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Relating Protein–Protein Interactions and Aggregation Rates From Low to High Concentrations

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At low protein concentrations (c_2), non-native protein aggregation rates are known to be sensitive to changes in conformational stability and "weak" or "colloidal" protein–protein interactions. Protein–protein interactions are also known to be strong functions of c_2. In the present work, protein–protein interactions and rates of aggregation were quantified systematically for a monoclonal antibody (MAb) across a broad range of c_2 at pH 5.1 and 6.5, with or without 5 wt/wt % sucrose or 100 mM NaCl present. Aggregation rates were determined from initial-rate analysis with size-exclusion chromatography, and interactions were quantified with static and dynamic laser light scattering. A number of hypotheses were tested regarding whether changes in protein–protein interactions can be predictive of changes in aggregation rates versus c_2. Hypotheses were based on (i) changes in thermodynamic activity; (ii) statistical mechanical fluctuation theory; and (iii) surface-contact probabilities. Arguments based on (i) and (ii) were qualitatively inconsistent with experimental rates and scattering. Hypothesis (iii) was reasonably successful and resulted in a semiquantitative correlation between rates and protein–protein interactions across almost 2 orders of magnitude in c_2. However, (iii) requires one to assume that the concentration-dependent protein–protein Kirkwood–Buff integral is a reasonable surrogate for contact probabilities.

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Introduction

Proteins are inherently labile molecules that have marginally stable structures and can be degraded and/or inactivated readily under reasonably mild *in vitro* and *in vivo* conditions. Non-native protein aggregation (hereafter referred to simply as aggregation) denotes the processes by which natively folded proteins form aggregates via interprotein contacts that involve secondary and/or tertiary structures that are measurably perturbed from the native or folded monomer state.[1-3] In this context, aggregates are typically net irreversible under the conditions that they form, and therefore the rates or kinetics of aggregation are a key quantity in determining product quality and shelf life. As aggregation is under kinetic control, the mechanism(s) of aggregation ultimately dictate the observed aggregation rates. Aggregation of therapeutic proteins is a long-standing issue during drug product development and commercial manufacturing[3,4] and is also problematic to control during protein expression *in vivo*.[2] Studies at relatively low protein concentration (c_2) have led to a reasonably common viewpoint, in which aggregation rates or kinetics are mediated by at least 3 main factors:[5-7] (1) protein conformational stability or conformational changes that expose stretches of hydrophobic amino acids or so-called aggregation "hot spots"; (2) "weak" or "colloidal" attractions (repulsions) that facilitate (inhibit) reversible protein self-association; and (3) intrinsic aggregation propensity, that is, strong interactions between exposed sequence "hot spots" that create net irreversible contacts between proteins, often via formation of interprotein beta sheet structures.

Environmental factors that greatly alter conformational stability include temperature, pressure, solution composition, and adsorption to solid–liquid interfaces.[3,8,9] Protein conformational stability is theoretically predicted to be sensitive to c_2, but direct experimental evidence for the magnitude of the effect is very limited.[10,11] Similarly, factors that have been found to greatly alter protein–protein interactions include c_2 and solution conditions such as pH and the concentration of commonly employed cosolutes, for example, salts, amino acids, and polyhydroxy

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compounds such as sucrose.[12,13] The intrinsic aggregation propensity of a protein when it is unfolded is putatively dictated by its primary sequence and is not an easy variable to control once a given protein candidate has been selected for product development and testing in patients.[7,14]

Accurate prediction of aggregation rates is a long-standing challenge in the biotechnology industry. For low c_2 conditions, phenomenological and mechanistic models have been developed in an attempt to provide quantitative prediction of aggregation rates for natively unfolded polypeptides under physiological conditions[14,15] and for therapeutic proteins under typical formulation conditions.[16-19] In all cases, the models either interpolate or extrapolate rate data from one experimental condition or protein sequence to another. These models have focused primarily on the effects of conformational stability and the inherent aggregation propensity of "hot spot" sequences. Weak or colloidal interactions have been shown to correlate, at least qualitatively, with aggregation rates at low c_2.[12,20] However, in a number of cases, it is an inverse correlation because changes in conformational stability outweigh changes in protein–protein interactions as one alters formulation conditions.[21-23] In contrast, relatively little has been done to quantitatively or semiquantitatively connect changes in protein–protein interactions with aggregation rates at high c_2.[24-26] In part, this may be due to the relatively small amount of published data for aggregation rates across a broad range of concentration as most studies either focus on only high-c_2 or low-c_2 data or provide only a small number of different c_2 conditions.[16-19,21,23,26]

The present study focuses on a systematic comparison of protein–protein interactions and accelerated aggregation rates (elevated temperature, 50°C) for a monoclonal antibody (MAb) of the IgG1 subclass, over a broad range of c_2 (~1-10^2 g/L) at pH 5.1 and 6.5, with and without either sucrose (5 wt/wt %) or NaCl (100 mM) present. Aggregation rates are based on loss of monomer, as quantified *ex situ* with size-exclusion chromatography (SEC). Protein–protein interactions are quantified *in situ* with static and dynamic light scattering (DLS) for the same c_2 as the measured aggregation rates. To the extent possible within experimental limits, changes in conformational stability are inferred from differential scanning calorimetry at low c_2.

