Cold denaturation of monoclonal antibodies

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Abbreviations: mAb1, monoclonal antibody 1; mAb2, monoclonal antibody 2; IgG, immunoglobulin type G; CDRs, complementarity-determining regions; ∆G, free energy of unfolding between the native and denatured states of a protein; GuHCl, guanidine hydrochloride; CD, circular dichroism; PPII, polyproline II helical structure; Tm, heat denaturation (melting) temperature; TCD, cold denaturation temperature; Tm, temperature of maximal protein stability; ΔHref, molar enthalpy change between the native and denatured states of a protein at a reference temperature; ΔSref, molar entropy change between the native and denatured states of a protein at a reference temperature; ΔCp, molar heat capacity change between native and denatured states of a protein; ΔGm, molar gibbs free-energy change between the native and denatured states of a protein at temperature T; ΔG0, free energy of unfolding in the absence of denaturant; LEM, linear extrapolation method; Wes, Van, abbreviations of family names of patients

The susceptibility of monoclonal antibodies (mAbs) to undergo cold denaturation remains unexplored. In this study, the phenomenon of cold denaturation was investigated for a mAb, mAb1, through thermodynamic and spectroscopic analyses. Tryptophan fluorescence and circular dichroism (CD) spectra were recorded for the guanidine hydrochloride (GuHCl)-induced unfolding of mAb1 at pH 6.3 at temperatures ranging from -5°C to 50°C. A three-state unfolding model incorporating the linear extrapolation method was fit to the fluorescence data to obtain an apparent free energy of unfolding, ΔG0 at each temperature. CD studies revealed that mAb1 exhibited polyproline II helical structure at low temperatures and at high GuHCl concentrations. The Gibbs-Helmholtz expression fit to the ΔG0 versus temperature data from fluorescence gave a ΔCp of 8.0 kcal mol-1 K-1, a maximum apparent stability of 23.7 kcal mol-1 at 18°C, and an apparent cold denaturation temperature (TCD) of -23°C. ΔCp values for another mAb (mAb2) with a similar framework exhibited less stability at low temperatures, suggesting a depressed protein stability curve and a higher relative TCD. Direct experimental evidence of the susceptibility of mAb1 and mAb2 to undergo cold denaturation in the absence of denaturant was confirmed at pH 2.5. Thus, mAbs have a potential to undergo cold denaturation at storage temperatures near -20°C (pH 6.3), and this potential needs to be evaluated independently for individual mAbs.

Introduction
Monoclonal antibodies (mAbs) have found use in diverse applications including research, diagnostics, immunochemistry/immunohistochemistry and as therapeutics against a variety of cancers and immunological-based disorders. For these various applications, mAbs are often stored frozen at temperatures ranging from -20°C to -80°C. The advantages of frozen storage over liquid storage include increased stability and reduced microbial growth. However, under these storage conditions, protein destabilization and aggregation can occur, either during frozen storage or during the freeze-thawing step. The destabilization during frozen storage may arise from cryoconcentration of protein and co-solutes, protein denaturation at the ice-water interface, undesirable crystallization of co-solutes, pH shifts associated with buffer crystallization and cold denaturation. Destabilization during freeze-thawing may arise from aggregation of misfolded/partially folded protein species that form as a result of the above-mentioned phenomena during frozen storage along with interaction of the protein with surfaces.

It is known that proteins can unfold spontaneously through the phenomenon of cold denaturation. The susceptibility of a protein towards unfolding in a given solution and as a function of temperature is described by the Gibbs-Helmholtz expression, which relates the free energy of unfolding, ΔGm, to the temperature-independent heat capacity change (ΔCp). The molar Gibbs free-energy change (ΔGm) between the native and denatured states of a protein at temperature T is defined as follows:

\[
ΔG_m = ΔH_{ref} + ΔC_p(T - T_{ref}) - T[ΔS_{ref} + ΔC_p \ln(T/T_{ref})]
\]

(1)

Tref is any reference temperature and ΔHref and ΔSref are the changes in the enthalpy and entropy at that temperature, respectively. Thermodynamic analysis of a range of small, globular proteins showing 2-state behavior reveals that the ΔGm under near-physiological conditions is usually in the range of 3–15 kcal mol-1. The temperatures at which ΔGm equals zero define

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the heat- and the cold-induced denaturation temperatures. The mechanism of such temperature-induced denaturation, although still not fully understood, is largely believed to be controlled by changes in the structure of water.\textsuperscript{10,15,16} The phenomenon of cold denaturation is a thermodynamic consequence of the large positive $\Delta C_v$ of unfolding for proteins, which results in curvature of the $\Delta G$ versus temperature plot.\textsuperscript{7} The cold denaturation temperature ($T_{cd}$) is dependent on a number of factors, e.g., the concentration of the protein, the presence of co-solutes, and the pH of the solution.\textsuperscript{15,16} Cold denaturation has been observed for several proteins, including $\beta$-lactoglobulin, myoglobin, phosphoglycerate kinase, ribonuclease A, ubiquitin and yeast frataxin.\textsuperscript{17-22} It is often difficult to observe $T_{cd}$ experimentally because cold denaturation usually occurs at temperatures well below the freezing point of water. Therefore, destabilizing perturbations are often used to promote cold denaturation at higher temperatures, including addition of chemical denaturants such as guanidine hydrochloride (GuHCl) and urea, alteration of the pH and application of high pressure to lower the freezing point of water.\textsuperscript{10}

Protein thermodynamic stability is most often studied by exploring the thermal and chemical denaturation of a protein. The experimental tools that are employed to look at thermodynamic stability include spectroscopy (UV-Vis, infrared absorption, circular dichroism (CD), fluorescence, NMR) and differential scanning calorimetry (DSC). For a two-state protein with reversible unfolding, thermal denaturation data from DSC can be used to estimate the thermodynamic parameters associated with unfolding such as the melting temperature ($T_m$), the heat capacity change ($\Delta C_p$) between the native and denatured states, and the enthalpy ($\Delta H$) and entropy ($\Delta S$) changes at the melting temperature. However, the estimation of thermodynamic stability using data from DSC is confounded for proteins that undergo irreversible changes following heat-induced unfolding. Antibodies, which are multidomain proteins, typically unfold and aggregate irreversibly following thermal unfolding. Therefore, data from thermal denaturation cannot be used to obtain these important thermodynamic parameters.

