Global and Local Conformation of human IgG Antibody Variants Rationalises Loss of Thermodynamic Stability

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 (Adalimumab, Infliximab, Rituximab, Bevacizumab and Trastuzumab) and another a fusion of an antibody Fc region to the ligand binding portion of human tumour necrosis factor (TNF) receptor (Etanercept). 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 novel 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.

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 (e.g. 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.

Central to the drive to better understand the relationship between molecular structure, dynamics and stability is the development of analytical techniques that are spatially and conformationally resolved. 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 due 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 coupled to mass spectrometry (IM-MS) and hydrogen/deuterium-exchange mass spectrometry (HDX-MS).

It is becoming increasingly common to use IM-MS to obtain information regarding molecular (global) conformation since the technique can provide further information on the conformational ensemble that a protein can adopt.[8] IM-MS is also amenable to intrinsically disordered proteins, and has previously been used to investigate the differences in the conformational dynamics of intact, and Fc-fragments, for IgG1 and IgG4.[7e]

HDX-MS has become established as a powerful technique for investigating the sub-molecular (local) structure and dynamics of proteins.[9] By incubating a protein in a solution containing deuterium oxide (D_2O), the resulting hydrogen/deuterium-exchange can be measured as an increase in mass (+1.006 Da per D). Each labile hydrogen atom in a polypeptide can exchange with those in solvent with a unique, observable rate constant (k_{ex}), related to the structure and dynamics of the protein's local environment.[10]

In this study, IM-MS and HDX-MS approaches have been utilised 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, 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 or 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[13](

Figure Legends

Figure 1). 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 (Figure S1 in Supporting Information). 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 ($\Delta\Delta G_{D-N}$). 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 *via* 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 (Figure S3 in Supporting Information).

From native MS experiments, the observed charge state distribution was consistent between all four variants (Figure S4 in Supporting Information). In all instances 11-14^+ charge states were observed at similar relative intensities, consistent with previous studies on native IgG1 fragments.[7e] Resolving the populations of conformations adopted by these charge-states, IMS-MS also suggests there is little global conformational difference between the mutants; the most native-like 11^+ charge state shows no change in estimated CCS values and arrival time distribution (ATD) between mutant variants, consistent with the molecules adopting a similar conformational ensemble. This is summarised in Figure 2. Native MS analysis suggests that the Fc structure of these antibodies displays no significant conformational change as a result of mutation.

Increasing the charge on a protein induces unfolding, which is illustrated in Figure 2 for the four Fc variants. Each follows a common unfolding transition, with similar CCS values estimated for each structure. The CCS estimates for each drift time were consistent between variants, however the ATD profiles show differences between variants, primarily for the 13^+ and 14^+ charge states. At these charge states there is a higher relative intensity for the more unfolded structures in the TM and YTE variants. This becomes more pronounced in the TM/YTE variant. This is consistent with a proportionally lower unfolding energy barrier being present for these molecules as they more readily adopt their gas-phase unfolded structures relative to the wild type. This observation is in agreement with the trend evident from DSC data. Native MS experiments indicate that the Fc regions of these antibodies do not show significant differences in their global conformational dynamics.

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 (Figure S5 in Supporting Information). Each peptide in the map was monitored for deuterium incorporation following dilution into 95% D_2O.

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 (**Error! Reference source not found.**6). 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 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 – 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 (red). (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 methionine in WT and TM and tyrosine in YTE and TM/YTE.). Each show that TM and YTE mutations result in increased rates of deuterium incorporation relative to wild type. This observation is increased when the mutations are present together (Tables S1-4 in Supporting Information). 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 *i.e.* $A_{slow} \approx {}^{TM/YTE}A_{slow} + {}^{TM/YTE}A_{inter}$. 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 suggests that, cooperatively, the TM/YTE mutations may destabilise the hydrogen-bonding network and/or increase the rate of structural dynamics in this region of the protein (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 (red). (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 methionine in WT and TM and tyrosine in YTE and TM/YTE.).

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 the dominant class of medicine today: therapeutic monoclonal antibodies. Together, these techniques have been used to rationalise the destabilising effect 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). No significant experimental changes to the global conformational ensemble was observed using the native IM-MS approach. There was however a suggestion that the gas phase energy barrier to unfolding may have been reduced in the dual TM/YTE mutant. HDX-MS experiments reveal significant changes in the Fc region of the antibody for all the mutants studied. A number of long range disruptions to protein conformation which were observed may have been interpreted as global conformational changes without the benefit of the IM-MS data. The largest changes in conformational dynamics were at the interior face of the C_H2 domain (β1-α1), suggesting, upon further statistical analysis, that there was 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 destabilise the C_H2 interior surface, without significantly disturbing the global conformation of 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 uninformative when used to study molecules of this size and mobility. 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.

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Figure Legends

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.

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 Supporting Information for experimental detail).

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 (red). (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 methionine in WT and TM and tyrosine in YTE and TM/YTE.