Concentration-dependent aggregation rates are used to scrutinize general mass-action arguments that have been developed primarily for low-c_2 conditions and that encompass a family of models for aggregation kinetics.[27] Based on previous work and new arguments presented here, a number of hypotheses are tested with respect to both qualitative and quantitative agreements or "predictability" for experimental aggregation rates in terms of measureable protein–protein interactions. These include changes in thermodynamic activity or chemical potential, local concentration fluctuations, and the probability of protein surfaces coming into contact.

The results highlight a number of limitations for existing mass action models of protein aggregation, as well as hypotheses for how to quantitatively link protein–protein interactions to aggregation rates at high c_2. The protein–protein Kirkwood–Buff integral (G_{22}) is offered as a surrogate for protein–protein contact probabilities that are intuitively expected to influence aggregation rates at high concentration. It is found that a reasonable correlation exists between k_{obs} and G_{22} if one has a reference data set against which to normalize. Finally, outstanding challenges are discussed for predicting and/or measuring the thermodynamic properties of proteins (e.g., native protein chemical potential) and the conformational stability of proteins at elevated concentrations. Overcoming these challenges will require significant advancements in terms of experimental capabilities and modeling approaches.

Materials and Methods

Sample Preparation

Purified IgG1 was provided by Bristol-Myers Squibb at a starting concentration of ~54 mg/mL. Stock solutions were dialyzed against a given buffer condition (Spectra/Por 7 tubing, 10 kDa MWCO; Spectrum Laboratories, Santa Clara, CA) using four 12 h buffer exchanges of 500 mL each, in refrigerated conditions. Buffer-only conditions were either 10 mM sodium acetate at pH 4.0 or 10 mM histidine HCl at pH 6.35. Glacial acetic acid (Fisher Scientific) and histidine HCl (Sigma) were used for preparation of dialysis buffers. After dialysis, protein solutions were concentrated using Amicon (Millipore, Billerica, MA) ultracentrifugation tubes with a molecular weight cutoff of 10 kDa. The concentrate was collected at different stages of centrifugation so as to monitor solution pH and c_2 until the desired pH (pH 5.1 ± 0.1 for acetate buffer and pH 6.5 ± 0.1 for histidine buffer) and concentration (>170 mg/mL) were achieved. Supplementary data include illustrative results from repeated sample preparations. As expected, the solution pH shifted during the protein concentrating step because of self-buffering by the protein.[28] The lower starting pH values (before the concentration step) were selected based on trial runs, so as to achieve the desired final pH values in the concentrated stocks. Solutions at lower c_2 were subsequently prepared by dilution of the concentrated stock at either pH 5.1 or pH 6.5, using the corresponding buffer solution and independently confirmed to maintain the desired pH after dilution.

Samples with either sucrose or NaCl were prepared by gravimetrically diluting concentrated stock solutions to lower concentration. This was done using the appropriate ratios of stock solution, buffer-only solution (pH 5.1 or pH 6.5), and buffer with either 500 mM NaCl or 25% (wt/wt) sucrose. This was done to achieve the desired c_2 and a final NaCl concentration of 100 mM or a final sucrose concentration of 5% (wt/wt). Table 1 lists the set of final solution conditions. Final pH was verified for all samples, and all solutions were filtered using 0.22 μm low protein binding filters (Millipore). Final concentrations were confirmed via UV absorbance (Agilent Technologies, Santa Clara, CA) at 280 nm, with an extinction coefficient of 1.54 mL mg^{-1} cm^{-1}.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) experiments were performed using a VP-DSC instrument (Microcal, Northhampton, MA) for antibody solutions with 1 mg/mL protein at each of the different solution conditions in Table 1. Multiple buffer–buffer scans were performed to obtain baseline values and establish thermal history on the instrument immediately before sample scans. Thermal scans were performed over 20°C-90°C with a scan rate of 60°C/h. The average of the buffer scans was subtracted from the subsequent

Table 1
Summary of Formulation Conditions

Formulation Description	Additional Excipient (Final Concentration)	Colors for Curves/ Symbols in Figures[a]
pH 5, buffer only[b]	none	Black
pH 5, buffer + NaCl	NaCl (100 mM)	Blue
pH 5, buffer + sucrose	Sucrose (5% wt/wt)	Red
pH 6.5, buffer only[c]	none	Black
pH 6.5, buffer + NaCl	NaCl (100 mM)	Blue
pH 6.5, buffer + sucrose	Sucrose (5% wt/wt)	Red

[a] Figures 5, 6, and 8 use closed symbols for pH 5, and open symbols for pH 6.5.
[b] 10-mM sodium acetate, pH 5.0 ± 0.1.
[c] 10-mM histidine HCl, pH 6.5 ± 0.1.

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