In this study, we explore the phenomenon of cold denaturation of mAb1, a full-length mAb of the IgG1 class. The susceptibility of mAb1 to undergo cold denaturation was investigated using chemical denaturation with GuHCl. Fluorescence data generated from the GuHCl-induced denaturation of mAb1 was used to investigate the temperature dependence of $\Delta G$, and subsequently, the susceptibility of mAbs to undergo cold denaturation. We employed the linear extrapolation method (LEM) to generate the protein stability curve ($\Delta G$ v. $T$) from the GuHCl-induced unfolding of mAb1.\textsuperscript{23,24} The LEM has been used in the past to determine the free energy of unfolding of several proteins showing multiphase transitions with the assumption that the free energy is linearly dependent on denaturant concentration in each phase of the unfolding curve.\textsuperscript{25-31} Cold denaturation of mAb1 and mAb2 was further explored at pH 2.5 (destabilizing to mAbs) in an effort to obtain direct experimental evidence of this phenomenon.

Through chemical denaturation and low pH destabilization studies, we show that mAbs exhibit a temperature of maximal thermodynamic stability around ambient temperature, and have a potential to undergo cold denaturation at subzero temperatures. We also show that two mAbs, mAb1 and mAb2, which differ only in the complementarity-determining regions (CDRs) and a few residues flanking the CDRs, show different thermodynamic stabilities at low temperature and are predicted to have different cold denaturation temperatures.

### Results

**Reversibility of mAb/GuHCl denaturation.** 1 mg/ml mAb1 and mAb2 were denatured overnight in 6 M GuHCl, 25 mM potassium phosphate buffer, pH 6.3. Following gradual dilution of these solutions, tryptophan emission spectra were collected on the resulting 0.1 mg/ml mAb/0.6 M GuHCl solutions to measure the extent of reversibility. The intensity ratio $I_{325 \text{ nm}}/I_{375 \text{ nm}}$ was calculated from the emission spectra for both mAbs and compared with this ratio from the spectrum of the native protein to track the shift in $\lambda_{max}$. Based on the recovery of the $I_{325 \text{ nm}}/I_{375 \text{ nm}}$ ratio following renaturation, mAb1 and mAb2 showed 86% and 75% reversibility, respectively. When the 0.6 M GuHCl containing mAb solutions were further dialyzed to remove the remaining GuHCl, the samples showed almost complete reversibility by fluorescence (96% and 88% for mAb1 and mAb2, respectively).

**GuHCl-induced unfolding of mAb1 investigated by intrinsic tryptophan fluorescence.** We assessed the loss of tertiary structure with GuHCl denaturation using intrinsic tryptophan fluorescence. MAb1/GuHCl samples were equilibrated at several temperatures (-5, 1, 10, 20, 30, 40 and 50°C) before obtaining fluorescence data at each temperature. Unfolding curves were generated by plotting the fluorescence intensity ratio $I_{325 \text{ nm}}/I_{375 \text{ nm}}$ as a function of GuHCl concentration. Figure 1A shows representative unfolding curves at -5, 10, 30 and 50°C. The curves reveal a temperature dependence with a greater concentration of GuHCl required to unfold the protein near ambient temperature (10 and 30°C) and less GuHCl required to unfold the protein at -5 and 50°C. Figure 1B shows the parabolic temperature dependence of the $I_{325 \text{ nm}}/I_{375 \text{ nm}}$ ratio at three GuHCl concentrations in the unfolding transition (2, 2.2 and 2.4 M).

Since a clear transition at intermediate GuHCl concentrations was observed in the unfolding curves at most temperatures, a three-state model, defined by a native state (N), an intermediate state (I) and an unfolded state (U), was used to fit the fluorescence data obtained at all temperatures studied. The non-linear least squares fit of the data at each temperature and the corresponding residuals are summarized in Figure 2. The values of the parameters from the three-state fit of the mAb1/GuHCl data are given in Table 1.

**Comparison of mAb1/GuHCl denaturation data collected by fluorescence and absorbance at a single temperature (40°C).** We compared the absorbance at 292 nm in mAb1 with increasing amounts of GuHCl at 40°C to the unfolding curve obtained by fluorescence at 40°C (Fig. 3). Both of these techniques primarily monitor the tryptophan environment and probe the loss in tertiary structure. The data overlay well, supporting the analysis of the fluorescence data. We attempted to fit the three-state model...
to the absorbance data and saw large errors in the $\Delta C_p$ due to a lower signal-to-noise associated with the data. For this reason, the absorbance data was not used for further thermodynamic analysis.

Circular dichroism of mAb1 in the presence of GuHCl. In addition to fluorescence and absorbance, samples of mAb1 with GuHCl were also analyzed using far-UV CD spectroscopy. Samples were incubated with GuHCl at multiple temperatures and studied by CD to monitor the loss of secondary structure. The change in CD signal at 220 nm was used to generate GuHCl-induced unfolding curves. The unfolding curves of mAb1 with 0–7 M GuHCl at 10, 20 and 30°C are shown in Figure 4A.

