

# **Thermal and Chemical Unfolding of a recombinant monoclonal IgG1 antibody: Application of the Multi-State Zimm-Bragg Theory**

P. Garidel\*, A. Eiperle, M. Blech, and J. Seelig\*

## **Statement of significance**

First quantitative thermodynamic study of an antibody with differential scanning calorimetry and analyzed with the multi-state Zimm-Bragg theory.

## Abbreviations

mAb	monoclonal antibody
Gnd	guanidineHCl
DSC	differential scanning calorimetry
ITC	isothermal titration calorimetry
HPSEC	High Pressure Size Exclusion Chromatography
N	native protein
U	unfolded protein
n	native amino acid residue
u	unfolded amino acid residue
$C_p$	heat capacity of the unfolding process
$T_0$	temperature of the $C_p$ maximum (midpoint temperature of N→U transition)
$\Delta H_{cal}$	calorimetric determined unfolding enthalpy of the transition
$\Delta H_{vH}$	van't Hoff enthalpy
h	unfolding enthalpy per amino acid residue
$\sigma$	cooperativity parameter
q(T)	equilibrium parameter for n → u transition
v	number of amino acid residues participating in the unfolding reaction
$g_{nu}$	free energy of the n → u transition of a single residue

## Abstract

The thermal unfolding of a recombinant monoclonal antibody IgG1 (mAb) was measured with differential scanning calorimetry (DSC). The DSC thermograms reveal a pre-transition at 72 °C with an unfolding enthalpy of  $\Delta H_{cal} \sim 200\text{-}300$  kcal/mol and a main transition at 85 °C with an enthalpy of  $\sim 900 - 1000$  kcal/mol. In contrast to single-domain molecules, mAb unfolding is a complex reaction that is analysed with the multi-state Zimm-Bragg theory. For the investigated mAb, unfolding is characterised by a cooperativity parameter  $\sigma \sim 10^{-4}$  and a Gibbs free energy of unfolding of  $g_{nu} \sim 100$  cal/mol per amino acid. The enthalpy of unfolding provides the number of amino acid residues  $v$  participating in the unfolding reaction. On average,  $v \sim 220 \pm 50$  amino acids are involved in the pre-transition and  $v \sim 850 \pm 30$  in the main transition, accounting for  $\sim 90\%$  of all amino acids. Thermal unfolding was further studied in the presence of guanidineHCl. The chemical denaturant reduces the unfolding enthalpy  $\Delta H_{cal}$  and lowers the midpoint temperature  $T_0$ . Both parameters depend linearly on the concentration of denaturant. The guanidineHCl concentrations needed to unfold mAb at 25 °C are predicted to be 2-3 M for the pre-transition and 5-7 M for the main transition, varying with pH. GuanidineHCl binds to mAb with an exothermic binding enthalpy, which partially compensates the endothermic mAb unfolding enthalpy. The number of guanidineHCL molecules bound upon unfolding is deduced from the DSC thermograms. The bound guanidineHCl-to-unfolded amino acid ratio is 0.79 for the pre-transition and 0.55 for the main transition. The pre-transition binds more denaturant molecules and is more easily destabilised than the main transition.

Overall, the current study shows the strength of the Zimm-Bragg model for the quantitative description of unfolding events of large, therapeutic proteins, such as a monoclonal antibody.

## Introduction

The standard unfolding model for small proteins (e.g. single-domain molecules) is the 2-state model. Only two types of molecules exist in solution, the native protein (N) and its structural unfolded conformation (U) (all-or-none model) (1). However, "peptides that form helices in solution do not show a simple 2-state equilibrium between a fully folded and a fully unfolded structure. Instead they form a complex mixture of all helix, all coil or, most frequently central helices with frayed coil ends" (2). A more realistic model is the multi-state Zimm-Bragg theory, originally developed for the temperature-induced coil-to- $\alpha$ -helix transition (3; 4). It has been applied successfully to describe the cooperative thermal unfolding of a variety of proteins (5). In fact, the Zimm-Bragg theory provides a perfect quantitative description of the thermal unfolding of apolipoprotein A-1, a protein with a high  $\alpha$ -helix content (~50%) (6; 7) as well as for a large number of other globular proteins (5). In this study, the theory is extended to the unfolding of a large multi-domain protein, a monoclonal antibody of molecular weight 143 kDa where the 2-state model fails completely. The thermal unfolding of a monoclonal antibody mAb was investigated with differential scanning calorimetry (DSC) in buffer and in the presence of the chemical denaturant guanidineHCl. GuanidineHCl is one of the most commonly used chemicals to induce protein unfolding. Increasing the concentration of denaturant shifts the folding equilibrium towards the unfolded state. The molecular mechanism of chemical denaturation is still discussed controversially (8). One theory postulates an indirect mechanism by which chemical denaturants change the water structure and thereby reduce the magnitude of the hydrophobic effect. The alternative view is a direct interaction of the denaturant with the protein (9; 10). Strong support for this mechanism comes from isothermal titration calorimetry (ITC) which provides evidence for an exothermic binding reaction of guanidineHCl with proteins (11). Molecular dynamics simulations (12) and X-ray studies (13) also support a direct interaction mechanism.

