Relative stabilities of IgG1 and IgG4 Fab domains: Influence of the light–heavy interchain disulfide bond architecture

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Abstract: The stability of therapeutic antibodies is a prime pharmaceutical concern. In this work we examined thermal stability differences between human IgG1 and IgG4 Fab domains containing the same variable regions using the thermofluor assay. It was found that the IgG1 Fab domain is up to 11°C more stable than the IgG4 Fab domain containing the same variable region. We investigated the cause of this difference with the aim of developing a molecule with the enhanced stability of the IgG1 Fab and the biological properties of an IgG4 Fc. We found that replacing the seven residues, which differ between IgG1 C_H1 and IgG4 C_H1 domains, while retaining the native IgG1 light-heavy interchain disulfide (L–H) bond, did not affect thermal stability. Introducing the IgG1 type L–H interchain disulfide bond (DSB) into the IgG4 Fab resulted in an increase in thermal stability to levels observed in the IgG1 Fab with the same variable region. Conversely, replacement of the IgG1 L–H interchain DSB with the IgG4 type L–H interchain DSB reduced the thermal stability. We utilized the increased stability of the IgG1 Fab and designed a hybrid antibody with an IgG1 C_H1 linked to an IgG4 Fc via an IgG1 hinge. This construct has the expected biophysical properties of both the IgG4 Fc and IgG1 Fab domains and may therefore be a pharmaceutically relevant format.

Keywords: disulfide bond; light chain; heavy chain; IgG4; IgG1; thermofluor; thermal stability

Introduction

IgG molecules are divided into four subclasses (IgG1 – IgG4), which differ in their heavy chain sequences, hinge lengths and interchain disulfide bond (DSB) patterns. They are hetero-tetramers of ~150 kDa (170 kDa for the IgG3 subclass), which are composed of a pair of identical heavy and light chain polypeptides linked by DSBs. The heavy chain contains the variable domain (V_H domain) and three constant domains, C_H1, C_H2, and C_H3 domains. The light chain consists of two types, Lambda (λ) and Kappa (K) and contains a variable (V_L) and a single constant (C_L) domain. In contrast to the constant domains, which are invariant, the variable domains display extensive sequence diversity that enables the recognition of antigen.[1,2] The four polypeptide chains are linked by DSBs in the hinge region, with two identical Fab domains (containing the variable and C_H1 domains) and the Fc segment (containing the C_H2 and C_H3 domains) forming the classical "Y" shaped molecule.[3]

Abbreviations: Ab, antibody; ADCC, antibody dependent cell cytotoxicity; CDC, cell dependant cell cytotoxicity; C_H1, heavy chain constant domain 1; C_H2, heavy chain constant domain 2; C_H3, heavy chain constant domain 3; L–H interchain DSB, light heavy interchain disulfide bond; mAb, monoclonal antibody; SEC, size exclusion chromatography.

Additional Supporting Information may be found in the online version of this article.

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Table I. *Average T_m of 22 Humanized IgG1 Fab and 22 Humanized IgG4 Fab Domains*

Isotype	Sample set	Mean fab T_m (°C)
IgG1	22	80.7 ± 5.4
IgG4	22	75.0 ± 4.0

Samples tested in PBS pH = 7.4.

The differences between the four IgG subclasses include the length of the hinge region, the number of inter hinge DSBs and the position of the cysteine residues that form the DSB between the light and heavy chains. IgG1 and IgG4 both have two inter heavy chain DSBs in the hinge region, IgG2 has four and IgG3 has 11 inter heavy chain hinge DSBs in this region.[4] IgG4, IgG3, and IgG2 antibodies share a similar arrangement for linking the heavy chain to the light chain, however it has recently been established that the IgG2 H–L chain DSB arrangement can alter over time.[5] The IgG4 C_H1 domain has a Cys at position 127 (Kabat numbering), which is used for the formation of a DSB with a Cys residue at the C-terminus of the light chain. In contrast, the IgG1 heavy chain has a Ser at position 127; the DSB to the light chain is formed via Cys 233 in the hinge. Although the sequence position of the L–H interchain DSB varies between IgG1 and IgG4, the spatial location is similar in the folded structure.[5–7]

