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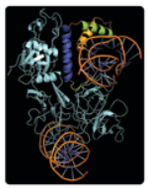

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REPORT

Modification of the kinetic stability of immunoglobulin G by solvent additives

Jonas V. Schaefer[a,§], Erik Sedlák [a,b,§], Florian Kast[a], Michal Nemergut[c], and Andreas Plückthun [a]

[a]Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, Zurich, Switzerland; [b]Center for Interdisciplinary Biosciences, P.J. Šafárik University, Jesenná 5, Košice, Slovakia; [c]Department of Biophysics, P.J. Šafárik University, Jesenná 5, Košice, Slovakia

ABSTRACT

Biophysical properties of antibody-based biopharmaceuticals are a critical part of their release criteria. In this context, finding the appropriate formulation is equally important as optimizing their intrinsic biophysical properties through protein engineering, and both are mutually dependent. Most previous studies have empirically tested the impact of additives on measures of colloidal stability, while mechanistic aspects have usually been limited to only the *thermodynamic* stability of the protein. Here we emphasize the *kinetic* impact of additives on the irreversible denaturation steps of immunoglobulins G (IgG) and their antigen-binding fragments (Fabs), as these are the key committed steps preceding aggregation, and thus especially informative in elucidating the molecular parameters of activity loss. We examined the effects of ten additives on the conformational kinetic stability by differential scanning calorimetry (DSC), using a recently developed three-step model containing both reversible and irreversible steps. The data highlight and help to rationalize different effects of the additives on the properties of full-length IgG, analyzed by onset and aggregation temperatures as well as by kinetic parameters derived from our model. Our results further help to explain the observation that stabilizing mutations in the antigen-binding fragment (Fab) significantly affect the kinetic parameters of its thermal denaturation, but not the aggregation properties of the full-length IgGs. We show that the proper analysis of DSC scans for full-length IgGs and their corresponding Fabs not only helps in ranking their stability in different formats and formulations, but provides important mechanistic insights for improving the conformational kinetic stability of IgGs.

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Introduction

To become a pharmaceutical product, therapeutic proteins must be resistant to a multitude of stresses encountered during production, long-term storage and handling, such as interfacial stress, freeze-thaw cycles, shear agitation, temperature changes and photodegradation. The question of conformational stability is arguably one of the most important ones in the development of immunoglobulin G (IgG) proteins for pharmaceutical applications. Moreover, the putative connection between the protein's conformational stability and colloidal stability continues to play a major role in guiding protein engineering and formulation design efforts for antibodies.[1-4] Today, great efforts are made to determine these properties early in development, if not already in the discovery phase, due to their pivotal importance in deciding whether a particular IgG can progress toward clinical trials. Besides the molecule itself, its formulation plays an important role in determining the conformational and colloidal stabilities. Despite its obvious importance, the mechanistic and quantitative understanding of these phenomena has lagged far behind, and the quantitative relationship between different measures and definitions of stability has remained largely obscure. Recently, we developed a new approach for quantitatively analyzing the stepwise *irreversible* denaturation of IgGs by differential scanning calorimetry (DSC), which may help in the untangling of these phenomena.[5,6]

Many monoclonal antibodies that have not been engineered for biophysical properties often show a high susceptibility to aggregation and cannot be developed despite their high specificity and affinity. This characteristic is frequently termed low "stability". In this context, it has to be stressed that the term "protein stability" is frequently used with different meanings, often leading to confusion and ambiguity when comparing results from the literature.[6] *Thermodynamic stability*, represented by either the free energy of unfolding, an unfolding equilibrium constant, or the melting temperature of the protein under conditions of reversibility describes the *reversible* transitions of the protein. On the other hand, *kinetic stability* refers to the length of time a protein remains active under given conditions before undergoing *irreversible* denaturation. The most commonly reported measure for kinetic stability is the half-life of denaturation under defined conditions. This number is also a measure for the stability under assay conditions or even *in vivo*, where it is one of many factors influencing pharmacokinetics.

Indeed, one of the most important determinants that affect the aggregation propensity of protein biopharmaceutics in

CONTACT Andreas Plückthun pluckthun@bioc.uzh.ch Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland.

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§ Both authors contributed equally to this work.

general is their thermodynamic stability.[7-13] Although the tendency of antibodies to aggregate is not directly related to their thermodynamic stability and does not exclusively depend on this parameter,[14-17] more stably folded antibodies typically also have lower aggregation propensities.[18-21] Higher stabilities of proteins significantly limit the population of aggregation-prone intermediates when exposed to destabilizing environmental factors such as, e.g., temperature, pH and pressure. Several studies concluded that an increased conformational stability was predictive of a longer colloidal stability of antibodies.[22-24]

In accordance with this conclusion, all models attempting to describe or predict irreversible aggregation processes are based on the assumption of the existence of non-native form(s) of proteins prone to aggregate.[25,26] On the other hand, besides this hypothesis, there are findings suggesting that protein self-association is controlled by the solubility of the native state, accompanied by only minimal protein unfolding.[27] This question has not been fully resolved and it is certainly possible that both mechanisms may play a role in different proteins.

