Using DSC to study antibody Fab region stability for improved biotherapeutic development

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Introduction

Stability is a crucial issue for the development of therapeutic antibodies and genetically tailored antibody fragments. Poor stability may affect antibody expression levels in various cell types, result in fractional populations of non-functional or misfolded material, or lead to the formation of large and potentially immunogenic protein aggregates over time.

Antibodies and antibody-like proteins represent a large and growing number of molecular entities entering human clinical trials in virtually all disease indications. Antibodies are heterotetramers composed of heavy and light chains. Constant domains (C_H and C_L chains) contain invariable amino acid sequences which depend upon the antibody isotype and subclass. In contrast, antibody variable domains are extraordinarily diverse, enabling the recognition of virtually any foreign antigen with high specificity and affinity. Variable domain diversity is derived by the pairing of variable heavy (V_H) and variable light (V_L, kappa or lambda) chains, numerous linker sequences between variable and constant domains, and hypersomatic mutation. Diversity within variable domains may also lead to stability variations from one antibody to another.

Until recently, there has been a relative paucity of stability data regarding full-length antibodies, potentially due to the fact that they are complicated, multi-domain proteins. Here, we investigated the domain-dependent unfolding properties of 17 human or humanized IgG_1 and IgG_4 antibodies using Differential Scanning Calorimetry DSC (1). These included Tysabri™ (clinically approved for the treatment of multiple sclerosis), seven molecules in phase I or II trials, four molecules on track for investigational new drug (IND) filing with the FDA in 2007, and six molecules in research. The dataset can be used as a reference guide to help researchers in the field of antibody discovery understand whether thermal stability may be an issue for any given antibody molecule.

The second part of this application note describes a process to engineer stability back into a particularly unstable antibody. A combination of stabilizing mutations resulted in a Fab with a midpoint of thermal unfolding (T_m) of 92°C measured by DSC. Stabilization resulted in greatly enhanced functional expression of the antibody fragment in E. coli (2).

Materials and methods

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Materials and methods used in these studies were described previously (1,2). DSC work was performed using a Malvern MicroCal VP-Capillary DSC system.
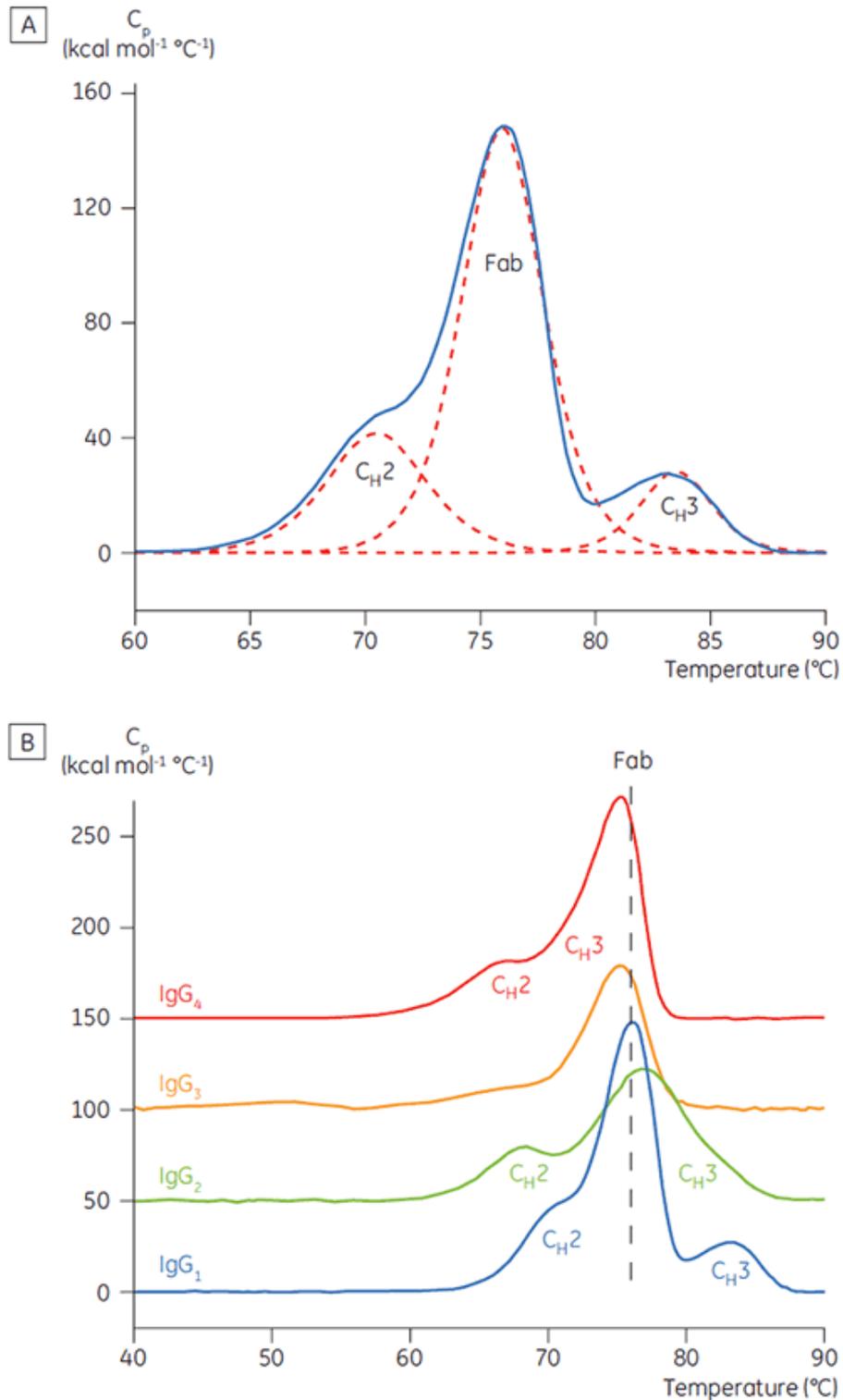