Development of an IgG therapeutic requires a selection from the subclasses based on the required interaction with the human immune system. The IgG1 subclass is used when the required mechanism of action relies on antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). The IgG3 subclass is comparable to IgG1 in ADCC and CDC activity[3,8] but is not considered to be the most viable therapeutic option due to its relatively short half life and the complexity of the hinge region.[9–11] Both IgG2 and IgG4 antibodies demonstrate significantly weaker ADCC activity and are therefore often the therapeutic format of choice when there is no requirement for effector function engagement.[12]

IgG2 antibodies form covalent dimers *in vivo*,[13] and it has yet to be determined if there are clinical safety consequences of this.[4] Furthermore, they adopt disulfide isoforms over time[14] which may alter their antigen binding characteristics.[15] IgG4 molecules have been shown to form significant levels of non-covalently associated molecules consisting of H–L chain dimers which lack inter heavy chain DSBs and form intra heavy chain DSBs. These molecules are able re-associate to form a hybrid molecule with dual antigen specificity. The precise mechanism for this chain exchange phenomena is unclear but it is known to involve the hinge region and also motifs in the C_H3 domain.[16–18] Changing the CPSC motif in the hinge region of the IgG4 to the IgG1 sequence (CPPC) dramatically reduces the rate of half molecule formation.[19,20]

The stability of antibodies (and antibody fragments) is increasingly an important factor in the selection process of therapeutic candidates. Numerous studies have examined methods to improve thermal stability.[21–25] Most of this work has examined antibody fragments, since the variable domain is typically responsible for the Fab stability.[26] Willuda et al.[27] demonstrated that increased thermal stability translated to improved *in vivo* performance of tumor targeting antibody fragments. Chennamestty et al.[28] stabilized full length antibodies by identifying and mutating regions with a propensity for aggregation. Their results revealed a correlation between mutations that increased thermal stability and reduced aggregation; unsurprisingly the authors also noted that several mutations in the CDRs that increased stability resulted in a loss of efficacy. It would clearly be preferable to increase stability without directly interfering with the CDRs.

Our observations of the different inherent stabilities of IgG1 and IgG4 antibodies led to us to examine in greater detail the structural differences between the two isotypes. Here we report the basis for the thermal stability difference between IgG1 and IgG4 Fab domains and discuss the implications for generation of a more stable hybrid molecule.

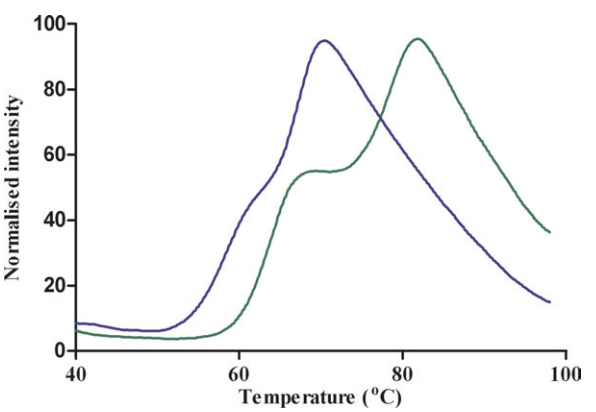

Figure 1. Thermograms of IgG1 and IgG4 polyclonal antibodies in 50 m*M* sodium acetate 125 m*M* sodium chloride pH 5.0. IgG1 thermogram (green trace): The first unfolding event is consistent with the expected T_m of the IgG1 C_H2 domain, and the second unfolding event is the Fab domain, which has a T_m of 78.9 ± 0.6°C. The IgG4 thermogram (blue trace): The first unfolding event is consistent with the expected T_m of the IgG4 C_H2 domain, and the second unfolding event is the Fab domain, which has a T_m of 67.8 ± 0.1°C.