Reliable conclusions from protein stability investigations are strongly dependent on appropriate analytical methods. Particularly, stability studies on multi-domain proteins are challenging due to the complex transitions accompanying their denaturation. IgGs belong to such complex multi-domain proteins.[28] They consist of four polypeptide chains forming twelve domains, which are grouped together in different segments: the two identical antigen-binding fragments (Fabs) are connected via the hinge region with the crystallizable fragment (Fc) that consists of the two homodimeric domains C_H2 and C_H3, giving rise to the well-known Y-shaped conformation. The close spatial proximity of the Fabs within the full-length IgG significantly increases their effective local concentration. This, together with high hinge flexibility, increases their potential *intramolecular* interaction within the full-length IgG. In the process of thermal denaturation, the interactions between partially unfolded domains apparently can affect the kinetic stability of IgG, as indicated by observations that the kinetic stability of individual Fabs is generally higher in isolation than in the context of a full-length IgG.[5] Indeed, findings of some recent studies suggest that increasing the stability of Fabs may be critical for the pharmaceutical storage stability of antibodies.[24,29-32]

Due to the absence of a theoretical basis for predicting the kinetic mechanisms of activity loss without further experimentation, very often empirical approaches are used in assessing suitable antibody modifications and formulations. Typically, multiple methods are employed, often in a high-throughput format.[28,33-36] The methods commonly used in such analyses are size-exclusion chromatography, multi-angle laser light scattering, DSC, circular dichroism, fluorescence and mass spectrometry.[2] Experiments are usually based on thermal scanning, extended incubation at elevated temperature or exposure of samples to mechanical stress.[13,37,38] In general, the purpose of applying these methods is to determine parameters such as onset temperatures, transition (melting) temperatures and the second virial coefficients that would enable predictions of the long-term stability of antibodies. The methods can also be applied to detect chemical and physical factors that influence antibody stability, such as interfacial shear stress in the process of manufacturing and handling. None of these approaches, however, is capable of providing a reasonable prediction of the long-term stability of antibodies.

Thermal denaturation of full-length IgGs is a fairly complex process consisting of three consecutive steps: (1) the reversible denaturation of the C_H2 domains; (2) the cooperatively irreversible unfolding of the Fabs; and (3) the cooperatively irreversible denaturation of the C_H3 domains.[39] For IgGs, the determination of the onset temperature and/or the transition temperature that are related to the first reversible transition, that of the C_H2 domains, only has a minor significance for predicting the rate of the subsequent irreversible step(s) in antibody inactivation.[24] The determination of the onset and midpoint temperatures in unfolding transitions solely provides convenient means to rank samples, and in high-throughput setups helps to determine an approximate temperature to perform accelerated stability testing.[40,41] On the other hand, in highly concentrated antibody preparations, unfolding of the C_H2 domain, accompanied by the exposure of hydrophobic regions to solvent, may affect the colloidal stability of such preparations. In fact, several studies have suggested that decreasing conformational stability of the C_H2 domain correlates with increasing aggregation propensity of the antibody.[12,42-44]

The optimization of antibody conditions by real time storage is time consuming and expensive. Conversely, DSC measurements may yield valuable information regarding protein kinetic stabilities, but only if all thermal transitions are correctly analyzed. The great advantage of DSC lies also in its ability to discernibly distinguish transitions that are difficult to differentiate or are even undetectable by other methods, such as spectroscopic techniques.[45-47] However, the thermal denaturation of IgG molecules by DSC is usually analyzed by assuming that all steps are *reversible*, merely because the application of fitting routines is then relatively simple. Obviously, this approach completely ignores the experimental reality that the unfolding of IgGs is *irreversible* and therefore gives no information about the half-life of the molecules. Conversely, the loss of activity of IgGs is often measured under “accelerated stress conditions”, but, in the absence of a theoretical framework, the power of such measurements to predict other conditions is very limited. Recently, we therefore developed a three-step model of IgG thermal denaturation that enables the extraction of meaningful parameters, properly describing the individual steps of thermal denaturation of IgGs.[5,6]

In principle, an improvement of protein stability (thermodynamic and kinetic) is possible by structural modification through protein engineering, usually by adding stabilizing mutations in the Fab of the IgG, or by choosing molecules with these properties from screening hits.[48-50] Furthermore, the solvent properties of the formulation comprise an important additional parameter, but despite many empirical data, the influence of the formulation on the denaturation pathway has not been studied systematically, mostly due to the lack of suitable methods with a theoretical framework.

For our study, we therefore took advantage of our irreversible unfolding model to characterize the effect of ten selected additives on the kinetic stabilities of both Fabs and full-length IgGs. The additives were selected based either on

their use in formulations of IgGs[23,51,52] or, for various salts, on their position in the Hofmeister series, having well-documented effects on thermodynamic stabilities of proteins.[53,54] Thus, we studied the effect of polyols (such as sorbitol, sucrose and trehalose), salts (sodium perchlorate, chloride and sulfate), arginine as well as methylamines (sarcosine, betaine and trimethylamine N-oxide (TMAO)). Our results show that the effects of additives on the kinetic stabilities of Fabs and IgGs generally correlate well with their impact on the thermodynamic stabilities of the proteins, albeit with some notable exceptions. Moreover, observed differences between the kinetic stability of Fabs, when in isolation or after their incorporation into the IgG scaffold further stress the role of additives in suppressing interactions between these fragments in the IgG format, thus influencing the kinetic stabilities of full-length IgGs. This mechanistic and comparative study of the effect of additives may thus contribute to a more rational understanding of kinetic stability.