Fig 1. (A) DSC curve of the BIIB7 IgG_1. Above the curves are structures of a full-length human IgG_1 antibody (13). (B) DSC curves representing the four human IgG subclasses. Reprinted from (1) with the permission of Elsevier.
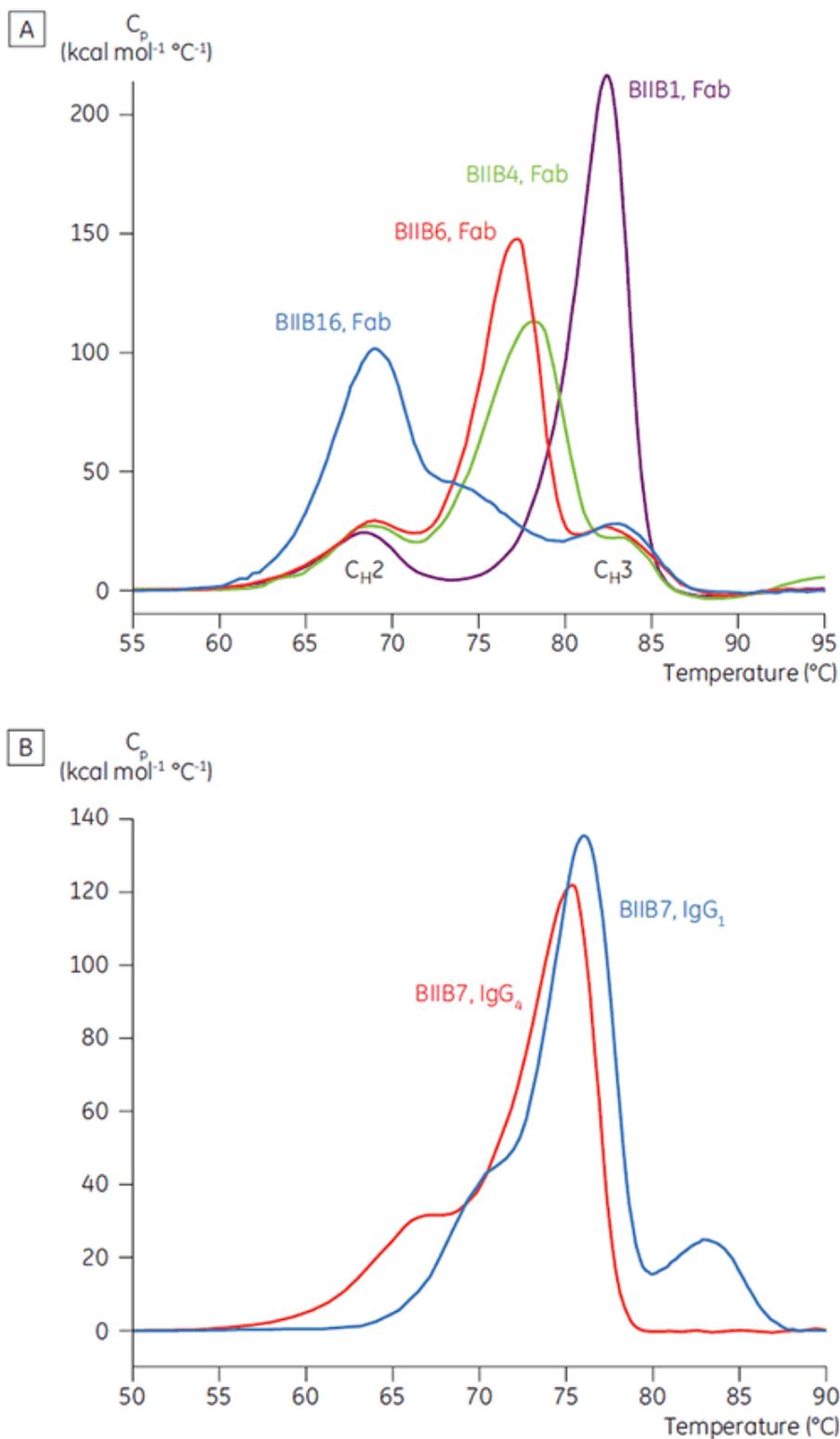
Fig 2. (A) DSC thermal unfolding curves of four human or humanized IgG$_1$ antibodies, BIIB16, BIIB6, BIIB4, and BIIB1. (B) DSC unfolding curves of the BIIB7 antibody in both the IgG$_1$ and IgG$_4$ formats. Reprinted from (1) with the permission of Elsevier.