In the present study, we used DSC to investigate the stability of mAb as a function of temperature, solvent pH, and guanidineHCl concentration. The thermograms were analysed with the multi-state Zimm-Bragg theory. The investigated mAb exhibits two transitions, a first transition denoted as pre-transition and a second transition at higher temperature connected to a higher transition enthalpy denoted as main transition. These transitions are characterised by their enthalpy, midpoint transition temperature, and molar heat capacity. The transitions result from the denaturation of specific domains of the monoclonal antibody. For the investigated IgG1, the first transition is probably related to the reversible transition of C<sub>H2</sub> domains whereas the large, irreversible transition at high temperature is tentatively assigned to Fab and C<sub>H3</sub> domains (14; 15).

GuanidineHCl was added up to a concentration of 2.5 M. The chemical denaturant destabilized the antibody, decreasing midpoint temperatures  $T_0$  and unfolding enthalpy  $\Delta H_{cal}$ . The molecular mechanism behind destabilization is deduced from the unfolding enthalpy.

## Materials and Methods

### *mAb sample preparation*

The humanized recombinant monoclonal antibody (mAb) of IgG1 isotype was produced by mammalian cell culture technology and purified accordingly (16; 17). The concentration of the IgG1 sample solutions were determined by UV-measurement at 280 nm using an extinction coefficient of 1.32 for a 1 mg/ml solution (path length  $d = 1$  cm).

Purity was determined by size exclusion chromatography. The monomer content as measured by HPSEC was >99% (18).

The pH of the sample was varied by titrating HCl, respective NaOH, to obtain the target pH value as described in the text. GuanidineHCl was added to the protein sample to generate a concentration range from 0 to 2.5 M. If necessary, the pH was readjusted after the addition of guanidineHCl.

### *Analysis of mAb differential scanning calorimetry (DSC) thermograms*

Protein concentrations were typically 3 mg/mL corresponding to ~20  $\mu$ M. Starting at 5 °C the thermal unfolding of mAb was measured by increasing the temperature to 95 °C at a heating scan rate of 1 K/min. DSC experiments were performed with a VP-DSC instrument (Microcal, Northampton, MA). Protein solutions were degassed, and the reference cell was filled with buffer. The cell volume was 0.51161 mL. Several authors have reported thermal unfolding of monoclonal antibodies with DSC, focusing on the midpoint temperature  $T_0$ (19; 20). However, no data on the enthalpy of unfolding or on the effect of chemical denaturant are yet available. To the best of our knowledge, the present study is the first where thermal unfolding of an antibody is combined with chemical denaturation and analysed with respect to heat capacity and enthalpy.

In a DSC scan unfolding appears as an endothermic event that can be approximated as a Gaussian distribution curve. The temperature of the peak maximum is the midpoint temperature  $T_0$  and the area under the peak is the enthalpy change  $\Delta H_{\text{cal}}$  of unfolding.

During the unfolding process, the heat capacity  $C_p$  of a protein goes through a maximum at the midpoint of the conformational transition. In addition, the post-transitional heat capacity is larger than that of the native protein by  $\Delta C_p$ .

The calorimetric unfolding enthalpy  $\Delta H_{\text{cal}}$  is thus composed of the conformational enthalpy proper,  $\Delta H_{\text{NU}}^0$  (often called van't Hoff enthalpy  $\Delta H_{\text{vH}}$ ), and the enthalpy increase  $\Delta H_{\Delta C_p}^0$ , caused by the increased molar heat capacity  $\Delta C_p^0$  of the unfolded protein.

$$\Delta H_{\text{cal}} = \Delta H_{\text{NU}}^0 + \Delta H_{\Delta C_p}^0 \quad (1)$$

Antibodies exhibit a manifold of thermogram (19; 20). Due to the fact that monoclonal antibodies are complex, multi-domain protein, mAb unfolding is characterised by several

independent unfolding domains (e.g. C<sub>H2</sub>, C<sub>H3</sub>, Fab). Proteins that contain multiple domains with different inherent stability require multiple Gaussians for empirical fitting of the thermograms. Protein denaturation is highly cooperative with many intermediates. For proteins/domains of 10-20 kDa size a simple 2-state model (all-or-none folding) is used as an approximation. When applied to antibodies (size ~150 kDa) a single 2-state model leads to unacceptable results. Hence, the superposition of several 2-state models could be employed to generate an optimal fit. Empirical scaling factors must be used in addition and little insight into the molecular events is gained. In the present analysis we use a quantitative model based on the Zimm-Bragg theory (5). The model allows a truly cooperative analysis of antibody unfolding with deconvolution of individual domains.

It is common for therapeutic antibodies to aggregate and precipitate after unfolding. Especially at higher protein concentrations, after the main unfolding transition is passed, the heat capacity may drop sharply and then become negative. It is then not possible to obtain a correct post-unfolding baseline and, in turn, impossible to accurately evaluate the enthalpy of unfolding. The proper choice of the DSC baseline is an essential step in the quantitative analysis of DSC scans.

## Theory

### *Thermal unfolding: Multi-state model (Zimm-Bragg theory)*

We use N and U to denote the native and the unfolded conformation of the antibody, whereas n and u refer to a single amino acid residue.

We describe protein unfolding as a multi-state equilibrium between “native (n)” and “unfolded (u)” amino acid residues/peptide units (discussed in detail in a recent review(5)). A quantitative analysis is possible with the Zimm-Bragg theory (3; 4; 21). The essential



parameters are the protein cooperativity  $\sigma$  and the equilibrium parameter  $q(T)$  of the native(n)  $\rightarrow$  unfolded (u) transition of a single amino acid residue:

$$q(T) = e^{\frac{h}{R} \left( \frac{1}{T} - \frac{1}{T_{\infty}} \right)} \quad (2)$$

The enthalpy  $h$  of the  $n \rightarrow u$  unfolding reaction is endothermic and is about 1.1 kcal/mol.  $h$  is an average value, comprising van-der-Waals interactions, electrostatic interactions and hydrogen bond formation (22; 23; 5).

