Protein Thermal Stability

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Global and Local Conformation of Human IgG Antibody Variants Rationalizes Loss of Thermodynamic Stability

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Abstract: *Immunoglobulin G (IgG) monoclonal antibodies (mAbs) are a major class of medicines, with high specificity and affinity towards targets spanning many disease areas. The antibody Fc (fragment crystallizable) region is a vital component of existing antibody therapeutics, as well as many next generation biologic medicines. Thermodynamic stability is a critical property for the development of stable and effective therapeutic proteins. Herein, a combination of ion-mobility mass spectrometry (IM-MS) and hydrogen/deuterium exchange mass spectrometry (HDX-MS) approaches have been used to inform on the global and local conformation and dynamics of engineered IgG Fc variants with reduced thermodynamic stability. The changes in conformation and dynamics have been correlated with their thermodynamic stability to better understand the destabilising effect of functional IgG Fc mutations and to inform engineering of future therapeutic proteins.*

In 2013, seven of the top ten selling drugs were therapeutic proteins, each grossing more than $5.5 billion annually.[1] Five of these medicines were monoclonal antibodies and another a fusion of an antibody Fc region to the ligand-binding portion of human tumor necrosis factor (TNF) receptor. These drugs have been used to treat a number of different conditions including autoimmune disorders (Adalimumab, Etanercept, and Infliximab) and cancers (Trastuzumab and Bevacizumab). There has been much recent interest in the treatment of cancer using antibody-based approaches, such as effector-enhanced, polyspecific, and toxin-carrying antibodies. Molecular engineering efforts to modulate antibody properties, such as immune system recruitment, serum half-life extension, and cytotoxic payload attachment are typically focused in the Fc region of IgG antibodies. This versatility demonstrates why the Fc region is a vital component of many protein drug molecules in clinical development.

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In contrast to small-molecule drugs, protein therapeutics are much larger and consequently have significantly more dynamic freedom.[2] Only a subset of these conformations will possess the desired activities (such as target binding), and some will be unstable.[3] These conformational dynamics are intrinsically related to thermodynamic stability and are implicated in the colloidal stability of the protein: for instance, structures may be populated that expose aggregation-prone motifs.[4] These fluctuations may not be appreciably reversible, and can lead to a degradation of the protein drug.

The development of spatially and conformationally resolved analytical techniques is essential to better understand the relationship between protein molecular structure, dynamics, and stability. Large and flexible molecules, such as monoclonal antibodies, are often difficult to study using high-resolution techniques, such as X-ray crystallography[5] and NMR.[6] Mass spectrometry (MS) is a core technology in the study of protein therapeutics owing to its high selectivity, specificity and sensitivity.[7] Higher order structural information can be obtained by utilising emerging structural mass spectrometry techniques, including ion mobility and hydrogen/deuterium-exchange coupled to mass spectrometry (IM-MS and HDX-MS, respectively).

IM-MS is a gas-phase separation technique that informs on shape-defining parameters of the molecule, which has been previously applied to the study of IgGs.[7e,8] HDX-MS reveals the local environmental structure and dynamics of the protein by monitoring labile hydrogen atoms in a polypeptide as they exchange with those in the solvent.[9,10]

In this study, IM-MS and HDX-MS approaches have been utilized that, in combination, yield information on the global and local conformational dynamics of the antibody Fc region. Functional IgG1 mutants, which have been engineered for reduced immune system recruitment and increased serum half-life, have been studied here and the thermodynamic destabilisation exhibited when the mutations are combined has been examined.

We compared the molecular (global) and sub-molecular (local) conformational dynamics of four human IgG1 Fc variants: a variant containing a triple mutation (TM) in the C_H2 domain (L234F/L235E/P331S) that results in a decrease in affinity for Fc γ-receptor type IIIA (FcγRIIIA) and abrogates antibody-dependent cellular cytotoxicity (ADCC);[11] a YTE variant, containing a triple mutation in the C_H2 domain (M252Y/S254T/T256E) that results in an increase in neonatal Fc receptor (FcRn) affinity at endosomal pH (~6.6), leading to a 4-fold increase in serum half-life in humans;[12] a TM/YTE variant, which contains both TM and

YTE mutations; and a wild type (WT) IgG1 antibody, which contains neither TM nor YTE mutations.