Table II. *T_m Data of IgG1 Antibody A and IgG4 Antibody B Fab Domains and Their Fab Fragments with the Same Variable Region*

Sample	Fab domain T_m (°C)
Antibody A IgG1	77.8 ± 0.2
Fab A IgG1 Fab	77.4 ± 0.6
Antibody B IgG4	73.2 ± 0.3
Fab B IgG4	73.7 ± 0.4

Antibody B IgG4 and Fab B IgG4 tested in 50 m*M* sodium phosphate, 300 m*M* sodium chloride, 250 m*M* imidazole, pH 8. Antibody A IgG1 and Fab A IgG1 tested in PBS pH 7.4. T_m Results demonstrate IgG1 and IgG4 Fab domain thermal stability is not influenced by the presence of the lower hinge region or Fc domain.

Results

Thermal stability analysis of humanized antibodies

A thermofluor assay was used to determine the thermal stabilities of 44 humanized antibodies with unique variable regions (the sample set contained 22 IgG1 and 22 IgG4 unmatched antibodies). We observed a large range of thermal stabilities for both isotypes. The highest and lowest IgG1 Fab T_m was 93.5°C and 70.5°C respectively. The highest Fab T_m observed for the IgG4 sample set was 84.9°C and the lowest 66.3°C (Supporting Information Table S1). The average Fab T_m for the IgG1 sample set was 80.7°C the IgG4 sample set had an average Fab T_m of 75.0°C, an average stability difference of 5.7°C (Table I). Comparison of purified human IgG1 polyclonal antibodies, with IgG4 polyclonal antibody Fab domains, revealed an even greater thermal stability difference, with an observed stability gain of 11°C over the polyclonal IgG4 Fab domains (Fig. 1).

Analyses of differences between IgG1 and IgG4 antibodies

In order to rule out stabilization effects of the lower hinge and Fc domains we compared Fab fragments with full-length antibodies with the same variable regions of both IgG1 and IgG4 isotypes (Table II). Antibody A was produced as both a full length IgG1 and Fab fragment; both Fab domains had equivalent thermal stabilities (77.4 ± 0.6°C and 77.8 ± 0.2°C). Antibody B was produced as an IgG4 antibody and IgG4 Fab fragment; again both Fab domains had equivalent thermal stabilities (73.2 ± 0.3°C and 73.7 ± 0.4°C), indicating that in both IgG1 and IgG4 isotypes, the lower hinge and Fc regions do not confer any thermal stability to their respective Fab domains.

There are seven residues that differ between the IgG1 and IgG4 C_H1 domain (omitting the residues that are involved in DSB arrangements). In order to determine whether these seven residues contributed towards thermal stability, three mutants of IgG1 Fab C were generated that contained differing numbers of the IgG4 specific residues. Mutant M7 contained all 7 residue differences (K129R, G135E, G136S, Q203K, I207T, N211D, K222R), mutant M3 contained three-residue differences (K129R, G135E, G136S), and mutant M4 contained four-residue differences (Q203K, I207T, N211D, K222R). These mutant Fabs were examined in the thermofluor assay. No measurable difference in thermal stability was observed when compared to the wild type IgG1 Fab (Table III) strongly suggesting that the non L–H interchain DSB C_H1 sequence differences were not responsible for the thermal stability differences between IgG1 and IgG4 Fab domains.

