J., ed.), pp. 63–102, John Wiley & Sons
- 20 Arosio, P. *et al.* (2011) Aggregation stability of a monoclonal antibody during downstream processing. *Pharmaceutical Research* 28, 1884–1894
- 21 Brader, M.L. *et al.* (2015) Examination of thermal unfolding and aggregation profiles of a series of developable therapeutic monoclonal antibodies. *Mol. Pharm.* 12, 1005–1017
- 22 Schon, A. *et al.* (2015) Denatured state aggregation parameters derived from concentration dependence of protein stability. *Anal. Biochem.* 488, 45–50
- 23 Todd, M.J. *et al.* (1998) The structural stability of the HIV-1 protease. *J. Mol. Biol.* 283, 475–488
- 24 Kasimova, M.R. *et al.* (1998) The conformational equilibrium of human growth hormone. *J. Mol. Biol.* 277, 409–418
- 25 Ionescu, R.M. *et al.* (2008) Contribution of variable domains to the stability of humanized IgG1 monoclonal antibodies. *J. Pharm. Sci.* 97, 1414–1426
- 26 Mehta, S.B. *et al.* (2014) Partial unfolding of a monoclonal antibody: role of a single domain in driving protein aggregation. *Biochemistry* 53, 3367–3377

Figure 1. Typical protein denaturation situations encountered in the laboratory. **The top panel illustrates** 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 multi-domain proteins with highly cooperative inter-domain interactions. **The bottom panel illustrates** the situation in which denaturation occurs with the presence of a significantly populated partially denatured state. This situation often occurs with multi-domain proteins.

Figure 2. Differential scanning calorimetry (DSC) and isothermal chemical denaturation (ICD) 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.

Figure 3. Self-association of the native state is characterized by an increase in ΔG upon increasing protein concentration. For this effect to occur the association process and the resulting aggregates must be fully or partially reversible, even though they may initiate the subsequent formation of irreversible aggregates. Self-association of the native state can be functionally relevant (oligomeric proteins) or detrimental (aggregation of monoclonal antibodies). **The inset** shows the example of the HIV-1 protease which is a dimer in the native state and dissociates upon unfolding. Denaturation experiments were performed using a linear gradient of guanidine hydrochloride from 0 to 5.5 M (The Hunk Automated Protein Denaturation System, Unchained Labs, Norton, MA). The buffer was 10 mM sodium formate, pH 3.4. The final concentration of protease was varied from 0.4 to 12 μ M. The exact concentration was determined from the absorbance at 280 nm using an extinction coefficient of 25,530 $M^{-1} cm^{-1}$ [23]. The gold arrow indicates that the equilibrium is shifted towards the native conformations and that chemical or temperature denaturation occur at higher values.

Figure 4. Partially denatured conformations might have a higher tendency to aggregate than the native or fully denatured state, in which case aggregation occurs when the partially denatured state becomes populated. In this case, dissociation occurs upon full denaturation. This behavior is exhibited by recombinant human growth hormone at low pH [24]. Gold arrows indicate that the equilibrium is shifted towards the partially denatured conformations. The effect of this behavior is shown in Figure 5..

Figure 5. Left panel: The heat capacity function of recombinant human growth hormone at pH 3 (10 mM glycine buffer) as reported by Kasimova *et al.* [24]. The blue curve corresponds to a concentration of 10mg/mL and the green curve to a concentration of 2mg/mL. Upon increasing concentration (blue curve) the first transition (native state to partially denatured state) occurs at a lower T_m and the second transition (partially denatured state to denatured state) occurs at a higher T_m . **Right panel:** Simulation of expected chemical denaturation of recombinant human growth hormone. Upon increasing protein concentration (blue curve) the first transition (native state to partially denatured state) is shifted to lower denaturant concentrations (lower ΔG) and the second transition (partially denatured state to denatured state) to higher denaturant concentrations (higher ΔG).

Figure 6. Denatured state aggregation will pull the equilibrium to the right and decrease ΔG as the protein concentration increases. The gold arrow indicates that the equilibrium is shifted towards the denatured state. . The decrease in ΔG indicates a lower stability of the native state. **The inset** illustrates this situation by depicting the concentration dependence of ΔG for cetuximab in urea denaturation experiments. Chemical denaturation experiments were performed using a linear gradient of urea from 0 - 9 M (The Hunk Automated Protein Denaturation System, Unchained Labs, Norton, MA). The buffer was 10 mM PBS, pH 7.5.

Figure 7. Situation in which a partially denatured state exhibits a tendency to self-associate but full denaturation does not lead to dissociation as in Figure 4. Even though the denatured high stability domain may not exhibit an intrinsic tendency to self-associate, the entire protein remains aggregated through the denatured low stability domain. In this situation, the ΔG of the first transition decreases with protein concentration but the second one remains constant. This appears to be the case observed for the guanidinium hydrochloride denaturation of cetuximab obtained at 12 and 48 Mg/mL (**inset in the figure**). Chemical denaturation experiments were performed using a linear gradient of guanidine hydrochloride from 0 - 5.5 M with same buffer as urea denaturation experiments (The Hunk Automated Protein Denaturation System, Unchained Labs, Norton, MA).

Table 1. Title^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

^aKnowledge of ΔG allows calculation of the population of denatured protein or domains. Since denatured conformations are prone to aggregation, ΔG should play a critical role in the selection of protein variants and formulation optimization.

Figure 1

(A)

(B)

Figure 2

(B)

Figure 3

Figure

Figure 5

Figure 6

Figure 7

(B)

Biologics exist in equilibrium between different conformational states (native, partially denatured or fully denatured states). Any of these conformations may have a propensity to aggregate. An important goal in the development of protein therapeutics is to identify the protein variants and/or the formulations that optimize the stability/aggregation profile. Since conformational stability and self-association are thermodynamically linked functions, the concentration dependence of conformational stability reveal aggregation tendencies at very early stages and provide critical information for accelerated development.