Unlike the fluorescence data, the curves show a shift in the baseline at high concentrations of GuHCl. Subsequent analysis of the far-UV CD spectra of mAb1 with 7 M GuHCl revealed the formation of polyproline II (PPII) helical structure (Fig. 4B) with a positive band around 220 nm and a strong negative band that is centered between 195 nm and 210 nm.39-41 A characteristic PPII helix CD spectrum with a strong negative band centered at 210 nm was observed using a shorter path length cuvette (0.1 mm instead of 1 mm) at 1°C with 4 mg/ml protein in 6 M GuHCl, 25 mM potassium phosphate, pH 6.3 (data not shown), supporting the presence of PPII helical structure at high GuHCl concentrations. It has been reported that the CD spectra of proteins denatured in GuHCl and urea resemble the PPII conformation.42,43 Formation of the PPII helix showed a temperature dependence, with more pronounced PPII helix formation at lower temperatures (Fig. 4B).33

Due to the shift in the baseline at higher GuHCl concentrations resulting from the temperature-dependent PPII helix formation, a thermodynamic analysis of the CD data was not performed. For similar reasons, no further experiments were carried out to collect far-UV CD data on samples containing mAb2 and GuHCl. An attempt was made to follow the change in the tryptophan CD signal of mAb1 with GuHCl using near-UV CD spectroscopy. The data did not show a unidirectional change in the CD signal with increasing GuHCl concentrations, thereby preventing a thermodynamic analysis or a qualitative data comparison.

Gibbs-Helmholtz fit of $\Delta G_u$ vs. T data obtained by fluorescence. The $\Delta G$ values obtained from fitting a three-state model to the fluorescence data were added to give a net apparent $\Delta G_u$ ($\Delta G_u = \Delta G_{1u} + \Delta G_{2u}$) for mAb1 unfolding at each temperature. The $\Delta G_u$ versus temperature data are shown in Figure 5. The Gibbs-Helmholtz expression (Eq. 1) was fit to the $\Delta G_u$ versus temperature data using a reference temperature of 283 K to give a $\Delta C_p$ of 8.0 ± 1.6 kcal mol$^{-1}$ K$^{-1}$, a $\Delta H$ of -38.7 ± 21.2 kcal mol$^{-1}$ and a $\Delta S$ of -0.22 ± 0.08 kcal mol$^{-1}$ K$^{-1}$ for mAb1. The Gibbs-Helmholtz expression assumes a temperature-independent $\Delta C_p$ between the native and denatured states of a protein, yet it has been reported that the $\Delta C_p$ only remains constant in the range 20–80°C.44 Outside of this temperature range, there is a temperature dependent decrease in $\Delta C_p$.10 It is common in the literature to assume a constant $\Delta C_p$ at temperatures that lie below this range and to calculate a $T_{mCD}$ based on this assumption.13 Using the Gibbs-Helmholtz expression and the calculated values of $\Delta C_p$, $\Delta H$ and $\Delta S$, we extrapolated $\Delta G_u$ across T to determine an apparent cold denaturation temperature ($T_{mCD} = -23^\circ$C) and an apparent melting temperature ($T_m = 60^\circ$C) for mAb1 (Fig. 5). From the extrapolation, we determined a maximum apparent stability ($T_{mCD}$) of 23.7 kcal mol$^{-1}$ at 18°C. Analysis at the upper and lower bounds of $\Delta C_p$, $\Delta H$ and $\Delta S$ did not significantly affect estimation of $T_{mCD}$. The $T_{mCD}$ values obtained from extrapolating $\Delta G_u$ across T with the upper and lower bounds of $\Delta C_p$, $\Delta H$ and $\Delta S$ are -22 and -25°C, respectively.

Comparison of the thermodynamic stability of two mAbs differing only in the CDR regions. We also obtained the GuHCl-induced unfolding curves of another mAb, mAb2, with
Figure 2. Three-state fit of mAb1/GuHCl data collected from -5°C (268 K) to 50°C (323 K). The intermediate, $Y_I$, is clearly shown by the inflection in the unfolding curves. The open squares denote the raw data and the line shows the non-linear least squares fit of the data. The residuals between the experimental points and the fitted curve are shown in the graph below each unfolding curve.
acid conformation is stable. A similar observation was observed for a human IgG4 antibody incubated at pH 2.7 over 24 hours and monitored by near-UV CD.\textsuperscript{45} In addition, mAbs are typically purified using a Protein A affinity column which requires eluting the bound antibody at low pH. This temporary exposure of the protein to low pH has not posed a problem in the purification of mAbs. Finally, as discussed above, the chemical denaturation of tryptophan fluorescence at 1 and 10°C. We fit the three-state model to the mAb2/GuHCl data and found that the total $\Delta G_0$ values for mAb2 unfolding at 1 and 10°C are 13.6 kcal mol$^{-1}$ and 17.9 kcal mol$^{-1}$, respectively (Table 1). These values are lower than the $\Delta G_0$ values obtained for mAb1 unfolding at these temperatures, indicating that mAb1 is more stable than mAb2.

mAb1 and mAb2 at low pH investigated by intrinsic tryptophan fluorescence—further evidence for cold denaturation. In an effort to directly obtain evidence of cold denaturation of mAbs (in the absence of a denaturant), we assessed the susceptibility of mAb1 and mAb2 to undergo cold denaturation at low pH (2.5). It has been shown previously that acidic pH tends to destabilize the conformation of mAbs. Hence, our expectation was that at pH 2.5 the cold denaturation temperature will be raised sufficiently to allow us to directly observe cold denaturation without inducing perturbation by a chemical denaturant.