Results

To thoroughly study the effects of solvent formulation on the kinetic and thermodynamic stabilities of antibodies, we chose an antibody that has been well characterized biophysically. IgG6B3 is targeted against myoglobin, and both a wild type (WT) and a stability-engineered variant (M for mutant) were previously analyzed (Fig. S1).[49] These two variants were investigated by DSC, each as a full-length IgG and as the corresponding isolated Fab. Ten different additives, divisible into three distinct groups based on their chemical structures, were chosen for our analyses: (1) sugar-related polyols (sorbitol, sucrose and trehalose), (2) salts (sodium perchlorate, chloride and sulfate) including the amino acid arginine, and (3) methylamines (betaine, sarcosine and TMAO).

A detailed description of the quantitative DSC data analysis for irreversible denaturation, including the derivation of the relevant equations, is given in our recent studies[5,6] and summarized in the Materials and methods section of those reports. Briefly, the thermal transition of Fabs proceeds in an apparent one-step irreversible transition. In contrast, the thermal denaturation of full-length IgG molecules must be described by a multistep mechanism, and the detailed rate equations are found in Materials and methods (Equations 3 and 5).

The half-life $\tau_{1/2}$ generally expresses the kinetic stability of an irreversibly denaturing protein at a given temperature and is determined by the rate constant of the irreversible transition k at this particular temperature. However, in the case that the analyzed irreversible step is preceded by reversible or irreversible step(s), the value of the half-life is based on a more complex function involving all preceding steps.[6] In the present study, we compare the half-lives of Fabs in isolated form and as part of a full-length IgG, each in the presence of various additives. Therefore, we analyze the rate constants k_2 corresponding to the denaturation of the Fab in both formats. The values of the half-lives listed in Tables S1 and S2 are calculated as $\tau_{1/2} = ln2/k$. The rate constant of the irreversible step is usually expressed as:

$$k = \exp\left(-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T^*}\right)\right) \quad (1)$$

where E_a is the activation energy and T^* the temperature at which $k = 1\ \mathrm{min}^{-1}$. Using the so-called frequency factor Ff, expressed as $\exp(E_a/R/T^*)$, Equation 1 can be transformed into the following form:

$$k = Ff \cdot \exp\left(-\frac{E_a}{RT}\right) \quad (2)$$

This equation provides a simple way to assess both the *enthalpic* and the *entropic* contributions to the energetic barrier of any irreversible step: the activation energy (E_a) corresponds to the enthalpic part while the frequency factor (Ff) reflects the entropic contribution of the irreversible step.

As is obvious from Equations 1 and 2, a decrease in either T^* or in E_a will result in a diminished kinetic stability of the protein, measurable in a higher k or lower $\tau_{1/2}$ value. The situation gets more complex when one of these two parameters increases while the other one decreases. This scenario might happen in the presence of certain additives, and will especially influence the respective frequency factor, as this factor depends on both of these values. For the cases of diverging T^* and E_a, the overall effect on the protein's stability will mainly depend on which parameter is more influenced by the additives.

DSC of Fab_{WT} and Fab_M in the presence of additives

As expected, the thermal denaturation curves of isolated Fabs, both in their wild-type (WT) (Fab_{WT}) as well as their engineered version (Fab_M), only consist of a single transition (Figs. 1A-C and 2A-C). This irreversible process can be described by Equation 3 (see Materials and methods), and thus the experimental DSC data can be well fitted using Equation 4. The fits are indicated as solid lines in Figures 1 and 2 and the corresponding fitting parameters derived from Equation 4 are listed in Tables S1 and S2.

Both the activation energy and the frequency factor were evaluated for Fabs under all analyzed conditions and are shown as insets in Figures 1 and 2. Each of these values was put in relation to the corresponding parameters obtained in phosphate-buffered saline (PBS), i.e., ΔE_a expresses the difference between the activation energy of a Fab under a given condition and that in PBS buffer ($\Delta E_a = E_{a(\mathrm{additive})} - E_{a(\mathrm{PBS})}$). Similarly, the entropic contribution is expressed as the ratio of the frequency factors under a given condition compared to PBS buffer ($Ff_{\mathrm{rel}} = Ff_{\mathrm{additive}} / Ff_{\mathrm{PBS}}$). As these relative frequency factors are quite large, the insets in Figures 1 and 2 show the logarithm of these parameters. Since they are further inversely related to the half-life, the negative logarithm is plotted (i.e., $-\log(Ff_{\mathrm{rel}})$), such that bars above zero correspond to a longer half-life both for the activation energy and the frequency factor, while bars below zero indicate a contribution to a decreased half-life.