Fab stability measurements of 18 human or humanized IgG sequences

Since their constant domains are virtually identical, IgGs of similar subclass vary in stability based on differences in their Fv regions. To investigate the range of IgG Fab stabilities that exist for human or humanized antibodies, particularly IgG$_1$s, DSC studies were performed using a panel of 18 full-length human(ized) antibodies, denoted BIIB1–18. The Fab transitions generally had CP$_{max}$ values (i.e., the height of a DSC peak) approximately three-fold greater than that of the C$_H$2 or C$_H$3 domains as shown by the DSC trace of BIIB7 (Fig 1A). Characteristic DSC traces of the four human IgG subclasses are shown in Figure 1B with IgG$_1$ demonstrating the highest apparent antibody Fc stability. Aggregation during the unfolding process affects Fab T$_m$ measured by DSC (3,4); therefore, each DSC scan was performed under identical conditions for each antibody, and the information obtained was considered a relative measure of each domain's stability.

BIIB1-18 Fab transitions varied significantly in their relative thermal stability as a result of differences in their Fv regions. The DSC curves shown in Figure 2A illustrate how individual Fv regions can lead to different Fab unfolding behaviors. The range of Fab T$_m$ values for the 18 BIIB antibodies was 57.2°C to 81.6°C (Table 1). The three antibodies with the lowest Fab T$_m$ values, BIIB15-17, demonstrated multiple unfolding transitions for their Fabs suggesting a breakdown in the cooperativity of unfolding (see Fig 2A for BIIB16's DSC curve). The Fab peak for these antibodies was split into two transitions. The transitions with the lower T are listed in Table 1. A second transition, which may represent the unfolding of C$_H$1/C$_L$, occurred with T$_m$ values of 74°C, 72°C, and 70°C for BIIB15, BIIB16, and BIIB17, respectively.

Table 1: BIIB IgG Fab T_m values from DSC

IgG	Fab T_m (°C)	V_H sub-class	V_K sub-class
BIIB1	81.6	VH1	VK1
BIIB2	78.5	VH1	VK1
BIIB3	78.2	VH1	VK2
BIIB4	77.7	VH3	VK2
BIIB5	77.1	VH4	VK1
BIIB6	76.8	VH3	VK1
BIIB7	75.9	VH1	VK3
BIIB8	75.6	VH1	VK1
BIIB9	74.7	VH3	VK4
BIIB10	74.7	VH4	VK1
BIIB11	73.1	VH1	VK4
BIIB12	71.2	VH1	VK4
BIIB13	70.8	VH3	VK4
BIIB14	70.6	VH7	VK-[†]
BIIB15	68.5	VH3	VK1
BIIB16	68.0	VH3	VK3
BIIB17	57.2	VH3	VK2
BIIB18	-*	VH3	VK2

* Did not express

[†] Unusual kappa, germline undetermined.