The cooperativity parameter  $\sigma$  determines the steepness of the unfolding transition. A small  $\sigma$  corresponds to a high cooperativity. In the present study, the reference temperature  $T_{\infty}$  is identical with the midpoint temperature  $T_0$ .

The change in Gibbs free energy per amino acid for a temperature-induced unfolding in the interval  $T_{ini} \leq T_0 \leq T_{end}$  is:

$$g_{nu} = -RT_{end} \ln q(T_{end}) + RT_{ini} \ln q(T_{ini}) = h \frac{\Delta T}{T_{\infty}} \simeq h \frac{\Delta T}{T_0} \quad (3)$$

The free energy  $g_{nu}$  depends on the unfolding enthalpy  $h$ , the midpoint temperature  $T_0$ , and the width of the unfolding transition  $\Delta T = T_{ini} - T_{end}$ .

The central building block of the Zimm-Bragg theory is the partition function  $Z(T) = Z(\sigma, q(T))$ , which determines the statistical and thermodynamic properties of protein unfolding.  $Z(T)$  can be calculated with a matrix method (21):

$$Z(T) = (1 \quad 0) \begin{pmatrix} 1 & \sigma q(T) \\ 1 & q(T) \end{pmatrix}^v \begin{pmatrix} 1 \\ 1 \end{pmatrix} \quad (4)$$

$v$  is the number of amino acids involved in the unfolding reaction.  $q(T)$  is given by eq. (2). The fraction of native protein  $N$ , defined as  $\Theta_N = \Sigma n / v$ , is:

$$\Theta_N(T) = \frac{q(T)}{v} \frac{d(\ln Z(T))}{dT} \left( \frac{dq(T)}{dT} \right)^{-1} \quad (5)$$

### *Differential scanning calorimetry (DSC)*

The transition from native protein (N) to the unfolded protein (U) is associated with an endothermic enthalpy  $\Delta H_{NU}(T)$  with the temperature-dependence:

$$\Delta H_{NU}(T) = \Delta H_{NU}^0 + \Delta C_p^0(T - T_0) \quad (6)$$

$\Delta H_{NU}^0$  is the conformational enthalpy whereas the second term defines the contribution of the heat capacity increase  $\Delta C_p^0$ . In the thermal unfolding experiment  $\Delta H_{NU}(T)$  is convoluted with the extent of protein unfolding,  $\Theta_U(T) = 1 - \Theta_N(T)$ :

$$H_{NU}(T) = \Delta H_{NU}(T) \Theta_U(T) \quad (7)$$

Differential scanning calorimetry measures the heat capacity:

$$C_{p,NU}(T) = \frac{dH_{NU}(T)}{dT} = \Delta H_{NU}^0 \frac{d\Theta_U(T)}{dT} + \Delta C_p^0 \Theta_U(T) \quad (8)$$

The enthalpy and entropy of unfolding are thus given by

$$\Delta H_{cal} = \int_{T_{ini}}^{T_{end}} C_{p,NU}(T) dT \quad (9)$$

$$\Delta S_{cal} = \int_{T_{ini}}^{T_{end}} \frac{C_{p,NU}(T)}{T} dT \quad (10)$$

The contribution of the  $\Delta C_p^0$  term is

$$\Delta H_{\Delta C_p^0}^0 = \int_{T_{ini}}^{T_{end}} \Delta C_p^0 \Theta_U(T) dT \quad (11)$$

$\alpha$ -helix and  $\beta$ -sheet structures are usually assumed to require specific hydrogen bonds. Experimental studies on short alanine-based peptides contradict this classical view(24) as do free energy calculations using the CHARMM potential function.(22; 23) Apparently, hydrogen bonds contribute little to  $\alpha$ -helix/ $\beta$ -sheet stability since the major driving force favoring structure formation are enhanced van-der-Waals interactions and the hydrophobic effect.(22) Protein unfolding can thus be characterised by an *average* enthalpy  $h$  of approx. 1.1 kcal/mol per amino acid, independent of the specific protein conformation (5).

## Results

### *Thermal unfolding (DSC) of mAb in guanidineHCl solution*

Differential scanning calorimetry is the gold standard for thermodynamic analysis of protein unfolding, because thermodynamic data are directly obtained from the experiment. DSC measures the heat capacity  $C_p(T)$  as a function of temperature and, by integration, the unfolding enthalpy  $H_{NU}(T)$ . The present mAb unfolding experiments in guanidineHCl solution were performed at pH 4.0, 6.2 and 8.0. Figure 1 shows the DSC scan of mAb in 1.0 M guanidineHCl at pH 6.2. The thermogram displays a low-temperature pre-transition and a high-temperature main transition, the general pattern of the present mAb unfolding experiments. Pre- and main transition are each characterised by a midpoint temperature  $T_0$  and an unfolding enthalpy  $\Delta H_{cal}$ . The number of amino acids involved in unfolding is  $\nu \approx \Delta H_{cal} / h$ . The averages of all measurements are  $\nu = 220 \pm 50$  for the pre-transition and  $850 \pm 30$  for the main transition.