Alterations to the L–H interchain DSB

To verify the thermal stability effects of L–H interchain DSB architecture several His-tagged Fab mutants were made (His-tagged Fabs were confirmed to have the same thermal stabilities as non-His tagged samples, data not shown), the IgG1 L–H interchain DSB of Fab B, was replaced with the IgG4 L–H interchain DSB arrangement by mutating Ser 127 to Cys and Cys 233 to Ser. The T_m was then determined (Table IV). The T_m of the Fab domain was reduced from 80.1 ± 0.6°C to 74.5°C ± 0.7°C, ~2°C greater than the observed T_m for the native IgG4 Fab B with the same variable region. Conversely, replacing the IgG4 Fab L–H interchain DSB with the IgG1 Fab L–H interchain DSB arrangement (Cys 127 to Ser and Gly 230 to Cys) resulted in an increase in thermal stability, to that observed for native IgG1 Fab with the same variable region

Table III. *Mutations of Residues Within the IgG1 C_H1 Domain of Antibody C to Their Counterpoint Residues in the IgG4 C_H1 Domain (residues involved DSB formation not altered)*

Format	C_H1 sequence	Fab T_m (°C)	C_H2 T_m (°C)
IgG1	PSS<u>K</u>STSG<u>G</u>T…SLGT<u>Q</u>TY<u>I</u>CNV<u>N</u>HKPSNTKVDK<u>K</u>V	85.5 ± 0.3	68.4 ± 0.4
IgG4	PCS<u>R</u>STS<u>E</u>ST…SLGT<u>K</u>TY<u>T</u>CNV<u>D</u>HKPSNTKVDK<u>R</u>V	76.4 ± 0.3	64.0 ± 0.5
M7	PSS**R**STS**E**ST…SLGT**K**TY**T**CNV**D**HKPSNTKVDK**R**V	84.9 ± 0.6	69.5 ± 0.4
M3	PSS**R**STS**E**ST…SLGTKTYTCNVDHKPSNTKVDKRV	85.2 ± 0.3	69.2 ± 0.1
M4	PSSKSTSGGT…SLGT**K**TY**T**CNV**D**HKPSNTKVDK**R**V	85.5 ± 0.3	69.1± 0.5

Underlined residues represent differences between IgG1 and IgG4 isotypes, bold residues represent mutations, Kabat numbers for mutations are: K129R, G135E, G136S, Q203K, I207T, N211D, K222R. Thermal stability data show no measurable difference between the mutants and the native IgG1 antibody, the expected T_m difference between the IgG1 and IgG4 isotype is present. Samples tested in PBS pH 7.4.

Table IV. *Thermal Stability Data of Mutations to Antibody B L–H Interchain DSB Arrangements in a Fab Fragment (residues not involved in DSB formation unaltered)*

Format	C_H1 sequence	Upper hinge	Fab T_m (°C)
IgG1	PSSKSTSGGT...SLGTQTYICNVNHKPSNTKVDKKV	(E)PKSCDKTHT	80.1 ± 0.6
IgG4	P<u>C</u>SRSTSEST...SLGTKTYTCNVDHKPSNTKVDKRV	(E)SK<u>Y</u>GPP	72.2 ± 0.5
IgG1 + IgG4 L–H DSB	P<u>C</u>SKSTSGGT...SLGTQTYICNVNHKPSNTKVDKKV	(E)PKS**S**DKTHT	74.5 ± 0.7
IgG4 + IgG1 L–H DSB	P**S**SRSTSEST...SLGTKTYTCNVDHKPSNTKVDKRV	(E)SKY**C**PP	79.3 ± 1.0

Underlined residues represent differences between IgG1 and IgG4 L–H interchain DSB formation; bold residues represent mutations. Samples tested in 50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole pH 8.0.

in this assay. Further to this analysis we removed by mutagenesis the Cys residues which form the L–H interchain DSB in IgG1 (C233S) and IgG4 (C127S) Fabs (Table V). Removal of the IgG1 L–H interchain DSB resulted in a ~7°C reduction in thermal stability. The removal of the IgG4 L–H interchain DSB reduced the thermal stability by ~2°C