We investigated the possibility that the introduction of these mutations could lead to a loss of thermodynamic stability. The thermal unfolding transitions for the C_H2 domain from 44 IgG1 antibodies were obtained using differential scanning calorimetry (DSC). The midpoint of the first unfolding transition (T_{m1}) was taken as a measure of the equilibrium thermodynamic stability of the C_H2 domain (Figure 1).[13]

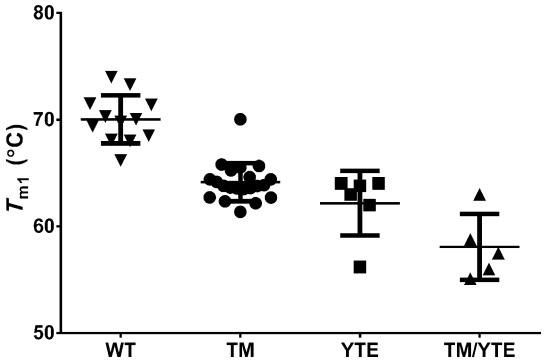

Figure 1. Thermal stability by DSC for WT, TM, YTE, and TM/YTE IgG1 antibodies. Midpoints of the first unfolding transition, assigned to the C_H2 domain, are shown. Bars denote mean ± 1 S.D.

Wild-type human C_H2 domains were found to be more stable (mean T_{m1} 70.1 ± 0.7 °C). Thermal stability was found to be reduced in both TM (mean T_{m1} 64.1 ± 0.4 °C) and YTE (mean T_{m1} 62.2 ± 1.2 °C) variants. The inclusion of all six mutations led to a further decrease in the observed thermal stability of the C_H2 domain (mean T_{m1} 58.1 ± 1.4 °C). This combined effect may indicate negative co-operativity, although a purely additive effect is within error (Supporting Information, Figure S1). If there is thermodynamic cooperativity between the two sets of mutations, then it is possible that a common site or sites within the protein may be responsible for translating the mutations into a difference in stability ($\Delta\Delta G_{D\text{-}N}$). This hypothesis formed the basis of the subsequent structural dynamics studies.

The global conformational dynamics of the IgG1 variant Fc regions were studied: shape and dynamics of the Fc region provide information on whether the mutations impact global stability through gross changes in structure and/or conformational dynamics. X-ray crystal models for the Fc region of the WT,[14] TM,[11a] and YTE[12a] variants reveal that the WT and YTE are highly similar, but that there are large C_H2 domain translation and rotation differences in the TM structure (Supporting Information, Figure S3).

The Fc variants exhibit a similar charge state distribution when analyzed by native-MS; in all cases the 11^+–14^+ are present (Supporting Information, Figure S4), consistent with previous studies on IgG1 Fc.[7e] The distribution of these charge states changes between mutants. The WT Fc has the narrowest distribution, centered on the 12^+ and 13^+ ions. The TM, YTE, and TM-YTE mutants exhibit an increasingly broader charge state distribution, with higher relative intensities of the 11^+ and 14^+ charge states. Proteins with intrinsic disorder are known to exhibit wider charge state distributions than highly structured proteins.[15] The observed widening of the charge state distribution suggests the mutations may act to destabilise the structure of the Fc domain.

IM-MS has been used to inform on the conformation adopted by each charge state (Figure 2). The 11^+ and 12^+ charges overlay, suggesting the ions have a similar molecular

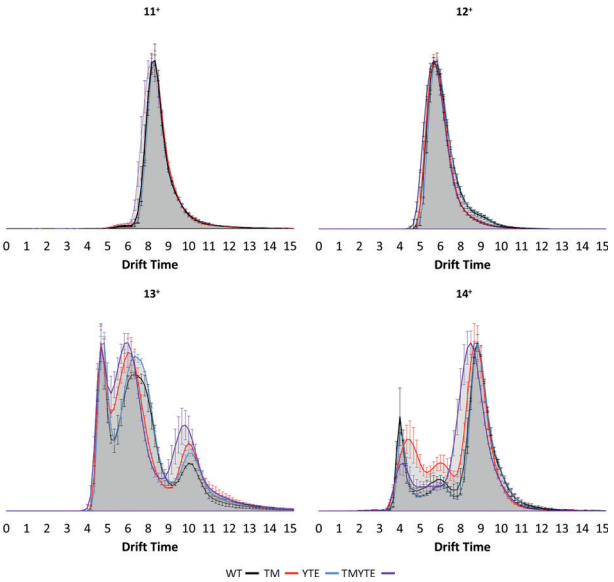

Figure 2. Global conformational ensembles for the Fc regions (54.7 kDa) of four engineered IgG1 variants. Collision cross section (CCS) variation with increasing molecular charge (11^+:14^+) were calculated from IM-MS drift time chromatograms under identical source and travelling wave conditions (see the Supporting Information for experimental details).