Figure 6 shows a temperature dependence for the tryptophan fluorescence of mAb1 and mAb2 in 25 mM potassium phosphate buffer, pH 2.5. Similar to the GuHCl-induced denaturation data, the mAbs exhibit a temperature of maximal stability near ambient temperature and show a temperature dependence, indicating that both mAbs have the tendency to undergo cold denaturation at subzero temperatures. As mentioned in the previous paragraph, it is known that acid induces conformational changes in antibodies and the change has been attributed to unfolding within the Fc portion of the antibody.\textsuperscript{45,46} To ensure that the change in the fluorescence signal is not due to chemical degradation of the protein, we collected near-UV CD spectra of mAb1 at low pH over the course of 24 hours and did not observe any change in the spectra (data not shown), indicating that the acid conformation is stable. A similar observation was observed for a human IgG4 antibody incubated at pH 2.7 over 24 hours and monitored by near-UV CD.\textsuperscript{45} In addition, mAbs are typically purified using a Protein A affinity column which requires eluting the bound antibody at low pH. This temporary exposure of the protein to low pH has not posed a problem in the purification of mAbs. Finally, as discussed above, the chemical denaturation

| mAb1  | 268  | 274  | 283  | 293  | 303  | 313  | 323  |
|-------|------|------|------|------|------|------|------|
| $Y_i$ | 1.8 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 | 1.0 ± 0.2 | 1.7 ± 0.1 | 1.2 ± 0.5 | 1.8 ± 0.1 |
| $Y_u$ | 0.55 ± 0.01 | 0.54 ± 0.01 | 0.54 ± 0.01 | 0.53 ± 0.01 | 0.55 ± 0.01 | 0.54 ± 0.01 | 0.57 ± 0.01 |

| $m_1$ (kcal mol$^{-1}$ M$^{-1}$) | 3.9 ± 0.5 | 2.5 ± 0.3 | 2.9 ± 0.2 | 2.2 ± 0.2 | 4.6 ± 0.8 | 2.3 ± 0.4 | 2.8 ± 0.6 |
| $m_2$ (kcal mol$^{-1}$ M$^{-1}$) | 3.5 ± 0.2 | 5.1 ± 1.0 | 6.4 ± 0.9 | 6.7 ± 2.4 | 4.0 ± 0.3 | 4.2 ± 0.6 | 3.7 ± 0.3 |
| $\Delta G_0$ (kcal mol$^{-1}$) | 6.5 ± 0.8 | 5.2 ± 0.5 | 6.3 ± 0.4 | 5.2 ± 0.4 | 8.9 ± 1.4 | 5.0 ± 0.5 | 3.5 ± 0.6 |
| $\Delta G_2$ (kcal mol$^{-1}$) | 8.6 ± 0.6 | 14.7 ± 2.8 | 18.7 ± 2.7 | 19.5 ± 7.1 | 10.4 ± 0.8 | 10.8 ± 1.7 | 7.8 ± 0.6 |
| Total $\Delta G$ (kcal mol$^{-1}$) | 15.1 | 19.9 | 25.0 | 24.7 | 19.3 | 15.8 | 11.3 |

| mAb2  | 274  | 283  |
|-------|------|------|
| $Y_i$ | 1.0 ± 0.1 | 0.81 ± 0.07 |
| $Y_u$ | 0.51 ± 0.01 | 0.52 ± 0.01 |
| $m_1$ (kcal mol$^{-1}$ M$^{-1}$) | 2.8 ± 0.2 | 2.4 ± 0.2 |
| $m_2$ (kcal mol$^{-1}$ M$^{-1}$) | 2.6 ± 0.5 | 3.9 ± 1.3 |
| $\Delta G_0$ (kcal mol$^{-1}$) | 6.0 ± 0.4 | 5.6 ± 0.4 |
| $\Delta G_2$ (kcal mol$^{-1}$) | 7.6 ± 1.6 | 12.3 ± 4.2 |
| Total $\Delta G$ (kcal mol$^{-1}$) | 13.6 | 17.9 |

The parameters were derived from a three-state fit of the fluorescence data using non-linear least squares analysis in Scientist software. The values are given with the errors obtained from the fit.
In this study, we explored the phenomenon of cold denaturation of full-length mAbs to gain insights into mechanisms that may result in instability of mAbs during cold/frozen storage. mAbs are multidomain proteins with at least six defined domains, i.e., C_{H3}, C_{H2}, C_{H1}, V_{H}, C_{L} and V_{L}, together forming a heavy chain and a light chain. It is reasonable to assume that each of these domains have intrinsic thermodynamic stabilities and thus have individual stability curves representing the dependence of the free energy of unfolding on temperature. It has been argued in the literature that the thermodynamic stability of individual domains of a multidomain protein may not translate to the thermodynamic stability of the full-length protein, and there may be an additional stability component due to interdomain interactions.

While studies to characterize the thermodynamic stability of full-length mAbs have not been reported, studies have been employed previously to determine the ΔG of single domains of antibodies and various regions in the Fab and Fc portions of IgG1 antibodies. For example, Rowe and Tanford estimated a ΔG of 5.5 kcal mol$^{-1}$ for the C_{L} and V_{L} domains of a human κ light chain derived from the human IgG1 myeloma protein Wes at pH 7.0 and 25°C in the presence of GuHCl. Goto et al. used the LEM to calculate ΔG values of 3.8 kcal mol$^{-1}$ and 4.7 kcal mol$^{-1}$ for the C_{L} and V_{L} fragments, respectively.

Our objective was primarily to understand the behavior of full-length mAbs to answer two questions; first, what is the temperature of maximal thermodynamic stability of a full-length mAb and second, where does the cold denaturation temperature of a mAb lie? It was expected that the answers to these questions would give some kind of average representing the thermodynamic stability of the individual domains with the strong likelihood that at least one domain of the total six domains would have these answers close to the observed average numbers. Therefore, the free energy values and the denaturation temperatures estimated in this study are referred to as apparent values.

We used a three-state model to fit the fluorescence data along with the linear extrapolation method to calculate net, apparent ΔG values ranging from 11.3 to 25.0 kcal mol$^{-1}$ for mAb1.