Interestingly, the effects of additives on the activation energy and the frequency factor of the irreversible thermal transition

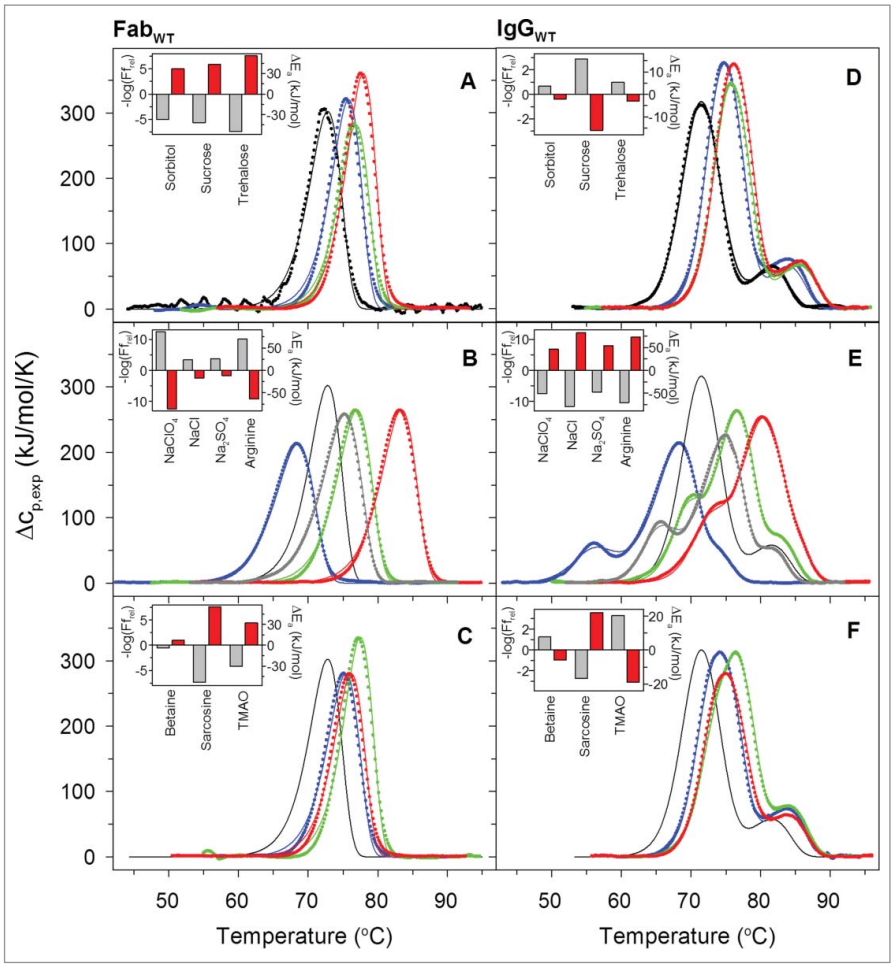

Figure 1. Effect of additives on the thermal denaturation of the Fab_{WT} fragment (left boxes) and IgG_{WT} (right boxes). DSC scans of Fab_{WT} and IgG_{WT} in PBS without additional substances are shown in black (in all boxes). (A) and (D) In the first row, effects of sorbitol (blue), sucrose (green) and trehalose (red) on DSC scans of Fab_{WT} (A) and IgG_{WT} (D) are shown. (B) and (E) Effects of $NaClO_4$ (blue), NaCl (green), Na_2SO_4 (red) and arginine (grey) on DSC scans of Fab_{WT} (B) and IgG_{WT} (E). (C) and (F) Effect of betaine (blue), sarcosine (green) and TMAO (red) on DSC scans of Fab_{WT} (C) and IgG_{WT} (F). Insets: Effect of additives on frequency factor, $log(Ff_{rel})$ (grey bars), and activation energy, ΔE_a (red bars), relative to the parameters obtained in PBS buffer. All DSC measurements were performed in PBS, pH 7.4, at a scan rate 1 K/min. The concentration of additives was 1 M.

of Fab_{WT} are very similar within all members of a respective group of additives (see insets in Figs. 1A-C). For example, all tested polyols increase both the frequency factor (larger negative value of -log(Ff_{rel})) as well as the activation energy. As, however, the increase in E_a is more pronounced than that of -log(Ff_{rel}), these additives result in a kinetic *stabilization* of Fab_{WT} (Fig. 1A, Table S1). Especially trehalose increases the Fab's half-life, actually being the one of all tested additives with the largest stabilizing effect for this construct. On the other hand, the tested salts, including arginine, decrease the frequency factor (such that -log(Ff_{rel}) is positive) and at the same time also reduce the activation energy of the irreversible step. Due to different extents of these effects, this leads to a kinetic *destabilization* of Fab_{WT} in the presence of sodium perchlorate and arginine, but a *stabilization* in the presence of sodium chloride and sodium sulfate, respectively (Fig. 1B, Table S1). Finally, the analyzed methylamines affect the parameters in a similar matter as the polyols: both the absolute value of -log(Ff_{rel}) as well as the activation energy of the irreversible step are increased (even to a larger extent), thus causing an improved kinetic stability of Fab_{WT} (Fig. 1C, Table S1). In this group, especially sarcosine has a clear beneficial effect on the kinetic stability, increasing it by a factor of more than 50, and thus almost as much as trehalose.

Our analyses of the effects of the tested additives on the engineered Fabs (Fab_M) revealed that they have similar effects (besides betaine) on both activation energy and frequency factor as for Fab_{WT} (insets of Figs. 2A-C). Consequently, the half-life of Fab_M under these conditions is similarly affected as that of Fab_{WT} (Table S2), but partly to different extents. For most additives, the increase in kinetic stability is actually slightly smaller for the engineered than for the WT Fab. The improvements caused by Na_2SO_4 or sucrose are even three to four times smaller for this stabilized Fab variant. Since also the effect of trehalose is slightly diminished, sarcosine is the additive with the largest effect on the stability of the engineered Fab.