The multi-state Zimm-Bragg theory provides an almost perfect simulation of the DSC thermogram (Figure1 smooth red line) resulting from the superposition of pre- and main transition. The heat capacity of the unfolded protein is  $\Delta C_p^0$  larger than that of the native mAb. Similar effects are well documented for thermograms of small proteins (25; 26).  $\Delta C_p^0$  is caused

by a restructuring of solvent molecules (25). The data in reference (25) suggest a linear relationship

$$\Delta C_p^0 (\text{kcal} / \text{molK}) = 18.6v - 300 \quad (12)$$

where  $v$  is the number of amino acid residues involved in unfolding. The heat capacity changes in Figure 1 are  $\Delta C_p^0 = 5.0 \text{ kcal/mol}\cdot\text{K}$  for the pre-transition and  $12.4 \text{ kcal/mol}\cdot\text{K}$  for the main transition. Using equation (12) the numbers of amino acid residues are estimated as  $v \sim 280$  for the pre-transition and 680 for the main transition, in broad agreement with the results derived from  $\Delta H_{\text{cal}}$  with  $v \sim 290$  and 890, respectively. Similar increases in the molar heat capacity of antibodies can be found in published DSC thermograms (e.g. (19; 27)).

Unfortunately, most DSC studies ignore the  $\Delta C_p^0$  effect. The change in heat capacity between native and unfolded protein is eliminated by applying a sigmoid baseline. This choice of baseline results in a reduced unfolding enthalpy (e.g. (28)). The enthalpy of this truncated heat capacity peak is usually considered to represent the conformational enthalpy proper (also called “van’t Hoff enthalpy” in the 2-state model). However, “it is clear that in considering the energetic characteristics of protein unfolding one has to take into account all energy which is accumulated upon heating and not only the very substantial heat effect associated with gross conformational transitions, that is, all the excess heat effects must be integrated” (26).

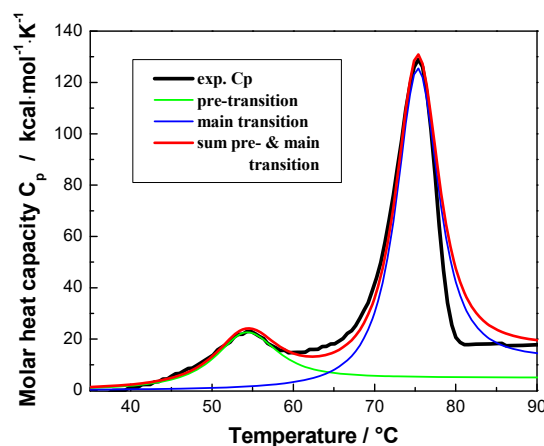


Figure 1. Thermal unfolding of mAb in 1.0 M guanidineHCl at pH 6.2, measured with differential scanning calorimetry (DSC). Molar heat capacity  $C_p(T)$  as a function of temperature. Black noisy line: experimental result. Smooth lines: simulations with the multi-state Zimm-Bragg theory (green: pre-transition; blue: main transition; red: sum of pre- and main transition).

Pre-transition parameters:  $T_0 = 54$  °C,  $\Delta H_{cal} = 322$  kcal/mol ( $\nu = \Delta H_{cal} / h = 293$ );  $\Delta C_p = 5.02$  kcal/mol·K,  $\sigma = 1.5 \times 10^{-4}$ .

Main transition parameters:  $T_0 = 75.4$  °C,  $\Delta H_{cal} = 976$  kcal/mol ( $\nu = 887$ );  $\Delta C_p = 12.42$  kcal/mol·K,  $\sigma = 7 \times 10^{-5}$ .

In some mAb experiments, particularly at pH 4.0 and low guanidineHCl concentrations, the heat capacity drops sharply and becomes negative after the main unfolding transition. This can be explained by the formation of aggregates and perhaps precipitation after denaturation.

#### Midpoint temperature $T_0$ as a function of the guanidineHCl concentration

The midpoint of the unfolding transitions  $T_0$ , defined by the  $C_p$  maximum, shifts linearly towards lower temperatures with increasing denaturant concentration (Figure 2).

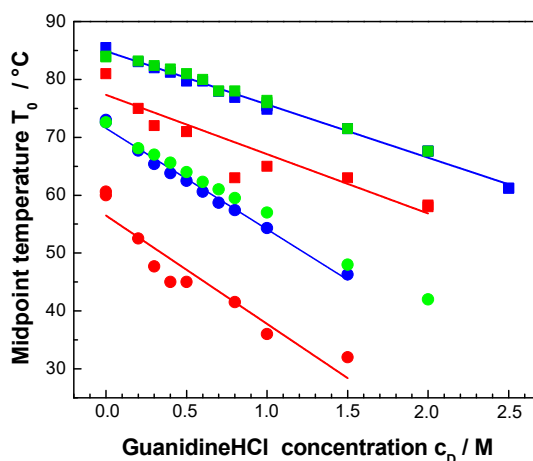


Figure 2. Midpoint temperature  $T_0$  as a function of denaturant concentration  $c_D$ . ■, ■, ■ Main transitions at pH 4.0, 6.2 and 8.0, respectively. ●, ●, ● Pre-transitions at pH 4.0, 6.2, and 8.0, respectively.

DSC thermograms at pH 6.2 and 8.0 show almost identical transition temperatures at a given guanidineHCl concentration. At pH 4.0, the antibody is destabilized. The unfolding temperatures of pre- and main transitions are reduced by 15 °C and 7 °C, respectively.