Figure 4. (A) CD signal at 220 nm at 10°C (●), 20°C (■) and 30°C (▲). All of the samples contained 0.5 mg/ml mAb1 with GuHCl in 25 mM potassium phosphate, pH 6.3 ± 0.2. (B) Far-UV CD of 0.5 mg/ml mAb1 in 7 M GuHCl, 25 mM potassium phosphate, pH 6.3 ± 0.2. Data are shown at 1°C (●), 10°C (■), 20°C (◆), 30°C (▲), 40°C (▽) and 50°C (□). The baseline shift in (A) with high concentrations of GuHCl is attributed to the temperature dependent formation of polyproline II helix with high concentrations of GuHCl.

Figure 4A shows the CD signal at 220 nm for mAb1 at different temperatures. Figure 4B shows the CD signal at different wavelengths for mAb1 in 7 M GuHCl at various temperatures.

The baseline shift in Figure 4A is attributed to the temperature dependent formation of polyproline II helix with high concentrations of GuHCl.

Figure 4B shows the CD signal at different wavelengths for mAb1 in 7 M GuHCl at various temperatures. The baseline shift in Figure 4B is attributed to the temperature dependent formation of polyproline II helix with high concentrations of GuHCl.

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We generated unfolding curves for two full-length mAbs, mAb1 and mAb2, using chemical denaturation with GuHCl and intrinsic tryptophan fluorescence to monitor unfolding. The GuHCl-induced denaturation of mAb1 and mAb2 both showed reversibility by tryptophan fluorescence following removal of GuHCl. Unfolding curves were generated from tryptophan fluorescence emission data for mAb1/GuHCl samples equilibrated at seven temperatures ranging from -5 to 50°C (Figs. 1 and 2).

The equilibration times varied, with longer equilibration times required at lower temperatures. The long equilibration times have been attributed to stable intermediates that form during the GuHCl-induced unfolding of mAbs. mAbs contain numerous disulfide bonds and there are significant noncovalent interactions between some neighboring domains. It is important to verify that mAb/GuHCl samples are equilibrated to ensure that the ΔG of the protein is not overestimated.

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temperature presumably because proteins exhibit flexibility and usually undergo conformational changes to perform their function under physiological conditions.

Extrapolation of the Gibbs-Helmholtz fit to $\Delta G_u = 0$ values yielded an apparent cold denaturation temperature ($T_{CD} = -23^\circ C$) and a melting temperature ($T_m = 60^\circ C$) for mAb1. The temperature of frozen storage for mAbs is typically in the temperature range of -20°C to -80°C.5 The predicted cold denaturation temperature for mAb1 suggests that cold denaturation may contribute to protein instability with a frozen storage temperature near -20°C. (Table 1). The range of $\Delta G_u$ values obtained by fluorescence are, in general, on the higher side of the range of $\Delta G_u$ values observed for many physiologically relevant proteins and demonstrate that mAbs are thermodynamically stable proteins, especially near ambient temperature. It should be noted that mAb1 contains the consensus frameworks $\text{V}_{H3}$ for the heavy chain and $\text{V}_{\kappa 1}$ for the light chain, similar to other known Genentech mAb IgG1 products.51,52 It has been shown that these light and heavy chain frameworks are among the most stable domains of the different framework families.53

The CD studies revealed that mAb1 exhibits a significant population of PPII helical content with high concentrations of GuHCl at low temperatures (Fig. 4). Therefore, it is evident that the GuHCl-induced denatured state acquires a different conformation at low temperatures compared to that at higher temperatures. It should be noted that PPII helical structure has been identified in the hinge region and the constant domains of native mAbs in the absence of a denaturant. NMR spectroscopy of the hinge region shows that the upper and lower hinge regions are highly flexible, while the proline rich middle region forms a more rigid polyproline II helix.54,55 An analysis of the crystal structures of immunoglobulin constant domains shows that PPII helices form interdomain links and are often located on domain surfaces.56

In our studies, the complex contributions of the PPII helical structure and other secondary structures, such as $\beta$-sheet, to the far-UV CD signal at a given wavelength and temperature prevented analyses of the CD data to obtain $\Delta G_u$ values. We believe, however, that the fluorescence data, through monitoring changes in the intrinsic tryptophan microenvironment as a result of conformational changes, provides a reasonable estimate of the apparent $\Delta G_u$ values. This stems from the fact that mAb samples at high concentrations of GuHCl showed a $\lambda_{max}$ of 355 nm at all temperatures studied (data not shown) indicating that the microenvironments of the tryptophans are independent of the presence of PPII helical structure. In other words, our data indicates that the tryptophan residues remain exposed to solvent even in the presence of PPII helical structure. Therefore, the $\Delta G_u$ values that we obtain reflect the free energy difference between the native state and the final, denatured state with the tryptophans completely exposed to solvent, irrespective of the final secondary structure. Our results also suggest that the final conformation of the denatured state for a given protein could be different for the heat-denatured state compared to the cold-denatured state. Therefore, estimation of the cold denaturation temperature and, in general, the free energy versus temperature curve from thermodynamic parameters derived from heat-induced unfolding, should be evaluated with caution since the cold-induced denatured state may exhibit different structural propensities.

With these considerations, a Gibbs-Helmholtz fit to the $\Delta G_u$ versus temperature curve (Fig. 5) yielded a temperature of maximal stability ($T_{max}$) of 18°C for mAb1, which is consistent with the temperature of maximal stability reported for mesophilic proteins. A recent compilation of studies involving 11 mesophilic proteins revealed an average $T_{max}$ of 18.1 ± 8.6°C.14 The temperature of maximal stability falls at a temperature below physiological temperature presumably because proteins exhibit flexibility and usually undergo conformational changes to perform their function under physiological conditions.