DSC of IgG_{WT} and IgG_M in the presence of additives

Recently, we derived a mathematical model that properly simulates all three consecutive transitions of the thermal denaturation of IgGs (one reversible followed by two irreversible transitions; refer to Equations 5 and 7 in Materials and

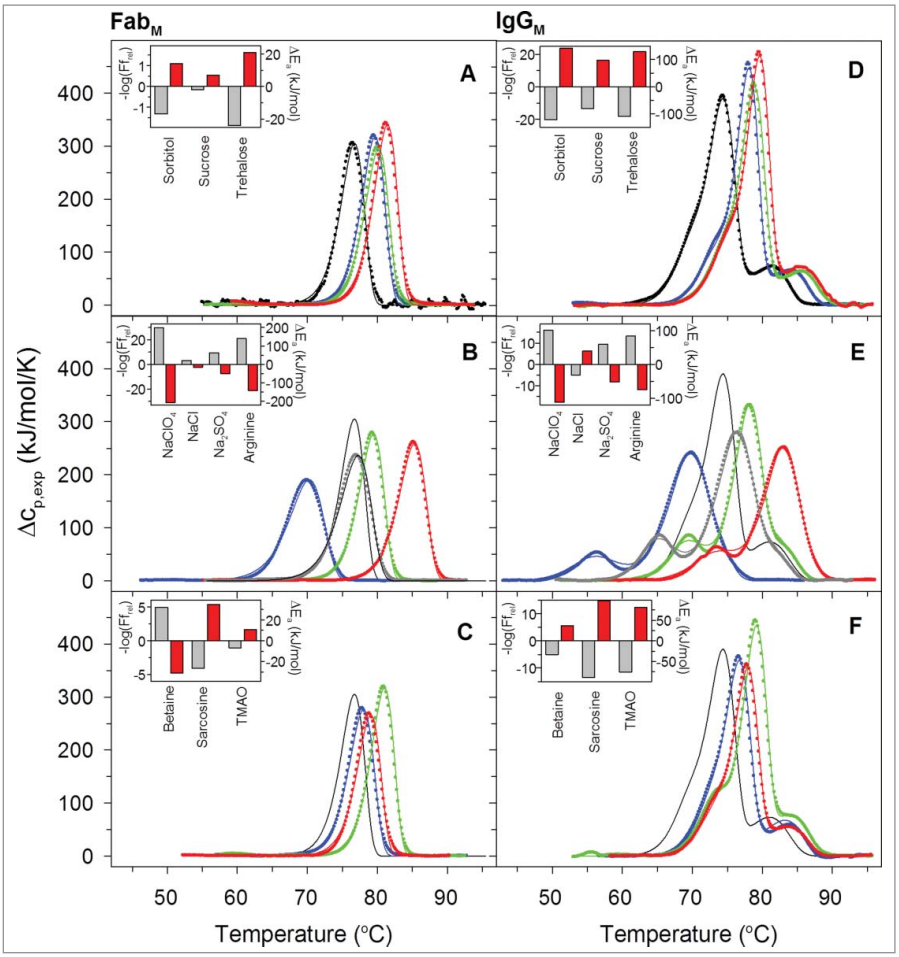

Figure 2. Effect of additives on thermal denaturation of the engineered Fab_M fragment (left boxes) and IgG_M (right boxes). DSC scan of Fab_M and IgG_M in PBS is shown in black (in all boxes). Effect of sorbitol (blue), sucrose (green) and trehalose (red) on DSC scans of Fab_M (A) and IgG_M (D). Effect of $NaClO_4$ (blue), NaCl (green), Na_2SO_4 (red) and arginine (grey) on DSC scans of Fab_M (B) and IgG_M (E). Effect of betaine (blue), sarcosine (green) and TMAO (red) on DSC scans of Fab_M (C) and IgG_M (F). Insets: Effect of additives on frequency factor, $log(Ff_{rel})$ (grey bars), and activation energy, ΔE_a (red bars), relative to the parameters obtained in PBS buffer. All DSC measurements were performed in PBS, pH 7.4, at a scan rate 1 K/min. The concentration of additives was 1 M.

methods).[5] DSC scans of IgG_{WT} and IgG_M under all studied conditions are shown in Figures 1D-F and 2D-F, respectively, with the corresponding fitting parameters being listed in Tables S1 and S2. The thermal denaturation curves of IgG_{WT} are clearly of a three-step nature in the presence of most salts (Fig. 1E), while in PBS buffer, as well as in the presence of polyols or methylamines (Figs. 1D and F), only two apparent transitions could be detected. An indication that these thermal transitions are, however, still composed of three steps, even under these conditions, follows from the graph shown in Figure S2, comparing the width of the first transition of IgG_{WT} (Fig. 1D) with that of the thermal transition of the Fab_{WT} fragment under identical conditions (Fig. 1A). The notably broader first peak of the intact IgG_{WT} indicates the presence of another transition "hidden" under this apparent one-step transition. On the other hand, thermal transitions of the engineered IgG_M are clearly three-step processes for all studied conditions, even if some of these steps overlap (Figs. 2D-F). As none of the additives change the ir/reversibility of the individual transitions, the thermal denaturation of all IgG molecules can be described by Equation 7 for these conditions.

The activation energies and frequency factors of the first irreversible transition (corresponding to the Fab denaturation) were evaluated for IgGs under all conditions and are shown in the insets of Figures 1D-F and 2D-F. Analogous to the analyses of Fabs, the values of the activation energies and the frequency factors of IgGs were compared to the corresponding parameters obtained in PBS buffer.