In order to take advantage of the thermal stability of IgG1 Fab and the significantly weaker ADCC activity of the IgG4 Fc,[12] a hybrid format containing a IgG1 Fab linked by an IgG1 hinge to a IgG4 Fc was generated. Overlapping PCR was used to link the coding sequences of IgG1 Fab and hinge to the Fc of an IgG4. Four antibodies, with a range of thermal stabilities, were made as IgG1, hybrid, and IgG4 molecules and the results are shown in Table VI. The hybrid molecules Fab T_m was consistent with their respective IgG1 parent molecules; but both IgG1 antibody C and IgG1 antibody D displayed thermal stability increases of ~10°C over their respective IgG4 isotypes. IgG1 antibody E showed a more moderate increase in stability of ~6°C compared to the IgG4 isotype, antibody F as an IgG1 isotype showed a 2°C increase in thermal stability over the IgG4 format. We have observed in other samples (data not shown) that when an IgG1 Fab domain has a relatively low $T_{m,}$ the magnitude of the thermal stability difference between the isotypes is reduced.

Discussion

Based on an analysis of 22 IgG4 and 22 IgG1 Fab domains with unique variable regions (no matched pairs), we found that the IgG1 Fab domains were, on average 5.7°C more thermally stable than IgG4 Fab domains. Whilst it is known that the variable regions contribute to the thermal stability[26] it was surprising to find such a large difference in thermal stabilities between the isotypes given the relatively large sample set tested. The objective of this study was to identify the molecular basis for the increased thermal stability of IgG1 Fab domains over IgG4 Fab domains. To confirm the thermal stability difference between IgG1 and IgG4 antibodies, isotype pairs (with the same variable regions) were produced. We found IgG1 Fabs were more stable than IgG4 Fabs with the same variable region, with differences of up to 11°C observed; a similar thermal stability difference to that found between the human polyclonal samples.

We have demonstrated through mutagenesis and Fab and Fc fragment analysis that the higher stability of the IgG1 Fab domain is predominantly due to the L–H interchain DSB arrangement in the C_H1 domain. Replacing the IgG1 L–H interchain DSB architecture, with the IgG4 L–H interchain DSB arrangement resulted in a large reduction in the Fab T_m. Conversely, replacing the IgG4 Fab L–H interchain DSB with the IgG1 Fab L–H interchain DSB arrangement resulted in an increase in thermal stability, consistent with the thermal stability of the native IgG1 Fab (with the same variable region). Furthermore, when Cys residues involved in the formation of the L–H interchain DSB in IgG1 and IgG4 isotypes were converted to Ser residues we found that the IgG1 Fab T_m decreased, consistent with the thermal stability of the IgG4 Fab domain, an ~8°C shift in thermal stability. A small reduction in stability (~2°C), was observed in the mutated IgG4 Fab suggesting the IgG4 L–H interchain DSB offers a relatively small thermal stability contribution compared to the IgG1 L–H interchain DSB arrangement.

Table V. *Effect of Removing L–H Interchain DSB on Thermal Stability in IgG4 and IgG1 His Tagged Fabs*

Construct	Fab T_m (°C)
Fab B IgG1	80.1 ± 0.6
Fab B IgG1 no L–H DSB	72.8 ± 0.9
Fab B IgG4 Fab	72.2 ± 0.5
Fab B IgG4 no L–H DSB	69.8 ± 1.0

Samples tested in 50 m*M* sodium phosphate, 300 m*M* sodium chloride, 250 m*M* imidazole pH 8.0.

Table VI. *Thermal Stabilities of Hybrid Molecules and Parent Isotypes Tested in 50 mM Sodium Acetate 125 mM Sodium Chloride pH 5.0*

	Fab T_m (°C)			
Format	Ab C	Ab D	Ab E	Ab F
IgG1	84.0 ± 0.5	82.4 ± 0.2	74.7 ± 0.6	70.2 ± 0.3
Hybrid	83.8 ± 0.3	81.7 ± 0.2	75.9 ± 0.5	71.3 ± 0.4
IgG4	74.0 ± 0.0	71.4 ± 0.2	68.2 ± 0.2	68.5 ± 0.4