Extrapolation of the Gibbs-Helmholtz fit to $\Delta G_u = 0$ values yielded an apparent cold denaturation temperature ($T_{CD} = -23^\circ C$) and a melting temperature ($T_m = 60^\circ C$) for mAb1. The temperature of frozen storage for mAbs is typically in the temperature range of -20°C to -80°C.5 The predicted cold denaturation temperature for mAb1 suggests that cold denaturation may contribute to protein instability with a frozen storage temperature near -20°C.
Thermodynamic parameters associated with the reversible unfolding of some antibody domains have been discussed in the literature and these parameters may be used in the Gibbs-Helmholz expression to estimate the cold denaturation temperature. A fragment of the constant domain of a type \( \lambda \) immunoglobulin light chain (\( C_{\text{H}} \), residues 109–212) was predicted to have a \( \Delta G \) of 1.8 kcal mol\(^{-1}\).\(^{-1}\), a \( \Delta H \) of 1.7 kcal mol\(^{-1}\) and a \( \Delta S \) of -0.006 kcal mol\(^{-1}\) K\(^{-1}\). This information was used to estimate a \( T_{\text{CD}} \) of -8°C for the unfolding of the \( C_{\text{H}} \) domain fragment. The thermodynamic parameters associated with the thermal unfolding of an Fc fragment from the human IgG1 myeloma protein Van at pH 8.0 have also been estimated. We used this information to predict \( T_{\text{CD}} \) values of -40°C and -19°C for the \( C_{\text{H}2} \) and \( C_{\text{H}3} \) domains, respectively.\(^{49}\) These predicted \( T_{\text{CD}} \) values lie in the vicinity of the \( T_{\text{CD}} \) estimated for mAb1 (-23°C), suggesting that the \( T_{\text{CD}} \) of the entire antibody represents an average value. The theoretical analysis also shows that a domain with a lower \( T_{\text{CD}} \), such as the \( C_{\text{H}2} \) domain, may not necessarily exhibit a higher \( T_{\text{CD}} \). While the \( C_{\text{H}3} \) domain has been reported to have a higher \( T_{\text{CD}} \) (more resistant to heat-induced denaturation), it is presumably less resistant to cold-induced denaturation, when compared to the \( C_{\text{H}2} \) domain.\(^{58}\)

In addition to GuHCl-induced denaturation, we obtained direct experimental evidence of cold denaturation of mAb1, in the absence of a denaturant, at pH 2.5, a pH value which intrinsically reduces the conformational stability of the antibody. Chemical denaturation and low pH destabilization revealed a temperature of maximal stability near ambient temperature and showed that the stability of the protein decreases as the temperature is raised or lowered from ambient temperature (Figs. 5 and 6).

Although a three-state model was used to fit the GuHCl-induced unfolding curve of mAb1, we realize that there are probably multiple transitions embedded within each of the two observed transitions. Upon closer inspection of the fluorescence unfolding curves from \( n = 3 - 5 \) M GuHCl, we noticed another small transition that suggested an additional intermediate. Therefore, it is possible that we are slightly underestimating \( \Delta G_{\text{m}} \) with the three-state fit of the denaturation data. We attempted to fit a four-state model to all of the data and found that the contribution of \( \Delta G_{\text{m}} \) to the total \( \Delta G \), is small and only on the order of a few kcal per mole, whereas the errors associated with the third transition \( (Y_{123}, \Delta G_{\text{m}} \) and \( m) \) are quite high at all temperatures (data not shown). In our analysis using a four-state model, the protein stability curve was only slightly shifted upward and did not have a large impact on the predicted \( T_{\text{m}} \), \( T_{\text{CD}} \) and \( T_{\text{m}} \) (data not shown).

Our data support the idea that cold denaturation must be considered when evaluating the storage temperature of mAb products. Further work needs to be done to determine the kinetics of denaturation in the vicinity of \( T_{\text{CD}} \) to evaluate the storage conditions that should be employed for the long-term storage of mAb products. In general, it is reasonable to believe that storage temperatures of -70°C to -80°C would provide a lower risk towards cold denaturation and subsequent aggregation due to very slow kinetics at these temperatures. On the other hand, storage in the vicinity of -20°C could present a higher risk of subsequent aggregation, if cold denaturation indeed occurs. It has been shown that the \( T_{\text{m}} \) of another protein, \( \beta \)-lactoglobulin, decreases significantly as the protein concentration increases, revealing that this protein is a self-stabilizer.\(^{59}\) Hence, it would be interesting to study the \( T_{\text{CD}} \) of mAbs at higher concentrations to determine if self-stabilization is observed. MAb solutions may also contain co-solutes that might increase or decrease the stability of the protein. A previous study exploring the effect of co-solutes (i.e., sucrose, trehalose and glycerol) on the cold denaturation of phosphoglycerate kinase (PGK) revealed that PGK shows a lower cold denaturation temperature in the presence of these stabilizers.\(^{59}\) Studies are ongoing in our laboratory to explore the effect of co-solutes and increasing concentrations on the \( T_{\text{CD}} \) of mAb products. Protein destabilization and aggregation at low temperature may also result from cryoconcentration of protein and co-solutes, adding complexity to the analysis of protein samples stored at subzero temperatures.\(^{7,8}\)

Finally, the data investigating the thermodynamic stability of mAb1 and mAb2 at low temperatures reveal that these molecules have the potential to undergo cold denaturation at different temperatures. MAb1 and mAb2 are both monoclonal antibodies constructed with an IgG1 human framework with \( \kappa \) light chains and differ only in the sequence of the CDRs and in seven residues flanking the CDRs. The \( \Delta G \) values for mAb1 and mAb2 unfolding at 1 and 10°C were compared (Table 1), revealing that mAb1 is 6–7 kcal mol\(^{-1}\) more stable than mAb2 at these temperatures. The lower \( \Delta G_{\text{m}} \) values for mAb2 at low temperature suggest a depressed protein stability curve, and that mAb2 would have a higher cold denaturation temperature than mAb1. This lessened stability is attributed to contributions from the CDR regions and suggests that the stability of each mAb needs to be determined independently when evaluating the potential to undergo cold denaturation.