The effects of additives on the activation energy and frequency factor of the first irreversible step of IgG_{WT}, analogous to the case of Fab_{WT}, are again very similar for all members of a respective group of additives (insets Figs. 1D-F). Actually, all tested additives result in an increased kinetic stability of this IgG, but to different extents. In contrast to their effect on Fabs, polyols slightly decrease the activation energy in the IgG context and consequently also the frequency factor (positive -log(Ff_{rel})), resulting in only a minor kinetic stabilization of IgG_{WT} (Fig. 1D, Table S1). Salts, including arginine, on the other hand, increase the frequency factor and at the same time significantly enhance the activation energy of the irreversible step. As the latter effect is dominant, these salts actually result in the largest kinetic stabilizations of IgG_{WT} of all tested additives (~50-150 fold increase for NaCl, Na_2SO_4 or arginine). It is noteworthy that this group of additives also induces the distinct separation of the first thermal transition (belonging to C_H2) from the second transition (the Fab), as can be seen in all

DSC scans of the respective group (Fig. 1E). For the last group of additives, the methylamines, no obvious general tendency — neither for the frequency factor nor for the activation energy — can be identified. However, the highest stabilization effect on the kinetic stability of IgG_{WT} (as already mentioned for the corresponding Fab) of this group is caused by the monomethylated amine compound sarcosine with an improvement factor of 10. Both trimethylated amines (betaine and TMAO) decrease both the frequency factors and the activation energies, with the result of a very mild stabilization or even no detectable effect on the kinetic stability of IgG_{WT}.

Corresponding experiments investigating the effects of the additives on the engineered IgG_M were also performed (Figs. 2D-F, Table S2). In contrast to their effect on the WT counterparts, all analyzed polyols and methylamines increase both the frequency factors and the activation energies of the first irreversible step in the thermal denaturation of IgG_M. In all cases, these changes result in an increased kinetic stability of IgG_M. Especially the polyols had a dramatic effect on the half-life, causing the greatest increase of all analyzed samples in this study (factor ~100-750). On the other hand, salts (with the exception of sodium chloride) clearly decrease the frequency factor as well as the activation energy of this step in IgG_M. Again, apart from NaCl, this is in agreement with Fab_M, but in contrast to IgG_{WT}. The changes in the parameters result in only a mild stabilization effect of sodium chloride and sulfate on the kinetic stability of IgG_M. Interestingly, the effect of these additives on the first (reversible) transition of IgG_M, corresponding to the unfolding of the C_H2 domain, is the opposite: chloride has a mild destabilizing effect while sulfate stabilizes this transition. In contrast, sodium perchlorate and arginine significantly decrease the kinetic stability of IgG_M by about four and two orders of magnitude, respectively, in comparison of the half-life of the same protein in PBS buffer (Table S2).

Effect of the incorporation of Fabs into IgGs on their kinetic stability

As our model enabled us to extract parameters describing the kinetic stability of the Fabs within the intact IgG, we next wanted to address the question of how the incorporation of Fabs into the IgG framework affects their kinetic stability under the conditions studied. Interestingly, under almost all conditions the half-life of Fabs is *lower* when they are part of the whole IgG molecule than for the isolated molecule (Tables S1 and S2). Only in the presence of sorbitol, this value is restored for the isolated and IgG-embedded Fab_M. This can be illustrated by plotting either the activation energies or the frequency factors (i.e., the parameters that determine the kinetic stability) of free Fabs versus those of the same fragments in the IgG context. In Figure 3, a comparison of these activation energies is plotted. The data for both the WT (Fig. 3A) and the engineered constructs (Fig. 3B) illustrate that the activation energy of the irreversible step is higher for the free Fabs than for the IgG counterparts under essentially all studied conditions. This tendency is more evident for Fab_{WT} then for Fab_M, and can be expressed as an average excess of all determined activation energies of free Fabs in comparison with the IgG-incorporated counterparts, equaling 128 ± 77 kJ/mol for Fab_{WT} and

