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Denatured state aggregation parameters derived from concentration dependence of protein stability

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ABSTRACT

Protein aggregation is a major issue affecting the long-term stability of protein preparations. Proteins exist in equilibrium between the native and denatured or partially denatured conformations. Often denatured or partially denatured conformations are prone to aggregate because they expose to solvent the hydrophobic core of the protein. The aggregation of denatured protein gradually shifts the protein equilibrium toward increasing amounts of denatured and ultimately aggregated protein. Recognizing and quantitating the presence of denatured protein and its aggregation at the earliest possible time will bring enormous benefits to the identification and selection of optimal solvent conditions or the engineering of proteins with the best stability/aggregation profile. In this article, a new approach that allows simultaneous determination of structural stability and the amount of denatured and aggregated protein is presented. This approach is based on the analysis of the concentration dependence of the Gibbs energy (ΔG) of protein stability. It is shown that three important quantities can be evaluated simultaneously: (i) the population of denatured protein, (ii) the population of aggregated protein, and (iii) the fraction of denatured protein that is aggregated.

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The identification of conditions that maximize the structural stability of the native state of proteins and prevent aggregation or other undesirable processes is essential for the development of protein therapeutics, protein formulations, process development conditions, and quality control as well as basic protein sciences [1–4]. In general, there are two situations in which the capability of identifying aggregation tendencies is critical: (i) the engineering or selection of the mutations or protein variants that exhibit the best stability/aggregation profile and, (ii) the identification of the solution (formulation) conditions that maximize conformational stability and minimize aggregation. Long-term stability of protein solutions is usually hampered by the presence of protein aggregates. Different mechanisms and kinetic processes account for the formation of these aggregates (see Refs. [5,6] for recent publications). Among the potential sources of aggregates is the presence of denatured protein, which is the subject of this article. Even if the amount of denatured protein is very small soon after a protein solution is prepared, it may have a tendency to aggregate, acting as a sink that eventually depletes the native state protein. This process has often been described in terms of the Lumry–Eyring model [7] or variants of this model [8]. In protein therapeutics, aggregation not only diminishes the amount of biologically active protein but also can trigger undesirable immune responses and other adverse effects [9]. Identifying the best protein variant or the best formulation requires the ability to measure both the concentration of denatured protein and the fraction of that protein that is aggregated in a timely fashion. Using this information, the protein clone or the formulation with the optimal stability and aggregation attributes can be rapidly determined.

Direct measurement of the population of denatured protein immediately after a formulation or protein solution is prepared is extremely difficult. The population of denatured protein in a fresh formulation is exceedingly small (usually <0.01% of the total protein) and escapes detection by conventional techniques. Commonly used accelerated stability or forced degradation assays essentially

Abbreviations: bCA-II, bovine carbonic anhydrase isozyme II; PBS, phosphate-buffered saline; GdnHCl, guanidinium HCl; L-Arg, L-arginine; DSC, differential scanning calorimetry.

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drive the denatured and aggregated population to measurable levels. An alternative method to determine the population of denatured protein relies on measurements of the structural equilibrium between native and denatured conformations. Determination of the Gibbs energy of stability (ΔG^o) allows calculation of the amount of denatured protein in a solution to the required levels (<0.01% of the total protein). Furthermore, because aggregation and conformational equilibrium are thermodynamically linked, it is possible to show that the protein concentration dependence of ΔG^o provides critical information to evaluate the degree of protein aggregation in the formulation. This was the main focus of this study.

Materials and methods

Proteins and reagents

Bovine carbonic anhydrase isozyme II (bCA-II) was purchased as a lyophilized powder from Sigma–Aldrich (cat. no. C2522, St. Louis, MO, USA) and was dissolved and further dialyzed into 20 mM succinate (pH 6.0). Cetuximab and trastuzumab were obtained from LGM Pharma (Nashville, TN, USA). Cetuximab was delivered as a solution of 10 mg/ml protein in phosphate buffer containing 0.005% (v/v) polysorbate, which was removed by dialysis against phosphate-buffered saline (PBS, pH 7.5) (Roche Diagnostics, Mannheim, Germany). Trastuzumab was delivered as a solution of 20 mg/ml protein in 5.42 mM histidine (pH 6.0) and 0.01% (v/v) polysorbate 20. Ultrapure urea was obtained from J. T. Baker (Center Valley, PA, USA), and molecular-grade guanidinium HCl (GdnHCl) was obtained from Promega (Madison, WI, USA). Sodium succinate, histidine, polysorbate 20, trehalose, and dextrose were obtained from Sigma–Aldrich.