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Although the DSC studies were performed with ideal heterotetrameric proteins as determined by size-exclusion chromatography, the antibody with the least stable Fab, BIIB17, expressed poorly and accumulated high molecular weight aggregates if left in solution at 2°C to 8°C for over 1 week. The C_H2 and C_H3 unfolding transitions of each IgG_1 superimposed well (representative DSC traces shown in Fig 2A). The same was true for the C_H2 and C_H3 transitions of the three IgG_4s (data not shown).

BIIB7 was constructed as both an IgG_1 and IgG_4. IgG_1 and IgG_4 C_H1 sequences have ten amino acid differences (six are conservative) and a different disulfide-bonding pattern with C_L. The apparent Fab T_m values of BIIB7 in the IgG_1 and IgG_4 format were similar ($\Delta T_m < 1$°C, Fig 2B) after deconvolution of the peaks. This similarity suggests that Fab thermostabilities between IgG_1 and IgG_4 can be directly compared.

Engineering Fab residues to improve stability

It is well-established that proteins can be stabilized with consensus design – the mutation of rare amino acids to the consensus amino acids found in homologous protein sequences. In support of this, the humanized BIIB antibodies with the poorest stability as determined by DSC, appeared to have the greatest levels of uncommon

amino acid types in their frameworks. In an effort to develop antibody and antibody fragment stabilization strategies, we selected a poorly-behaved Fab, and performed a mutagenesis campaign to engineer stability back into the Fab. We began with a Fab that recognized tetanus toxoid (αTT) and was derived from a human source (5). We chose 45 residue positions for randomization. Full details on this saturation mutagenesis campaign are described in (2).
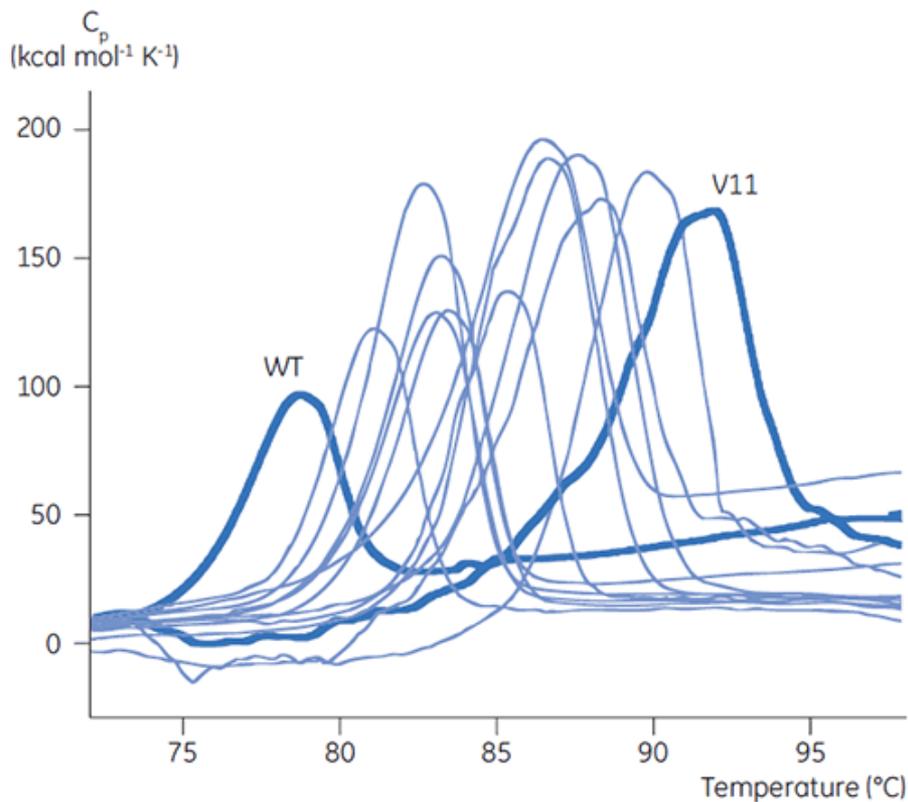

Fig 3. DSC analysis of the wild-type αTT Fab and all 12 Fabs containing various mutant combinations including V11, the most stable Fab. The identity of each curve can be derived based on the ΔT_m listed in Table 2. Reprinted from (2) with the permission of Oxford University Press.