size at these charge states. Increasing the charge on a protein adds a gas-phase unfolding force, observed in the four Fc variants. Each Fc follows a common unfolding transition, with similar CCS values estimated for each structure (Supporting Information, Table S5). The 13^+ and 14^+ charge states show significant difference in the arrival time distributions between mutants. For the 13^+ charge the TM and YTE variants show an increase in the intensity of the extended populations when compared to the wild-type, whilst for the 14^+ charge state the WT retains a higher intensity of compact conformation relative to the TM and YTE variants. The data suggests that the introduction of the mutations may serve to destabilize the protein, lowering the energy barrier required to induce gas-phase unfolding relative to the wild type. The differences observed in the TM and YTE variants become more pronounced in the TM/YTE variant; consistent with the DSC data, the TM and YTE mutations in tandem appear to have a cooperative effect on reducing the energy required to induce Fc unfolding.

To investigate whether the thermodynamic differences were related to local conformational dynamics for these engineered IgG variants, HDX-MS experiments were carried out on isolated Fc regions. Sub-molecular localisation was

provided by analysis of individual peptides that result from proteolytic digestion of the intact protein after deuterium labelling. The coverage and redundancy of the peptide map defines the detection and resolution at which structure and conformational dynamics may be observed. The peptide maps for all four Fc variants resulted in a sequence coverage of 93.8% and a redundancy of 5.4 (Supporting Information, Figure S5). Each peptide in the map was monitored for deuterium incorporation following dilution into 95% D2O.

Three mutations in the antibody sequence in either the upper or lower C_H2 domain (TM and YTE variants, respectively) have an impact on structure and/or conformational dynamics throughout the entire Fc region (Supporting Information, Figure S6). These effects are both local to the site of mutation and also long ranging, reaching the posterior surface of the C_H3 domain, a distance of 65 Å from the first TM mutation. The mutations predominantly induce deprotection to HDX, which results from increased exposure to solvent (as seen in a less compact conformational ensemble), and/or a reduction in intramolecular hydrogen-bonding. This is consistent with the trend observed in the DSC experiments; suggesting that the lower thermodynamic stability has a significant component derived from the protein (as opposed to solvent) enthalpy and entropy.

HDX-MS permits spatial resolution of the sites within the Fc that contribute to this effect. The experiments reveal that the interior C_H2 surface has the largest net change in deuterium incorporation between the wild type and the TM/YTE variant. Four degenerate peptides were identified that span β1-α1 of the C_H2 domain (Figure 3). Each show that TM and YTE mutations increased rates of deuterium incorporation relative to wild type. This observation is increased when the mutations are present together (Supporting Information, Tables S1–4). An F-test analysis indicated that, for the TM/YTE variant, a two-state model did not adequately fit the hydrogen-exchange observed in this region ($P < 0.0002$ for all peptides). Using a three-state model, backbone amide groups exchanging for deuterium at an intermediate rate were observed, with a concomitant decrease in the number of amides undergoing exchange at the slow rate, that is, $A_{slow} \approx {}^{TM/YTE}A_{slow} + {}^{TM/YTE}A_{inter}$. In the wild type and with TM or YTE mutations alone, a two-state model was adequate to describe the hydrogen-exchange data ($P \geq 0.93$). This small change is seen for multiple ions in all four overlapping peptides, significantly outside error. This suggests that, cooperatively, the TM/YTE mutations may increase the rate or extent of conformational dynamics in this region of the protein (Figure 3).