Isothermal chemical denaturation

All chemical denaturation experiments were carried out with an AVIA ICD 2304 Isothermal Chemical Denaturation System (Norton, MA, USA) using intrinsic protein fluorescence detection. The excitation wavelength was 280 nm, and scans of the emission intensity were recorded between 300 and 500 nm. The buffers used in the absence or presence of excipients were 20 mM succinate (pH 6.0) and PBS (pH 7.5) for bCA-II and cetuximab, respectively. For trastuzumab, the base buffer was 4.5 mM histidine and 0.009% polysorbate 20 (pH 6.0) with 5% (w/v) dextrose as excipient. The different concentrations of trehalose or L-arginine (L-Arg) solutions were automatically prepared by the instrument. For each denaturation experiment, protein, buffer, and 10 M urea were dispensed into 36 wells with a linear gradient of urea from 0 to 9 M generated by the instrument. The protein solution is diluted 12.5-fold on addition to the wells; therefore, the protein stocks were prepared at concentrations that accommodate this dilution. For experiments in which GdnHCl was used as denaturant, a similar procedure was used except that the gradient upper limit was different. To allow complete denaturation of cetuximab, the fluorescence intensities were recorded 18 h after incubation with urea. Trastuzumab denaturations were undertaken using 32-point linear 0 to 5.52-M GdnHCl gradients with 3 h of sample incubation in denaturant. Data processing was performed with the software provided by the instrument, and global analysis was performed with the software package DataFit (Oakdale Engineering, Oakdale, PA, USA).

Differential scanning calorimetry

Thermal denaturation experiments were performed using a VP-DSC microcalorimeter from MicroCal/Malvern Instruments (Northampton, MA, USA). Solutions of bCA-II were extensively dialyzed against buffer in the absence or presence of 300 mM L-Arg. The bCA-II concentration was 1.0 mg/ml. The reference cell was filled with dialysis buffer. The buffer was 20 mM succinate (pH 6.0). The samples were thoroughly degassed before loading of the calorimetric cell (~0.5 ml). Thermal denaturation scans were conducted from 10 to 100 °C at a rate of 1 °C/min.

Results and discussion

Population of denatured protein

Fig.1 illustrates the basic thermodynamic equilibrium model considered in this article. The native state (red) exists in equilibrium with the denatured state (green), which has a tendency to aggregate in an eventually irreversible manner. The population or fraction of denatured protein (F_{denat}) is equal to the total concentration of denatured protein (non-aggregated plus aggregated) divided by the total protein concentration:

$$F_{\text{denat}} = \frac{[D] + j[D_j]}{[N] + [D] + j[D_j]}, \tag{1}$$

where j is the average degree of oligomerization of the aggregates. Denatured state aggregation is not a process characterized by well-defined stoichiometries or structures. In our approach, it is represented by an ensemble of diverse aggregates characterized by a mean degree of oligomerization.

In the absence of aggregation, Eq. (1) reduces to the well-known equation

$$F_{\text{denat}} = \frac{[D]}{[N] + [D]} = \frac{K}{1 + K} = \frac{e^{-\frac{\Delta G^o}{RT}}}{1 + e^{-\frac{\Delta G^o}{RT}}}, \tag{2}$$

which, at any constant temperature, is only a function of the Gibbs energy of stability (ΔG^o). K is the equilibrium constant $\left(K = \frac{[D]}{[N]}\right)$, which is also defined in terms of ΔG^o. Eq. (2) can be easily generalized to the situation where a protein exhibits multiple transitions

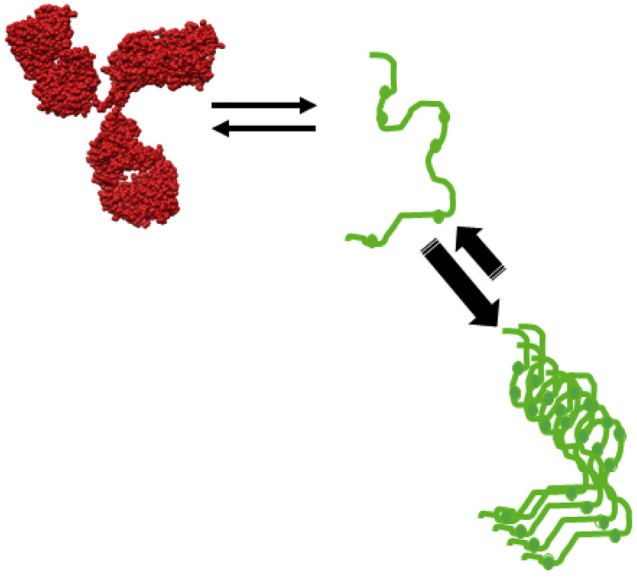

Fig.1. Proteins exist in conformational equilibrium between the native (red) and denatured (green) (unfolded or partially unfolded) states. Under some conditions, the denatured state may exhibit a tendency to associate or aggregate. This tendency to aggregate affects the conformational equilibrium in a concentration-dependent fashion. Analysis of the conformational equilibrium at different protein concentrations allows calculation of different aggregation parameters.

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