Thermal stability analyses of three reduced and alkylated IgG1 and IgG4 Fab pairs corroborated these stability losses (data not shown). The reduced and alkylated IgG1 Fab domains had a thermal stability similar to the non-reduced IgG4 Fab domain with the same variable region. Reduced and alkylated IgG4 Fab domains showed an average decrease in T_m of ~2°C. We presume that differences in the upper hinge sequences between the isotypes may account for the small T_m differences observed for IgG4 and IgG1 Fab domains lacking a L–H interchain DSB. Our results clearly demonstrated that the IgG1 DSB arrangement could confer a greater thermal stability than the IgG4 DSB arrangement. The most favorable placement of residues involved in the formation of the L–H interchain DSB in an IgG4 antibody has been investigated by others at UCB, their findings are consistent with our own observations that the IgG1 L–H interchain DSB arrangement shows greater thermal stability than the IgG4 L–H interchain DSB arrangement.[29]

Our findings suggest the potential thermal stability increase of the IgG1 L–H interchain DSB arrangement over the IgG4 L–H interchain DSB arrangement (with the same variable region) appears to be dependent on the absolute stability of the Fab. IgG1 Fabs that have thermal stabilities at some point below ~82°C exhibit reduced thermal stability differences compared to IgG4 Fab with the same variable region. IgG1 Fabs with a thermal stability above this threshold show a maximal T_m increase of ~11°C compared to the IgG4 Fab containing the same variable region. For example antibody C as an IgG1 has a Fab T_m of 84 ± 0.5°C; this variable region in an IgG4 Fab format gives a T_m of 74 ± 0.0°C. Antibody D has a Fab T_m of 82.4 ± 0.2°C; the same variable region in an IgG4 Fab format has a T_m of 71.4 ± 0.2°C. Antibody E as an IgG1 has a Fab T_m of 74.7 ± 0.6°C, whereas the IgG4 format with the same variable region has a Fab T_m of 68.2 ± 0.2°C (a difference of 6.5°C). Antibody F has a Fab T_m of 70.2 ± 0.3°C; the same v region in an IgG4 Fab format has a T_m of 68.5 ± 0.4°C, a difference of 1.7°C. This suggests that it is a combination of the L–H interchain DSB arrangement and variable region that determines the thermal stability of the Fab domain. The Fab domain unfolds co-operatively[30]; therefore the overall thermal stability of the Fab is dependent upon the stability of the variable domains as well as the constant domains. We presume that in cases where the variable domain stability is less than the constant domain, then the increased stability of the IgG1 C_H1 over the IgG4 C_H1 is not fully reflected.

Our observations differed from recent studies examining the thermal stabilities of IgG1 and IgG4 isotypes with the same variable regions.[31,32] These authors saw little difference in thermal stability of IgG1 and IgG4 Fab domains. The focus of these investigations was not on isotype differences, rather the effect of variable regions and pH on antibody stability respectively. Furthermore the sample sets were small and the IgG1 Fab T_ms relatively low, and therefore based on our observations, we would not expect their data to demonstrate the relatively large thermal stability difference that has been reported here.

How does the IgG1 L–H interchain DSB arrangement confer greater thermal stability compared to the IgG4 L–H interchain DSB? Our Fab T_m data show that both the IgG1 and IgG4 C_H1 domain can remain folded (in the context of a Fab) at temperatures greater than many of the IgG4 Fab T_ms recorded in this study as evidenced by a single unfolding transition observed for all the Fab domains tested, and that removal of the L–H interchain DSB results in a similar T_m for IgG1 and IgG4 Fab domains with a common variable region. We therefore presume that the arrangement of the L–H interchain DSB can positively (or negatively) alter the packing environment of the Fab domain. To assess the structural effect of the different L–H interchain DSB arrangements the crystal structures of a number of IgG4 and IgG1 Fab domains were examined. A search of the PDB (excluding structures that included ligands, chimerics, possessed lambda light chains or had no identifiable L–H interchain DSB) generated only four suitable IgG4 Fab domains, numerous IgG1 Fab domains were returned based on our search criteria and from them we selected four at random for comparison (no matched pairs with the same variable region were available). We note the sample set is small, and that crystal contacts may affect the findings, however when the distance between the C_H1 and C_L centre of mass were calculated, a small, but statistically significant difference between the two isotypes was observed (one-tailed *t*-test). IgG1 mean distance = 15.7 Å ±0.05, IgG4 mean distance = 16.1 Å ±0.1 (Supporting Information Table S2). We presume this difference is reflective of the different L–H interchain DSB arrangements and may therefore contribute to the altered thermal stabilities through subtle alterations in domain interfaces.