Linear regression analysis of the data shown in Figure 2 yields for the pre-transition:

$$\text{pH 4.0} \quad T_0 (\text{°C}) = -18.7 c_D (\text{M}) + 56.5 \quad (13a)$$

$$\text{pH 6.2} \quad T_0 (\text{°C}) = -17.5 c_D (\text{M}) + 71.6 \quad (13b)$$

$$\text{pH 8.0} \quad T_0 (\text{°C}) = -15.4 c_D (\text{M}) + 71.9 \quad (13c)$$

The guanidineHCL concentrations for mAb denaturation at 25 °C are predicted as 1.7 M (pH 4.0), 2.7 M (pH 6.2), and 3.0 M (pH 8.0).

The results for the main transition are:

$$\text{pH 4.0} \quad T_0 (\text{°C}) = -10.3 c_D (\text{M}) + 77.3 \quad (14a)$$

$$\text{pH 6.2} \quad T_0 (\text{°C}) = -9.21 c_D (\text{M}) + 84.9 \quad (14b)$$

$$\text{pH 8.0} \quad T_0 (\text{°C}) = -8.69 c_D (\text{M}) + 84.7 \quad (14c)$$

The guanidineHCL concentrations for denaturation of the mAb main transition at 25 °C are predicted as 5.1 M (pH 4.0), 6.5 M (pH 6.2), and 6.9 M (pH 8.0). The maximum solubility of guanidineHCL in water at room temperature is however only ~ 6 M.

The pre-transition is twice as sensitive to guanidineHCL denaturation as the main transition.

### *Unfolding enthalpy $\Delta H_{cal}$ as a function of guanidineHCL concentration*

The calorimetric unfolding enthalpy,  $\Delta H_{cal}$ , decreases with increasing denaturant concentration  $c_D$  (Figure 3).

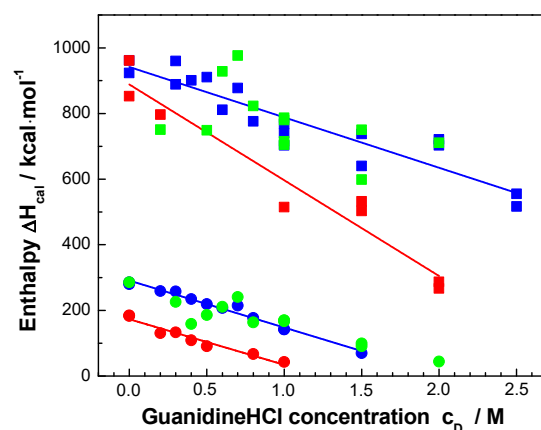


Figure 3. Unfolding enthalpy  $\Delta H_{cal}$  as a function of denaturant concentration  $c_D$ . ■, ■, ■ Main transitions at pH 4.0, 6.2 and 8.0, respectively. ●, ●, ● Pre-transitions at pH 4.0, 6.2, and 8.0, respectively.

Linear regression analysis yields for the pre-transition:

$$\text{pH 4.0} \quad \Delta H_{\text{cal}}(\text{kcal / mol}) = -139 c_{\text{D}}(\text{M}) + 173 \quad (15\text{a})$$

$$\text{pH 6.2} \quad \Delta H_{\text{cal}}(\text{kcal / mol}) = -143 c_{\text{D}}(\text{M}) + 291 \quad (15\text{b})$$

$$\text{pH 8.0} \quad \Delta H_{\text{cal}}(\text{kcal / mol}) = -107 c_{\text{D}}(\text{M}) + 261 \quad (15\text{c})$$

The number of amino acid residues involved in the unfolding transition can be estimated according to  $v = \Delta H_{\text{cal}}/h$  and are 157 (pH 4.0), 265 (pH 6.2), and 237 (pH 8.0) (Average:  $220 \pm 50$ ).

The results for the main transition are:

$$\text{pH 4.0} \quad \Delta H_{\text{cal}}(\text{kcal / mol}) = -292 c_{\text{D}}(\text{M}) + 884 \quad (16\text{a})$$

$$\text{pH 6.2} \quad \Delta H_{\text{cal}}(\text{kcal / mol}) = -153 c_{\text{D}}(\text{M}) + 941 \quad (16\text{b})$$

$$\text{pH 8.0} \quad \Delta H_{\text{cal}}(\text{kcal / mol}) = -174 c_{\text{D}}(\text{M}) + 971 \quad (16\text{c})$$

The number of amino acid residues is  $v = 808$  (pH 4.0),  $855$  (pH 6.2), and  $883$  (pH 8.0). (Average:  $849 \pm 30$ )

Antibody chains are divided into regions or domains consisting of around 110 amino acids. The pre-transition would thus represent the unfolding of about 2-3 domains, the main transition that of 8-9 domains. We tentatively assign the pre-transition to the reversible unfolding of  $C_{\text{H}2}$  domains and the main transition to Fab and  $C_{\text{H}3}$  domains.

#### *Unfolding enthalpy $\Delta H_{\text{cal}}$ as a function of midpoint temperature $T_0$*

The unfolding enthalpy  $\Delta H_{\text{cal}}$  and the midpoint temperature  $T_0$  correlate linearly with the denaturation concentration  $c_{\text{D}}$ . This predicts to a linear correlation between  $\Delta H_{\text{cal}}$  and  $T_0$ .