In this study we have applied a combination of emerging structural mass spectrometry techniques to investigate global (native IM-MS) and local (HDX-MS) conformational dynamics in a major class of medicines: therapeutic monoclonal antibodies. For the first time, these techniques have been used together to rationalize the destabilising effects of mutations in the IgG1 Fc region that serve to abrogate recruitment of natural killer cells (TM), or to enhance in vivo serum half-life (YTE). Native MS analysis suggested minor conformational changes upon mutation, evidenced by changes in the charge state distribution. IM-MS suggests that the gas phase energy barrier to unfolding may have been reduced in the dual TM/YTE mutant. HDX-MS experiments reveal a number of long range disruptions to protein conformation. The largest changes in conformational dynamics were at the interior face of the C_H2 domain (β1-α1). Further statistical analysis suggests that there is a cooperative effect on the local structure and dynamics at this location, brought about by combining structurally distant TM and YTE mutations. In combination, the biophysical tools have been able to show that the mutations locally destabilize the C_H2 interior surface, without significantly disturbing the global conformation of

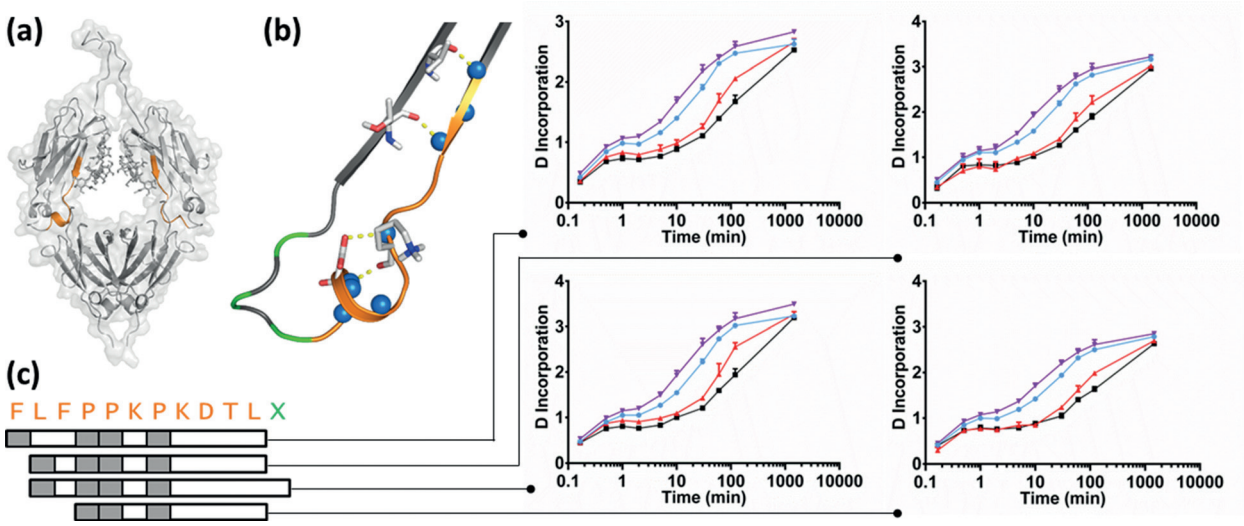

Figure 3. HDX of the IgG1 C_H2 domain interior surface. a) Four overlapping peptides span the region of largest magnitude change in HDX between WT and TM/YTE (orange). b) X-ray crystal structure of wild type Fc (3AVE) predicts that four of the amide nitrogen atoms (blue spheres) are involved in stable hydrogen bonds (sticks: acceptor amino acids), which would confer significant protection to HDX. Green: YTE mutations. c) D-label exchanged over time for the overlapping peptides for the four variants (black squares: WT; red triangles: TM; blue circles: YTE; purple triangles: TM/YTE). Unobservable amino acids (prolines and N-termini) in grey. X denotes Met in WT and TM and Tyr in YTE and TM/YTE.

the Fc region. Taken together, these findings may explain the clear thermodynamic trend among a large number of antibodies tested, and highlight a focused region of the molecule for future stability engineering work. The data presented here illustrate the power of MS-based technologies for the study of molecular structure and dynamics, notably in the field of therapeutic protein engineering and development where other, more established structural techniques have proven to be relatively intractable. We anticipate that these techniques will be used to focus mutagenesis campaigns in efforts to increase thermodynamic stability in next generation antibody and Fc-fusion protein therapeutics.

Keywords: antibodies · hydrogen exchange · immunoglobulin · ion mobility · mass spectrometry

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