The V_H/V_L and C_H1/C_L interfaces are large, densely packed and predominately hydrophobic, but in contrast to this, the V_H/C_H and V_L/C_L interfaces are packed loosely and contain fewer hydrophobic residues.[30] We suggest the V_H/C_H and V_L/C_L interfaces are more likely to be affected by the different L–H interchain DSB arrangements of the IgG1 and IgG4 isotypes. We find that the maximal stability difference between IgG1 and IgG4 Fab domains is ~11°C this might reflect the stabilizing limit of the different interface(s) between IgG1 and IgG4 Fabs, which is further dependent on the stability of the Fv region.

In summary, we have demonstrated that the L–H interchain DSB arrangement of IgG1 antibodies confers greater thermal stability when compared to the L–H interchain DSB arrangement found in IgG4 antibodies. We have generated a hybrid molecule which demonstrates how one could take advantage of this property of the IgG1 C_H1 domain while retaining the low ADCC activity of the IgG4 Fc for certain therapeutic applications. We believe that this, or similar molecules taking advantage of the L–H interchain DSB arrangement will provide an improved therapeutic option where a neutralizing, antibody is required.

Experimental Procedures

Samples

Human polyclonal antibodies sourced from AbDSerotec (Kidlington, UK), all other antibodies produced sourced from UCB Pharma.

Molecular biology

Site-directed mutagenesis of amino acid residues and the generation of the His-tagged Fab and IgG1–IgG4 hybrid antibody formats was performed using overlap extension PCR. The His-tagged Fab format was generated by creating a contiguous coding region encoding either IgG1 or IgG4 C_H1 (Kabat numbering 114–223), followed by a partial hinge (EPKSCDKTHT for IgG1 or ESKYGPP for IgG4) and a 6x Histidine tag. The IgG1–IgG4 hybrid antibody format was generated by replacing the C_H2 (Kabat numbering 244–360) and C_H3 (Kabat numbering 361–478) coding regions of IgG1 with those of IgG4.

His-tagged Fab expression

The heavy chains and light chains of all antibody formats used are encoded on individual propriety mammalian expression vectors under the control of the HCMV-MIE promoter and SV40E-polyA termination sequences. His-tagged Fab format was expressed by transient transfection of 5×10^6 HEK293 cells with 2ug each of heavy and light chain plasmid using 293fectin, used according to the manufacturer's instructions (Invitrogen Paisley, UK). The IgG1–IgG4 hybrid antibody was similarly expressed but on a 10-fold greater scale.

Fab and Antibody expression

Large scale transient transfections were carried out using electroporation, 2×10^8 CHO cells/ml were resuspended in Earles Balanced Salt Solution (Sigma, Poole, UK) and 400 mg of DNA was added. Cells were electroporated and then resuspended in 1 L of CD-CHO culture medium (Invitrogen) and incubated for 24 h. Incubation continued at 32°C for 13 days and at 4 days post-transfection sodium butyrate (3 m*M* final concentration) was added to the culture. On day 14 post-transfection, cell cultures were spun down for 1 h at 4000 RPM and filtered using a 0.22-μm Stericup filter (Millipore, Massachusetts).