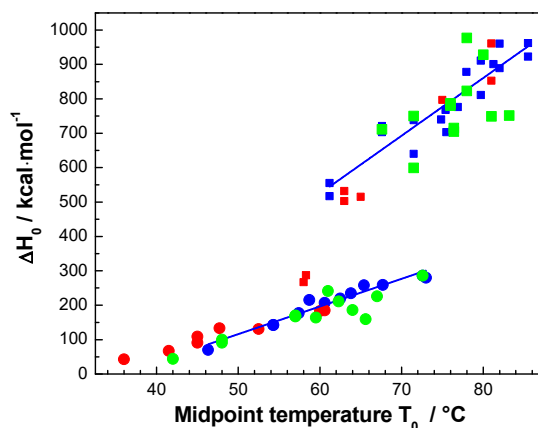


Figure 4. Unfolding enthalpy  $\Delta H_{cal}$  as a function of midpoint temperature  $T_0$ . ■, ■, ■ Main transitions at pH 4.0, 6.2 and 8.0, respectively. ●, ●, ● Pre-transitions at pH 4.0, 6.2, and 8.0, respectively.

As shown in Figure 4, the enthalpies of pre- and main transitions cluster in narrow intervals. The slopes of the  $\Delta H_{cal}$  versus  $T_0$  plots define a heat capacity  $C_{p,T_0}$ . The average values are  $C_{p,T_0} = 6.4 \pm 0.5$  kcal/mol·K for the pre-transitions and  $C_{p,T_0} = 21.0 \pm 4.2$  kcal/molK for the main transitions. The magnitude of the molar heat capacity  $C_{p,T_0}$  correlates with the number of amino acids  $v$  involved in the transition. In particular,  $C_{p,T_0}(\text{pre}) / C_{p,T_0}(\text{main}) = 0.27 \pm 0.3$  is identical within error to  $v(\text{pre}) / v(\text{main}) = 0.30$ .

### Cooperativity parameter $\sigma$

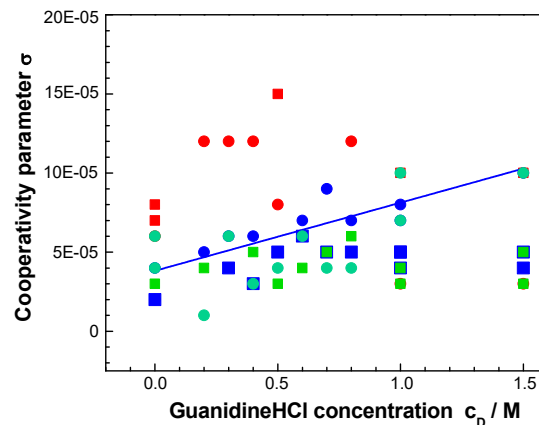


Figure 5. Cooperativity parameter  $\sigma$  as a function of guanidineHCl concentration  $c_D$ . ■, ■, ■ Main transitions at pH 4.0, 6.2 and 8.0, respectively. ●, ●, ● Pre-transitions at pH 4.0, 6.2, and 8.0, respectively.

Figure 5 summarizes the cooperativity parameters for pre-transitions and main transitions. The cooperativity parameter  $\sigma$  increases slightly with increasing denaturant concentration, that is, the cooperativity of protein unfolding decreases. Figure 5 further demonstrates that  $\sigma$  increases from pH 8.0 (green symbols) over pH 6.2 (blue symbols) to pH 4.0 (red symbols). The  $\sigma$  parameter varies between  $1.5 \times 10^{-5}$  and  $1.5 \times 10^{-4}$  and is thus 10 to 100 times larger than  $\sigma$  of small proteins such as ubiquitin or lysozyme (cf. reference(5), Table 3).

The cooperativity parameter  $\sigma$  determines the average length  $\langle l \rangle$  of a folded region according to  $\langle l \rangle \sim 1/\sqrt{\sigma}$ . A cooperativity parameter  $\sigma = 10^{-4}$  thus predicts an average length of  $\langle l \rangle = 100$  amino acid residues. Several domains of length  $\langle l \rangle$  will unfold independently and simultaneously upon heating mAb.

## Discussion

### *Analysis of the DSC thermograms with the multi-state Zimm-Bragg theory*

The 2-state model cannot fit the mAb pre- or main transition. In fact, it generally fails when applied to thermograms of large proteins. The unfolding of mAb is a multi-state transition with a large number of intermediates. In commercial instruments, an empirical curve-fitting software is applied without providing physical insight.

The multi-state Zimm-Bragg theory fits the mAb unfolding transition with 3 physical parameters: (i) the unfolding enthalpy  $h$  per amino acid residue, (ii) the cooperativity parameter  $\sigma$  and, (iii) the number  $v$  of amino acids residues involved in the transition. A small  $\sigma$  reflects a high protein cooperativity, which together with a large  $v \gg 1/\sqrt{\sigma}$  leads to a sharp DSC transition. In contrast, a large  $\sigma$  and/or a small  $v$  result in a broad transition with low cooperativity.

The Zimm-Bragg theory makes predictions about the average number  $\langle k \rangle$  and average length  $\langle l \rangle$  of segments that fold independently.(29) At the midpoint of the main transition of the thermogram shown in Figure 1 ( $T_0 = 75$  °C) the Zimm-Bragg theory predicts  $\langle k \rangle = 3.7$  segments  $\langle l \rangle \simeq 100$  amino acids. They are in dynamic equilibrium with interspaced unfolded regions.