Figures:

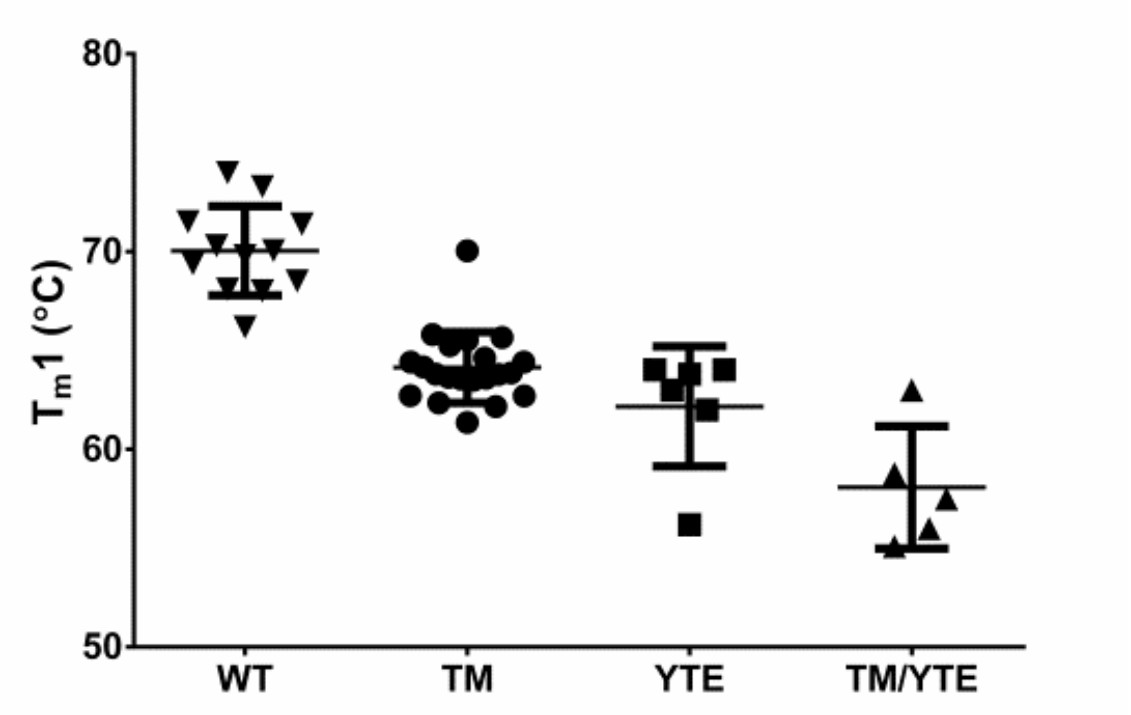

Figure 1

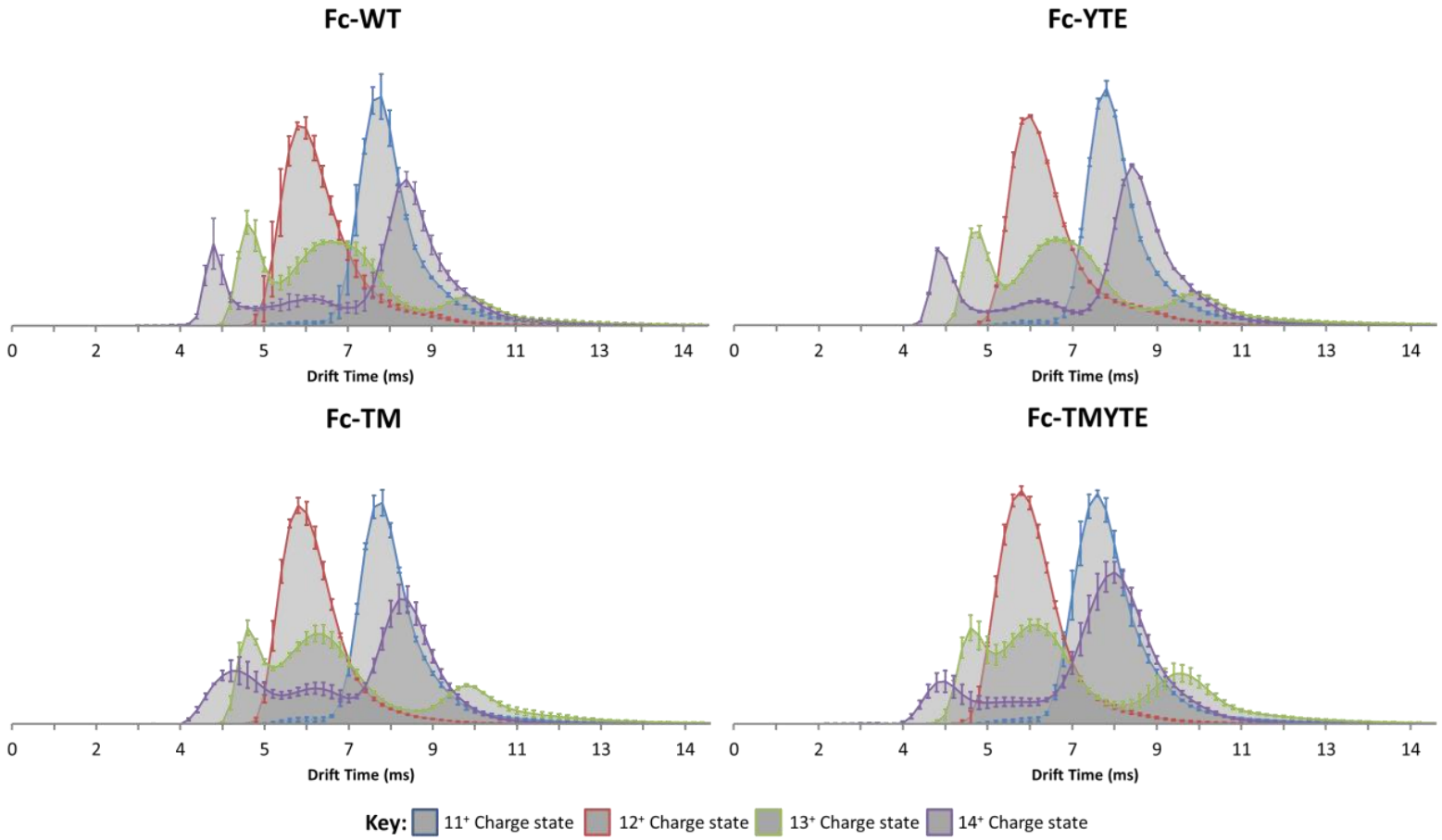
Fc-WT
Fc-YTE
Fc-TM
Fc-TMYTE
0 2 4 5 7 9 11 13 14
Drift Time (ms)
Key: 11+ Charge state 12+ Charge state 13+ Charge state 14+ Charge state