His tagged purification

His tagged samples were purified by nickel affinity chromatography in a plate based vacuum purification system consisting of a vacuum manifold (Millipore) and filter plate (Qiagen, Crawley, UK), a vacuum (−15 In. Hg) was applied following each buffer addition. A total of 150 μl of Ni-NTA 50% ethanol slurry (Qiagen) was dispensed into wells of the Filter plate. A total of 800 μl of resin preparation buffer (50 m*M* sodium phosphate, 300 m*M* sodium chloride, pH 8.0) followed by 800 μl of elution buffer (50 m*M* sodium phosphate, 300 m*M* sodium chloride, 250 m*M* imidazole, pH 8.0) followed by another two washes of 800 μl of resin preparation buffer. Samples were first mixed in a 1:1 ratio with sample preparation buffer and drawn through the resin, followed by three 800 μl aliquots of wash buffer (50 m*M* sodium phosphate, 300 m*M* sodium chloride, 20 m*M* imidazole, pH 8.0). Bound material was eluted with 100 μl of elution buffer. Elutant was collected in a deep well plate and stored at 4°C (His tagged samples were confirmed to have the same thermal stabilities as non-His tagged samples, data not shown).

Fab and antibody purification

MAb supernatants were purified using a Mab Select SuRe column (GE Healthcare, Amersham). Fab supernatants were purified using a protein G column (GE Healthcare) Supernatant was loaded onto the column and the column washed with PBS pH 7.4. Bound antibody was eluted in 0.1*M* sodium citrate, pH 3.0, bound Fab was eluted in 0.1*M* glycine—HCl pH = 2.7 and peak fractions were collected. Eluted antibody was neutralized by addition of 2*M* Tris-HCl, pH = 8.5. The level of aggregation was determined by size exclusion chromatography (SEC) HPLC on an analytical Zorbax GF-250 column (Agilent Technologies UK, Wokingham), developed with an isocratic gradient of 0.2*M* phosphate, pH 7.0 at 1.0 ml/min. Aggregate removal and final buffer exchange into PBS was performed by preparative gel filtration using a Superdex 200 XK16/60 column (GE Healthcare, Amersham) run at a flow rate of 1.0 ml/min.

Thermal stability measurement

A thermofluor assay was performed to assess the thermal stabilities of purified molecules. Fluorescence-based thermal shift assays (also referred to as thermofluor assays) were initially developed to detect ligand binding and were later used to test protein stabilizing buffer compositions.[33–35] Others

have demonstrated that the thermofluor derived T_ms correlate well with those determined by differential scanning calorimetry (DSC), the gold standard for thermal stability measurement.[36] The reaction mix contained 5 μl of 30x SYPRO® Orange dye (Invitrogen), diluted with water from 5000X stock solution and 45 μl of sample at 0.12 mg/ml, in one of three buffers: PBS pH 7.4, 50 m*M* sodium acetate 125 m*M* sodium chloride pH 5.0, or 50 m*M* sodium phosphate 300 m*M* sodium chloride, 250 m*M* imidazole pH 8.0. A total of 10 μl of the mix was dispensed in quadruplicate into a 384 PCR optical well plate and was run on a 7900HT Fast Real-Time PCR System (Agilent). The PCR system heating device was set at 20–99°C with a ramp rate of 1.1°C/min. A charge-coupled device monitored fluorescence changes in the wells. Intensity increases were plotted, the inflection point of the slope(s) was used to generate the T_m. Domain assignments were made by referring to the known T_ms of the C_H2 and C_H3 domains in a given buffer as demonstrated by Garber et al. and He et al.[31,36] In situations where one peak is generated for both the C_H2 and Fab domains a second experiment was performed in a lower pH buffer, typically 50 m*M* sodium acetate 125 m*M* sodium chloride pH 5.0. The C_H2 domain is less stable at this pH and loses approximately 5°C from its expected T_m in PBS (pH 7.4). The Fab domain is less sensitive to pH 5.0 and therefore the single peak is resolved into two, allowing the T_m of the Fab domain to be assigned.[37]

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