As a general approximation the Zimm-Bragg theory predicts the average length of a folded segment as  $\langle l \rangle \sim 1/\sqrt{\sigma}$ .

The analysis of the unfolding enthalpies of small proteins led to an average unfolding enthalpy per amino acid of  $h = 0.9-1.3$  kcal/mol (5). Hydrogen bonds have an unfolding enthalpy of 0.9 - 1.1 kcal/mol depending on the environment (4; 30). The present analysis used a constant value of  $h = 1.1$  kcal/mol. As mentioned above, the number of amino acid residues participating in the unfolding reaction can be calculated as  $v = \Delta H_{cal} / h$ . The exact value of  $v$  must not be known provided  $v \gg 1/\sqrt{\sigma}$ . This condition is fulfilled for both pre- and main

transitions, with cooperativity parameters of  $1.5 \times 10^{-5} \leq \sigma \leq 1.5 \times 10^{-4}$ . Cooperativity parameters of polypeptides and small proteins range between  $10^{-3}$  for a 50 amino acid peptide and  $5 \times 10^{-7}$  for highly cooperative lysozyme(5). Most proteins of molecular weight 7-20 kDa have a cooperativity parameter of  $\sigma \sim 10^{-6}$ .

The Zimm-Bragg theory can be applied equally well to small and large proteins. It allows a comparison of molecular systems of different structure and size in terms of the parameters mentioned above.

#### *Antibody stability and unfolding temperature $T_0$*

The development of antibodies for therapeutic use has led to an increased effort in determining the factors influencing their stability. The stability of an antibody is dependent on different interactions such as van-der-Waals interactions, hydrophobic forces, hydrogen bonds, salt bridges, electrostatics, etc. In a DSC scan, these interactions are disrupted and the sum of all enthalpy changes is  $\Delta H_{cal}$ . Published DSC thermograms are, however, often analysed exclusively in terms of transition temperatures whereas  $\Delta H_{cal}$  is ignored and not evaluated (19; 20; 27). Various attempts have been made to correlate the unfolding temperature  $T_0$  with structural characteristics of the antibody, assigning individual antibody domains to specific transition temperatures  $T_0$  (19; 20; 27). The associated enthalpy and entropy are however not considered even though these parameters are the essential factors in determining  $T_0$ .

At the midpoint of the unfolding transition the Gibbs free energy is zero,  $\Delta G(T_0) = \Delta H_{cal} - T_0 \Delta S_{cal} = 0$ . It thus follows:

$$T_0 = \frac{\Delta H_{cal}}{\Delta S_{cal}} \quad (17)$$

The transition temperature  $T_0$  is an indirect parameter, that is, it is the ratio of two thermodynamic quantities, the enthalpy  $\Delta H_{cal}$  and the entropy  $\Delta S_{cal}$ . A small change in either

enthalpy or entropy can lead to a significant change in  $T_0$ . As an example, we compare the pre- and main transition of mAb unfolding at pH 6.2 in the absence of denaturant. The pre-transition is centered at 71.6 °C (eq. (13b)), the main transition at 84.9 °C (eq. (14b)). The corresponding enthalpies are  $\Delta H_{\text{cal}} = 291$  kcal/mol (eq. (15b)) and 941 kcal/mol (eq. (16b)). The entropies calculated with eq. (17) are  $\Delta S_{\text{cal}} = 0.84$  kcal/mol·K and 2.63 kcal/mol·K, respectively. Next, the entropies are normalised with the number of amino acid residues involved. The entropy per amino acid residue is  $\Delta S_{\text{cal}}/v = 3.19$  cal/mol·K for the pre-transition and 3.07 cal/mol·K for the main transition. The larger entropy of the pre-transition explains its lower melting temperature compared to the main transition if the enthalpy  $h$  is identical in both transition.

#### *Unfolding enthalpy and the number of bound guanidineHCl molecules*

GuanidineHCl decreases the unfolding enthalpy  $\Delta H_{\text{cal}}$  of both the mAb pre- and main transition (Figure 3). In parallel, the transition temperature also decreases (Figure 2). According to  $T_0 = \Delta H_{\text{cal}}/\Delta S_{\text{cal}}$  this is only possible if the entropy  $\Delta S_{\text{cal}}$  changes less than the enthalpy  $\Delta H_{\text{cal}}$ .

The decrease of  $\Delta H_{\text{cal}}$  and  $T_0$  with guanidineHCl is a general phenomenon which has been reported in DSC studies of small proteins such as lysozyme (11; 31; 32), ribonuclease (11), ubiquitin (33), and apolipoprotein A-1 (29). The present experiments are the first report for a large protein.

Guanidine is fully charged in the pH range of 4 to 8. A strong electrostatic interaction with charged peptide side chains was found (34). Recent X-ray studies of lysozyme also showed that guanidine binds to protein backbone and side chains and replaces water from the protein's first solvent shell (13). GuanidineHCl binds to proteins with an exothermic binding enthalpy  $h_{\text{Gnd}} \simeq -2.63$  kcal/mol (11) compensating, in part, the endothermic unfolding enthalpy  $\Delta H_{\text{cal}}$ . For mAb a concentration increase of  $\Delta c_{\text{D}} = 1$  M reduces the enthalpy  $\Delta H_{\text{cal}}$  of the pre-transition by  $\delta\Delta H_{\text{cal}} = -143 \pm 5$  kcal/molM (eq. (15b)). The number of bound guanidine molecules can

thus be calculated as  $\Delta N_{\text{Gnd}} = \delta\Delta H_{\text{cal}}/h_{\text{Gnd}} = (54 \pm 2)/M$ . The corresponding results for the main transition are similar with  $\delta\Delta H_{\text{cal}} = -(153 \pm 10 \text{ kcal/molM})$  (eq. (16b)) and  $\Delta N_{\text{Gnd}} = (58 \pm 5)/M$ .