Table 2. Thermostabilizing mutations led to highly stable Fab variants with increased bacterial expression and apparent functionality

Fab	HC Mutants	LC Mutants	ΔT_m* (DSC)	Δexpress[†]	ΔMidpoint[‡] (µg/ml)
WT	-	-	0.0	1.0±0.4	63.0
V1	V1 V11L, S77T, F89I	-	5.0	2.3	25.0
V2	V11L, S77T, T72N, A84L, F89I, G118A	D9L, N22S, W50A, N152G	7.9	2.0	8.9
V3	S77T, T72N, A84L, F89I, G118A	D9L, N22S, W50A	7.8	3.8	3.5
V4	V11L, S77T, F89I	N22S, W50A	5.3	3.2	26.0
V5	V11L, S77T, F89T	N22S, W50A	3.9	2.6	50.0
V6	V11L, S77T, F89I	N22S, W50H	6.7	1.7	13.0
V7	S77T, G127A	D9L ,N22S, W50A	2.4	1.9	25.0
V8	V11L, S77T, T72N, A84P, F89I, G118A	D9L, N22S, W50A, N152G	8.9	3.9	10.0
V9	V11L, S77T, F89T	W50A	4.3	3.5	20.0
V10	V11L, S77T, T72N, A84P, F89I, G118A	D9L, W50A, N152G	9.7	3.3	7.1
V11	V11L, S77T, T72N, A84P, F89I, G118A, G24A	D9L, N22S, W50H, N152G	13.3	3.0±0.3	4.0
V12	V11L, S77T, T72N, A84P, F89I, G118A	D9L, W50H, N152G	11.1	2.6	2.8

* WT T_m = 78.7°C, scan rate = 1.0°C/min

[†] E. coli expression level normalized to 1.0 for the WT αTT Fab whose average expressed yield over three separate cultures was 0.56 ± 0.20 mg/l culture. The standard error is also provided for V11 which was expressed twice.

[‡] Fab concentration at the midpoint of the ELISA saturation curve for biotinylated tetanus toxoid. Reprinted from (2) with the permission of Oxford University Press.

Mutagenesis and thermotolerance screening of the αTT Fab library

The 45 mutant PCR reactions were individually transformed, and close to 4500 individual colonies were picked and cultured in 96-well plates containing expression media. Supernatants containing Fab were heat challenged at three separate temperatures; 70°C, 72°C, and 74°C (2). Variants that demonstrated enhanced thermostability were resubjected in duplicate to the thermotolerance screen to confirm their properties.

A complete list of stabilizing mutations is provided in (2). Approximately 1% of the variants in the library exhibited enhanced thermostability. Fourteen of the "hits" were located in the V_H domain, and the remaining four in the V_L domain. This result suggests the stability of the native Fab was limited by the marginal stability of the V_H. Surprisingly, no mutant within the ~2000 member constant domain library was found to stabilize the Fab as a whole. We speculate that stabilizing mutations within the constant domains really do occur, but that the limited stability of the V_H domain controls the temperature at which the Fab unfolds and denied our ability to observe such events, see DSC experiments below.

Another explanation for the inability to discover stabilizing mutations within the C_L/C_H1 region may be that the 10 minute unfolding period at elevated temperatures was not sufficient for fully unfolding the constant domains. Röthlisberger and coworkers found that unfolding of the C_L/C_H1 heterodimer is extremely slow (6) and that the stability of all four Fab domains benefit from this kinetic stabilization. It is possible therefore that the constant domains may not unfold during the relatively short period of the thermochallenge.

