well discussed in the literature (Chan & Dill, 1993; Cleland, 1993, Dill, Chan, & Yue, 1995; Dill, Ozkan, Shell, & Weikl, 2008; Dill, Ozkan, Weikl, Chodera, & Voelz, 2007; Dill & MacCallum, 2012). Early investigations based on unfolding of small globular proteins, such as RNase, led to the development of two-state models for protein unfolding. The early classic experiments by Anfinsen (for which he was awarded a Nobel Prize) on RNase showed that when solution conditions were changed that favored the unfolded state the protein lost its overall structure, but could be refolded after changing solution conditions back to initial conditions (Anfinsen, 1973). These studies led to the conclusion that all the information for maintaining the structure/conformation of the protein resided in the amino acid sequence, known as primary structure. Although the two-state model could account for the concerted unfolding process as monitored by spectroscopy such as circular dichroism, later kinetic measurements of the process suggested that there were intermediates present (Tsong, Baldwin, & Elson, 1971).

Solution variables that may determine conformational stability include temperature changes, shear forces, ice formation as a result of freezing and thawing, changes in ionic strength, and changes in protein–solvent interactions. Recent work investigating the response of mAb to typical shear forces encountered during processing suggests that shear forces are less of a contributor to protein/mAb aggregation (Bee et al., 2009).

Although most proteins undergo unfolding at temperatures below 70 °C, mAbs in general do not unfold completely until the temperature exceeds 70 °C. The unfolding process can be monitored by spectroscopic assays, such as fluorescence, UV absorption, circular dichroism, and Fourier transform infrared (FTIR) spectroscopy.

A nice example of the impact of processing conditions on protein conformation is the process of creating a sustained delivery formulation for recombinant human growth hormone (rhGH). In this process polymer microspheres containing rhGH were created by spray freeze-drying rhGH formulated with or without Zn in an organic solvent that contains poly(lactic-*co*-glycolic acid) copolymer (Johnson et al., 1996). Addition of Zn generates precipitates of rhGH and it was found that the precipitation did not alter rhGH secondary structure (Yang et al., 2000). However, after extraction from the microspheres it was shown that without Zn addition the rhGH underwent conformational changes as assessed by FTIR measurements, whereas precipitation of rhGH with Zn resulted in protection of the protein conformation (Yang et al., 1999). Apparently the exposure to organic solvents in the process perturbed the rhGH secondary structure, and generation of precipitates with Zn prior to the microencapsulation prevented the solvent-induced conformational change.

As previously discussed, one distinguishing feature of IgG1 mAbs is the large amount of flexibility in the hinge region, which connects up the two Fab domains with the Fc domain. Conformational changes in the hinge region could conceivably impact the flexibility of the mAb which may impact the binding to the antigen target. This appears to be the case in a study by Taschner et al. (2001) where a mAb (subtype not mentioned) lost its ability to bind to a targeted carbohydrate on a cancer cell after freeze-drying. Standard biochemical assays, such as SDS PAGE, gel sizing chromatography, and capillary zone electrophoresis, did not show any alterations compared to mAb before freeze-drying. Scanning transmission electron microscopy was used to compute the distribution of angles between Fab domains of the mAb before and after lyophilization. The results suggested that the nonlyophilized mAb had a wider