Relevant for the unfolding reaction is the number of bound denaturant molecules after unfolding is complete. The midpoint concentrations  $c_{\text{D}}^0$  of the mAb pre- and main transitions are predicted as  $c_{\text{D}}^0 = 2.7 \text{ M}$  and  $6.5 \text{ M}$ , respectively (25 °C, pH 6.2). The pre-transition is completed at  $\sim 3.5 \text{ M}$  and  $N_{\text{Gnd}} = 190$  guanidines are bound. The number of amino acid residues participating in the unfolding transition is  $N_{\text{aa}} = 265$  (pH 6.2) leading to a stoichiometry of guanidineHCl/amino acid residues  $N_{\text{Gnd}}/N_{\text{aa}} = 0.72$ . The main transition is completed at  $\sim 7.3 \text{ M}$ , resulting in  $N_{\text{Gnd}} = 424$ ,  $N_{\text{aa}} = 855$  (pH 6.2), and  $N_{\text{Gnd}}/N_{\text{aa}} = 0.50$ . The pre-transition binds relatively more guanidineHCl molecules than the main transition.

The same analysis applied to published DSC data predicts for lysozyme (11)  $N_{\text{Gnd}} = 49 \pm 3$ ,  $N_{\text{aa}} = 129$ ;  $N_{\text{Gnd}}/N_{\text{aa}} = 0.38$ ; ribonuclease (11)  $N_{\text{Gnd}} = 49$ ,  $N_{\text{aa}} = 124$ ,  $N_{\text{Gnd}}/N_{\text{aa}} = 0.4$ ; ubiquitin (33)  $N_{\text{Gnd}} = 15$ ,  $N_{\text{aa}} = 76$ ,  $N_{\text{Gnd}}/N_{\text{aa}} = 0.2$ ; and apolipoprotein A-1 (29)  $N_{\text{Gnd}} = 50$ ,  $N_{\text{aa}} = 110$ ,  $N_{\text{Gnd}}/N_{\text{aa}} = 0.45$ . Average  $N_{\text{Gnd}}/N_{\text{aa}} = 0.36 \pm 0.09$  ( $0.41 \pm 0.03$  without ubiquitin).

### *Free energy of unfolding*

The free energy  $g_{\text{nu}}$  of the  $n \rightarrow u$  transition of a single residue depends on the width  $\Delta T$  of the transition and the midpoint temperature  $T_0$  (eq. (3)). The width of pre- and main transition is  $\Delta T \simeq 30 - 35^\circ\text{C}$  with about 95 % unfolded protein at the higher temperature. The free energy is thus  $g_{\text{nu}} \simeq 95 - 110 \text{ cal/mol}$ , which is in agreement with results obtained previously with small proteins such as lysozyme or ubiquitin (5).

A completely different line of experiments supports the present results. The binding of amphipathic peptides/proteins to phospholipid membranes induces  $\alpha$ -helix- or  $\beta$ -sheet structure. The Gibbs free energy change of the folding reaction was found experimentally and model-independent to be  $-140$  to  $-400 \text{ cal/mol}$  per amino acid (35-40). This result is of similar

magnitude but of opposite sign than the mAb unfolding free energy  $g_{NU}$ . The binding of amphipathic peptides to phospholipid *promotes* structure formation, and the free energy change is *negative*. In contrast, the binding of denaturants *disrupts* protein structure and the free energy change is *positive*. The two processes are of different sign but of equal magnitude.

## Concluding remarks

Thermal unfolding of a monoclonal antibody mAb displays two independent folding domains. A smaller domain (~220 amino acids) centered at 72 °C and a larger domain (~850 amino acids) with a midpoint temperature at 85 °C. Together the two domains account for ~90% of the total amino acids of mAb. Unfolding is not a simple 2-state equilibrium between a fully folded and a fully unfolded domain (all-or-none equilibrium) but a complex reaction with many intermediates. The multi-state Zimm-Bragg theory provides an excellent description of the experimental data. The analysis of the DSC thermograms yields the unfolding enthalpy, the protein cooperative parameter  $\sigma$ , the number of residues participating in the unfolding, and the Gibbs free energy for the unfolding of a single amino acid residue. The theory predicts 10-12 segments of average length  $\langle l \rangle \sim 100$ , which fold independently and are in dynamic equilibrium. The addition of guanidineHCl up to 2.5 M has little influence on the protein cooperativity, but decreases drastically the unfolding enthalpy. The binding of guanidineHCl to the protein backbone and side chains is an exothermic reaction, which compensates in part the endothermic unfolding enthalpy. The decrease in the unfolding enthalpy yields the number of guanidineHCl molecules bound to each of the two domains. The stoichiometry guanidineHCl-to-amino acids is 0.72 for the small domain and 0.50 for the large domain. The small domain ( $C_{H2}$ ) is better accessible to the denaturant and thus easier to destabilize.

## **Author Contributions**

Antibody preparation and DSC measurements were performed by P.G., A.E., and M.B.

Theoretical analysis were made by J.S.

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