While many of the stabilizing V_H mutations did not match the overall consensus, the majority of the mutations did match the consensus for at least one or more of the other V_H germline subclasses (2). For example, position 72 within the αTT V_H was optimized by mutation to Asn. Its consensus residue in all germline V_H subfamilies is the isosteric amino acid Asp. After sequencing the library plate for residue 72, it was discovered that Asp was serendipitously not represented. Also, even though V89 is the overwhelming consensus in most human V_H subfamilies, mutation to Val did not improve Fab stability. The isosteric V_H2 consensus residue Thr was highly stabilizing in the screen ($\Delta T_m > 2°C$). The similarly beta-branched Ile was one of the most stabilizing mutations discovered in the screen and is found only occasionally at this position in human V_H sequences, but is not the consensus residue for any V_H subclass.

Four stabilizing mutants were discovered within the αTT V_L domain. Mutation of the V_K4 consensus residue W50 to Ala (the consensus residue for V_K1) or His was highly stabilizing. Histidine is rarely found at position 50 in human kappa variable domains, but is often found in human lambda variable domains. This residue is close to the V_H/V_L domain interface. We hypothesized that its contribution to Fab stability might be linked to potential buttressing of the V_H domain since the V_H domain in particular appears to limit the stability of the αTT Fab (2). However, studies with the isolated V_L domain suggest otherwise (data not shown). Mutation from Tyr to His at this position has been reported to improve the apparent stability and expression of a scFv lacking a disulfide (7). Thus, it appears that large aromatic groups at residue 50, the most C-terminal residue of V_L framework 2, were not favorable for Fv stability in multiple cases.

Results of the combination of stabilizing mutations with the αTT Fab

Twelve constructs were generated containing between three and eleven stabilizing mutations identified in the initial screen (Table 2). Various combinations were derived rationally to determine the apparent contribution each mutant provides toward Fab stabilization.

Interestingly, introduction of multiple stabilizing mutations to the αTT Fab increased Fab expression in a parallel transformation/expression experiment. The best constructs consistently exhibited >three-fold increases in expressed yield over wild-type (Table 2). The stability of each Fab was evaluated by DSC (Fig 3) and circular dichroism (CD) (data not shown). DSC and CD scans were universally irreversible and the solutions were highly turbid after heating. Due to the aggregation of the Fabs, the measured T_m values were used to rank order the apparent Fab stability.

Coupled unfolding of all four domains is a common although not universal feature of Fabs, provided they are fully disulfide-linked both intramolecularly and intermolecularly (1,3,6,8,9). Uncoupling of the unfolding reactions of multidomain proteins based on substantial differences between the intrinsic stabilities of the individual domains is not uncommon and depends on the overall affinity of the domains for one another and the total interaction surface between the domains (10,11). In the absence of any portion of the Fab, unfolding of the remaining domains may not be coupled. Rowe and Tanford have demonstrated that the V_L and C_L within a kappa light chain appear to denature in an uncoupled fashion when not attached to a heavy chain and instead unfold as isolated
domains (12). It appears additional contacts with a heavy chain must be required for cooperativity. Röthlisberger and coworkers have recently reported exhaustive and elegant experiments demonstrating how variable domains of differing stability can unfold separately from one another while variable domains with high and similar stabilities can unite the unfolding transitions of all four domains of the Fab (6). The most stable Fab construct described here (T_m = 92°C) appeared to be on the border of uncoupling the thermal unfolding reactions of its variable domains as well.

The thermal melting temperatures of all twelve Fab variants were used to deconvolute the individual stability contribution of each mutation. All mutations appeared to contribute additively to the stability of the Fab even though several of these residues are relatively close to one another both in primary sequence and within the tertiary fold of the Fab. The analysis indicated that four mutations account for ~86% of the Fab stabilization. G24A, T72N, and F89I mutations within the V_H domain individually increased the T_m of the Fab construct by 3.0°C, 2.8°C, and 2.8°C, respectively. W50H mutation within the V_L domain increased the thermostability of the Fab by 3.5°C.

Fab thermal stability correlates with the functional protein fraction expressed by E. coli

One important consideration subsequent to framework mutagenesis was the potential effect this might have on the antigen binding function of the αTT Fab. The thermostabilizing mutations were derived from the original screen using a quantitative ELISA detecting both the C_L domain and the histidine tag at the C-terminus of C_H1. It has often been speculated that the dynamics of an Fv domain can have a profound effect on antigen binding. We were concerned that the stabilizing mutations introduced

into the Fv framework regions may interrupt a dynamic balance important for docking the antigen.