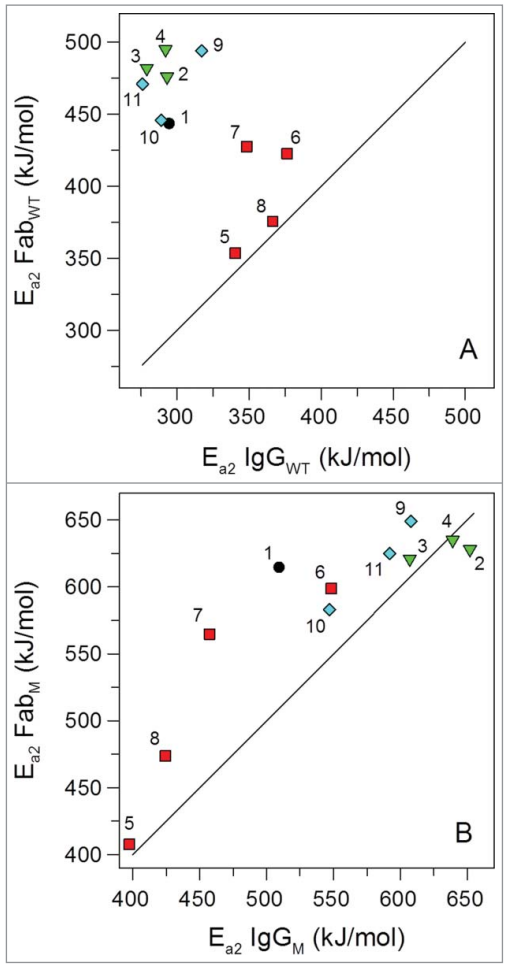

Figure 3. Correlation between values of activation energy (E_a) of the irreversible step of thermal denaturation of the Fab in isolation and in the context of the IgG molecule, for wild type (A) and mutant (B) formats. To distinguish effects of different additives, the parameters obtained in different groups of additives are numbered and shown in different colors: polyols (green triangles): sorbitol (2), sucrose (3), and trehalose (4); salts (red squares): $NaClO_4$ (5), NaCl (6), Na_2SO_4 (7), and arginine (8); and methylamines (blue diamonds): sarcosine (9), betaine (10), and TMAO (11). The parameters obtained in PBS buffer are shown as a black circle and designated by the number (1). The diagonal lines in the graphs show the ideal correlation between the parameters.

37 ± 40 kJ/mol for Fab_M. This substantial activation energy decrease of the irreversible step of thermal denaturation upon Fab incorporation into the whole IgG molecule may indicate an interaction of this fragment upon unfolding with other (partially) unfolded domains. These could be either the neighboring C_H2 domains or the other Fab in the neighboring arm in the full-length IgG (see below).

Influence of Fabs on the stability of the C_H2 domains

We were interested whether the thermodynamic parameters of unfolding of the C_H2 domains, which are only interacting with each other in an IgG1 through the sugar residues, are conversely influenced by the stabilizing mutations in the Fab under the conditions studied. In other words, we wanted to address the question whether and, if so, to what extent the C_H2 domain interacts with the Fabs under unfolding conditions. The thermodynamic parameters are listed in Tables S1 and S2. If the observed differences in the activation energies of the fragments

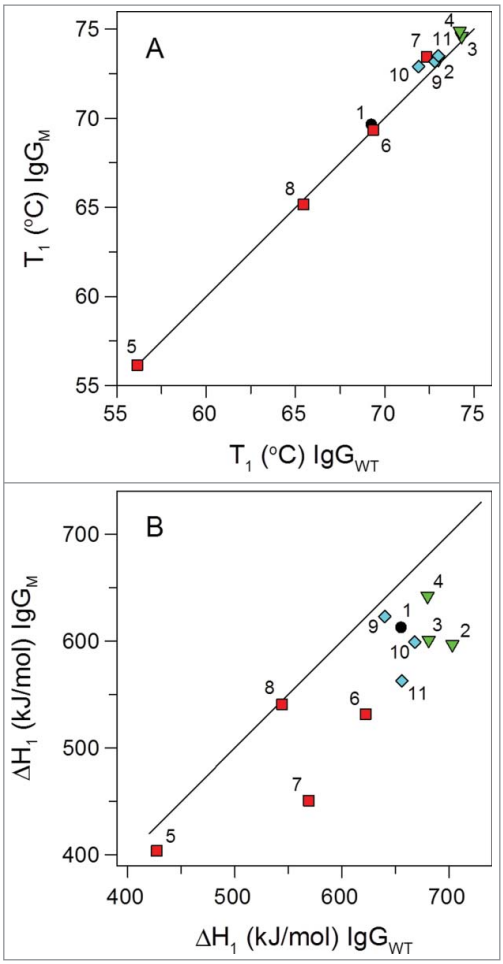

Figure 4. Correlation between values characterizing the reversible step, the unfolding of domain C_H2, in IgG_M and IgG_{WT} unfolding: transition temperature (T_1) and calorimetric enthalpy (ΔH_1). To distinguish effects of different additives, the parameters obtained in different groups of additives are numbered and shown in different colors: polyols (green triangles): sorbitol (2), sucrose (3), and trehalose (4); salts (red squares): $NaClO_4$ (5), NaCl (6), Na_2SO_4 (7), and arginine (8); and methylamines (blue diamonds): sarcosine (9), betaine (10), and TMAO (11). The parameters obtained in PBS buffer are shown as a black circle and designated by the number (1). The diagonal lines in the graphs show the ideal correlation between the parameters.

(Fab_{WT} vs. Fab_M) was due to their interactions with the C_H2 domain, the stability of the C_H2 domain itself should be affected as well.

Comparison of the influence of the mutations within the Fab of the IgG on the observed *transition temperature* of the C_H2 domain (T_1) does not indicate any such effect: the transition temperatures of the C_H2 domain in IgG_{WT} and in IgG_M are essentially identical (Fig. 4A). In contrast, however, the *transition enthalpy* of the C_H2 domain of the IgG_{WT} is always greater than that of the engineered variant IgG_M (Fig. 4B) for most conditions (except arginine). On average, the reversible transition enthalpy is 63 ± 39 kJ/mol larger in IgG_{WT} than in IgG_M for the studied conditions, consistent with an (indirect) interaction of the C_H2 domain during unfolding (see below).