Figure 4

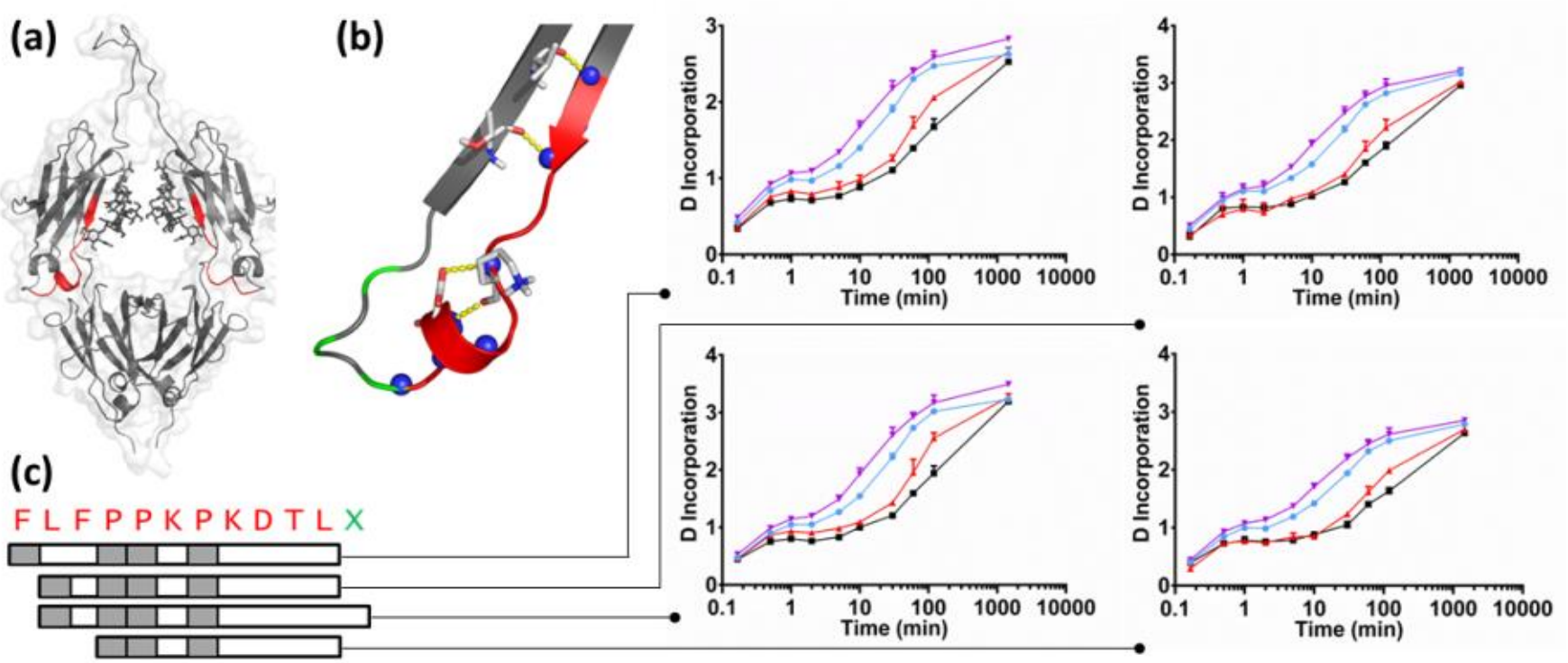
(a)
(b)
(c)
F L F P P K P K D T L X
D Incorporation
Time (min)
0.1
1
10
100
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Figure 3