The thermostabilizing mutations did not attenuate the function of the molecule expressed by E. coli, but in fact enhanced the apparent affinity of the αTT Fab in a functional ELISA (Table 2). Two separate wild-type Fab preparations did not titrate equally with the antigen in a functional ELISA, indicating that small variations during cell culture may subtly control the amount of functional Fab expression. Both wild-type preparations yielded less functional protein than most of the stabilized αTT Fab constructs. The functional capacity of each Fab variant appeared to be directly correlated to its stability as described by the T_m (Fig 4). As the mutations were distant from the predicted binding loops, it is unlikely that these residues directly enhance contact with the antigen.

Based on the behavior of the wild-type αTT Fab, we believe that a fraction of the Fabs secreted from E. coli are in a non-functional or misfolded form. This interpretation correlates well with the fact that the isolated V_L domain, when secreted from E. coli, was in a very different conformation than the V_L expressed in the oxidizing cytosolic environment of BL21trxB(DE3) (results not shown). This also explains the functional variation observed between batch productions of Fab.
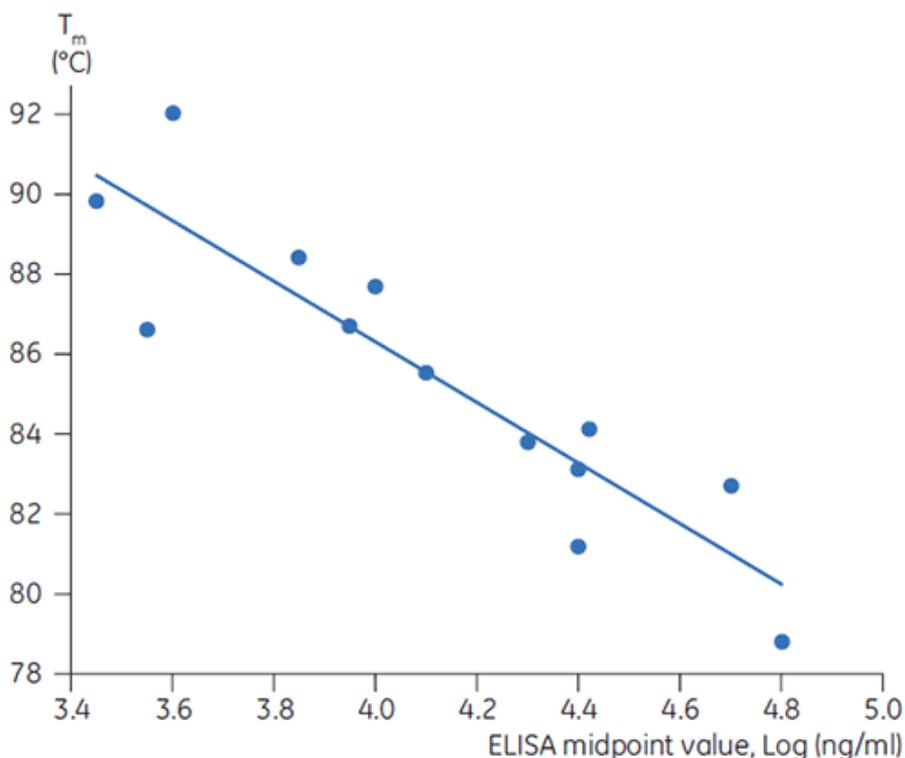

Fig 4. Plot comparing the T_m of each Fab against the midpoint concentration of each Fab's sigmoidal functional ELISA binding curve. Reprinted from (2) with the permission of Oxford University Press.

Summary

The thermal unfolding profiles of 18 human or humanized antibodies were measured using DSC and a large range of Fab stabilities were found. Using consensus design, we performed saturation mutagenesis of a particularly unstable Fab to engineer stability. Screening of the Fab libraries using a thermochallenge assay led to the

discovery of 19 stabilizing mutations within 11 sites of the Fab. Combination of stabilizing mutations led to increases in thermal stability (up to 92°C), increased bacterial expression, and a significantly reduced level of incorrectly-folded and nonfunctional material. DSC rapidly determines the factors that contribute to antibody stability, and is a valuable stability indicating technique that can be applied throughout biotherapeutic development.

Acknowledgement

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