**Materials and Methods**

mAb1 and mAb2 are humanized monoclonal antibodies constructed with an IgG1 human framework with \( \kappa \) light chains and differ only in the sequence of the CDRs and in seven amino acids flanking the CDRs. The antibodies were produced by expression in Chinese Hamster Ovary (CHO) cells and purified at Genentech (South San Francisco, CA). The isoelectric points of mAb1 and mAb2 are 9.0 and 7.6, respectively.

**Determination of protein concentrations and mAb sample preparation.** Protein concentrations were calculated using absorption coefficients of 1.45 ml/mg cm\(^{-1}\) and 1.60 ml/mg cm\(^{-1}\) at 280 nm for mAb1 and mAb2, respectively. mAb1 and mAb2 were dissolved in 25 mM potassium phosphate buffer, pH 2.5, for the temperature-controlled tryptophan fluorescence experiments at low pH. To prepare the mAb/GuHCl solutions, a GuHCl stock solution was made by dissolving GuHCl (Sigma-Aldrich) in 25 mM potassium phosphate buffer, pH 8.2. After all of the GuHCl dissolved, the pH of the GuHCl stock was checked and found to be pH 6.2. The concentration of the GuHCl stock solution was determined by refractive index from the relationship [GuHCl] = 57.147\(\Delta n \) + 38.68\(\Delta n \^2\) - 91.6\(\Delta n \)\(^3\).\(^{32}\) mAb/GuHCl \(0–7\)
M GuHCl) solutions were prepared by adding the appropriate volume of the GuHCl stock to a solution containing protein in 25 mM potassium phosphate buffer, pH 7.2. The final pH of the mAb/GuHCl solutions was controlled within the pH range 6.3 ± 0.2. mAb1/GuHCl samples were equilibrated at several temperatures (5, 1, 10, 20, 30, 40 and 50°C) prior to conducting the temperature-controlled tryptophan fluorescence experiments. Additional mAb1/GuHCl samples were equilibrated at some of these temperatures for far-UV CD and tryptophan absorbance experiments. mAb2/GuHCl samples were equilibrated at 1 and 10°C for temperature-controlled tryptophan fluorescence experiments, and the results were compared to the data obtained for mAb1/GuHCl samples at these temperatures. Samples were equilibrated for 38 days at -5°C, 18 days at 1°C, 14 days at 10°C and 20°C, ten days at 30°C, 2.5 days at 40°C and 18 hours at 50°C. These equilibration times were determined by monitoring the fluorescence signal over time for samples equilibrated at each temperature (data not shown). Long equilibration times for GuHCl-induced unfolding of Fab and Fc fragments of mAbs have been reported previously.33-36 The samples equilibrated at -5°C were dust free and did not freeze at this temperature.

Fluorescence measurements. mAb1 and mAb2 contain 22 and 24 tryptophan residues, respectively, which are well distributed throughout the entire antibody. Intrinsic tryptophan fluorescence emission spectra of mAb solutions at low pH or in the presence of GuHCl were obtained using a Horiba Jobin Yvon Fluoromax-4 Spectrofluorometer (Edison, NJ) equipped with a temperature-controlled water bath. For the low pH studies, the samples contained 0.075 mg/ml mAb1 or mAb2 in 25 mM potassium phosphate buffer, pH 2.5 ± 0.1, and for the chemical denaturation studies all samples contained 0.075 mg/ml mAb1 or mAb2 with GuHCl (0–6 M) in 25 mM potassium phosphate buffer, pH 6.3 ± 0.2. A tryptophan emission spectrum was collected from 300 to 450 nm upon excitation at 295 nm. The excitation and emission slit widths were both 3 nm and data were collected at 0.2 nm increments with a 0.2 s integration time. Emission spectra were obtained for mAb1 and mAb2 at pH 2.5 at temperatures ranging from -5 to 40°C. The low pH sample was equilibrated in the spectrofluorometer for 30 min at 30 and 40°C, 1 hour at 1, 10 and 20°C, and 19 hours at -5°C prior to collecting the fluorescence data. Emission spectra were collected for mAb1/GuHCl samples equilibrated at -5, 1, 10, 20, 30, 40 and 50°C and mAb2/GuHCl samples equilibrated at 1 and 10°C. The temperature inside the cuvettes was checked using a thermocouple inserted in a reference solution. The samples were stirred for one minute before data collection to insure mixing of the protein and GuHCl at all temperatures and the sample chamber was purged with nitrogen at temperatures below 20°C. All spectra were collected in a 1 cm quartz cuvette and a reference spectrum of the buffer containing GuHCl was subtracted from each protein/GuHCl spectrum.

The fluorescence intensity ratio I(325 nm)/I(375 nm) was used to monitor conformational changes in the mAbs since the intensity ratio provides a precise quantitative assessment of the shift in \( \lambda_{max} \) itself. The ratio I(325 nm)/I(375 nm) was chosen because this ratio provided the largest difference between the 0 M and the 6 M GuHCl containing samples of mAbs, thereby maximizing the signal-to-noise. We also used the ratio I(316 nm)/I(355 nm) to analyze the fluorescence data from the mAb1/GuHCl samples, and we obtained similar results from the fit (data not shown) indicating that the choice of wavelengths to obtain intensity ratios did not impact the results. \( \lambda_{316 \text{ nm}} \) and \( \lambda_{355 \text{ nm}} \) correspond to the \( \lambda_{max} \) for the fully native and unfolded protein, respectively. Unfolding curves were generated by plotting the fluorescence intensity ratio I(325 nm)/I(375 nm) as a function of GuHCl.

Reversibility of mAb/GuHCl denaturation. 1 mg/ml mAb1 or mAb2 was incubated overnight at room temperature in 6 M GuHCl, 25 mM potassium phosphate, pH 6.3. This solution was gradually diluted with buffer (six dilutions over six hours) to give a final concentration of 0.1 mg/ml mAb in 0.6 M GuHCl, 25 mM potassium phosphate, pH 6.3. An intrinsic tryptophan emission spectrum was collected on the spectrofluorometer at 20°C as described above. The intensity ratio I(325 nm)/I(375 nm) was calculated from the emission spectra for both mAbs and compared with this ratio from the spectrum of the native protein. The percent reversibility was calculated based on the recovery of the I(325 nm)/I(375 nm) ratio following renaturation. A small aliquot of the of the 0.1 mg/ml mAb/0.6 M GuHCl solution was dialyzed with 25 mM potassium phosphate buffer, pH 6.3, to remove the remaining GuHCl (Slide-A-Lyzer Dialysis Cassette, 10,000 MWCO, Thermo Scientific). Following dialysis the solution was concentrated by centrifugation in an Amicon centricron (10,000 MWCO, Millipore). 0.075 mg/ml solutions of mAb1 and mAb2 were prepared and tryptophan fluorescence emission spectra were collected. The percent reversibility was calculated as described above.

Data modeling. We fit a three-state model to the fluorescence data, which involves native (N), intermediate (I) and unfolded (U) states, and calculated a net, apparent \( \Delta G \) at each temperature. The three-state model is described as follows:

\[
N \leftrightarrow I \leftrightarrow U
\]

where \( K_i \) and \( K_u \) are equilibrium constants in the presence of GuHCl. The LEM was used to relate the denaturant concentration, \([D]\), to the \( \Delta G \) for each transition.23,24 LEM assumes a linear dependence of \( \Delta G \) on denaturant concentration and gives the \( m \) value, which corresponds to the slope of \( \Delta G \) vs. \([D]\) curve. The \( m \) value is related to the change in solvent accessible surface area upon unfolding. In general, the larger the protein the bigger the change in the solvent accessible surface area and the larger the \( m \) value.37 The equilibrium constant for each transition is defined by the following expression:

\[
K_i = \exp(-(\Delta G_i^0 - m[D])/RT)
\]

where \( K_i \) represents a general equilibrium constant with an associated \( m \) value, \([D]\) is the denaturant concentration, \( R \) is the gas constant, \( T \) is the temperature, and \( \Delta G_i^0 \) represents the free
energy of unfolding in the absence of denaturant. Assuming that the fluorescence intensity ratios from the native (N), intermediate (I) and unfolded (U) states are additive, then at any denaturant concentration, the observed fluorescence intensity ratio, Y, can be written as,

$$Y = Y_N f_N + Y_I f_I + Y_U f_U$$

(4)

where $Y_N$, $Y_I$, and $Y_U$ represent the signal of each of these states and $f_N$, $f_I$, and $f_U$ represent the fraction of each of these states. The fluorescence data at each temperature were fit to the following three-state model by non-linear least squares analysis in Scientist software (v. 2.01, Micromath, St. Louis, MO):

$$Y_{obt} = \frac{Y_N (Y_I K_I + Y_U K_U)}{1 + K_I + (K_N K_U)}$$

(5)

where,

$$K_1 = \exp\left(-\frac{(\Delta G_1^0 - m(D))}{RT}\right)$$

(6)

$$K_2 = \exp\left(-\frac{(\Delta G_2^0 - m(D))}{RT}\right)$$

(7)

Y is the dependent variable and the denaturant concentration, [D], is the independent variable. The gas constant, R, is 0.001987 kcal mol$^{-1}$ K$^{-1}$. $Y_N$ was fixed in the three-state model leaving the following parameters as unknowns: $\Delta G_1^0$, $\Delta G_2^0$, $m_1$, $m_2$, $Y_I$, and $Y_U$. The following constraints were used for the parameters: $m_1$ (0–10 kcal mol$^{-1}$ M$^{-1}$) and $\Delta G^0$ (0–30 kcal mol$^{-1}$). An apparent, net $\Delta G^0$ was obtained by adding up the AG values from the fitting analysis, $\Delta G = \Delta G_1^0 + \Delta G_2^0$. $Y_U$ was floated in the fitting analysis because this approach provided a better fit at higher GuHCl concentrations than fixing this parameter to the final signal at 6 M GuHCl.

**Absorbance measurements.** Absorbance spectra were collected for mAb1/GuHCl solutions at a single temperature (40°C) using an Agilent 8453 diode-array UV-Visible spectrophotometer (Palo Alto, CA). All samples contained 0.5 mg/ml mAb1 with GuHCl (0–6 M) in 25 mM potassium phosphate buffer, pH 6.3 ± 0.2. All spectra were collected in a 1 cm quartz cuvette. The absorbance at 292 nm was determined for each sample and an unfolding curve was generated. Correction for scattering was performed when the absorbance at 320 nm was greater than 1% of the absorbance value at 292 nm.\(^{38}\)

**Far-UV CD measurements.** CD spectra were collected using a Jasco J-815 CD Spectrometer (Easton, MD) equipped with a temperature-controlled holder. All samples contained 0.5 mg/ml mAb1 with GuHCl (0–7 M) in 25 mM potassium phosphate buffer, pH 6.3 ± 0.2. CD measurements were collected at intervals of 0.5 nm from 222 to 215 nm or 240 to 210 nm with an 8 s response time and a 1 nm bandwidth. All spectra were collected in a 1 mm quartz cuvette and the temperature was controlled using a thermostocouple inserted in a reference cell. Two scans were collected for the mAb/GuHCl solutions and the corresponding buffer containing GuHCl. The data were averaged and the buffer data were subtracted.

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