Kinetic stabilities of Fabs and full-length IgGs as a function of additives

The comparison of the influence of different additives on isolated Fabs with that in the context of the IgG may reveal the reason(s) for the kinetic destabilization of these fragments in the full-length format (Fig. S3). For this purpose, we compared the relative half-life of the Fab in the context of full-length IgGs and individual Fabs, within each condition and for each variant separately (WT or M), represented by the black and grey bars, respectively. The half-lives of Fabs were thus related to the half-life of a Fab in PBS at 37°C for WT (upper part) and for mutant (lower part).

For the stabilized mutant form (M; lower panel in Fig. S3), the kinetic stabilities of the engineered Fabs (in both forms, i.e., Fab_M and IgG_M) are comparable to each other within one order of magnitude in the presence of nearly all studied additives (similar grey and black bars within each condition) (lower part of Fig. S3). Notable exceptions are PBS buffer and Na_2SO_4. In the presence of these chemicals, the IgG-embedded Fab_M is significantly less kinetically stable than in its isolated form.

On the other hand, when comparing the kinetic stabilities of isolated Fab_{WT} with the Fab as part of IgG_{WT} within each condition, a large discrepancy can be noted, i.e., when relating grey and black bars. The kinetic stabilities of the IgG_{WT} are lower by at least two orders of magnitudes compared to the isolated Fab_{WT} fragments in the presence of almost all studied additives. The only exceptions are NaCl, $NaClO_4$ and arginine. In the presence of these reagents, the stabilities of isolated Fab_{WT} and Fab_{WT} in IgG are actually comparable with each other within one order of magnitude. However, in the case of arginine and $NaClO_4$ the half-life of the Fab_{WT} is *reduced* and thus approaches that of IgG_{WT}, because of a significant destabilization.

We next compared the overall effect of additives on a given construct type (Fab_{WT} vs. Fab_M, or IgG_{WT} vs. IgG_M) (Fig. S3). For both Fabs, arginine and $NaClO_4$ are unfavorable (i.e., grey bars are in the negative part of the plot). As a matter of fact, the qualitative effect of most additives is similar when comparing Fab_{WT} and Fab_M. In contrast, the effect of the additives is rather different when comparing IgG_{WT} and IgG_M, as analyzed in detail in the Discussion section.

Moreover, it is apparent from Figure S3 that some additives (sorbitol and trehalose) can increase the half-life of the stabilized Fab in the full-length IgG_M to a comparable level of the isolated stabilized Fab_M fragment. In contrast, the only approximation of Fab_{WT} to IgG_{WT} is at *decreased* half-lives, in the case of arginine and $NaClO_4$ (Fig. S3), as already mentioned above.

To compare the effect of the additives, we created a parameter expressing the relative half-life of the full-length IgG under given conditions, but weighted it for a negative effect of the respective additive on the Fab when in context of the IgG. The reference state for calculation of all relative half-lives — of the full-length IgG as well as the isolated Fab under a given condition — is the half-life of the Fab in PBS at 37°C.

The parameter is expressed as $\tau_{rel,IgG} \cdot \frac{\tau_{rel,\ IgG}}{\tau_{rel,Fab}}$, where $\tau_{\mathrm{rel,IgG}}$ and $\tau_{\mathrm{rel,Fab}}$ are the *relative* half-lives (comparing additive vs. PBS) of Fabs in the full-length IgG and in the isolated form, respectively. This dimensionless parameter can be formally divided into two parts: $\tau_{rel,IgG}$ (the observed relative change in half-life through the additive) and the weighting factor $\frac{\tau_{rel,IgG}}{\tau_{rel,Fab}}$. The latter factor takes into account an effect of a given additive on an intra-molecular interaction of the Fabs within the full-length IgG. For example, while the observed relative half-life of

the IgG_{WT} in sucrose and $NaClO_4$ is comparable, as shown in Figure S3 (black bars), our analysis gives the additional insight that $NaClO_4$ destabilizes the Fab less. This is presumably caused by better solubilization of partially unfolded intermediates, expressed as a higher ratio $\frac{\tau_{rel,IgG}}{\tau_{rel,Fab}}$ for $NaClO_4$ than for sucrose. Therefore, $NaClO_4$ may be preferred as an additive. Using this parameter to rank the additives for IgG_{WT} and IgG_M we obtained (cf. Table S3):

IgG_{WT}: Sucrose<TMAO<Trehalose<Sorbitol<Betaine <PBS<Sarcosine<$NaClO_4$<Na_2SO_4<NaCl<Arg

IgG_M: $NaClO_4$<Arg<PBS<TMAO<Betaine<Na_2SO_4 <NaCl<Sarcosine<Sucrose<Sorbitol<Trehalose

Ranking of IgG stability in the presence of additives by other parameters

We further analyzed the effect of the additives by parameters commonly used for ranking their effect on IgG, i.e., the onset temperature T_{onset} (reflecting the first reversible transition) and the aggregation temperature T_{agg} of IgG_{WT} and IgG_M (Table S3). T_{onset} and T_{agg} are defined as the temperatures at which the corresponding processes, i.e., the thermal denaturation and the temperature-induced aggregation start. The temperatures T_{onset} and T_{agg} were obtained either from DSC (Figs. 1 and 2) or by absorbance measurements (Fig. S4), respectively. They were plotted for IgG_{WT} and IgG_M for all studied additives (Fig. 5, Table S3), and they practically all fell on the diagonal line, indicating that the presence or absence of stabilizing mutations of the Fab neither affect T_{onset} nor T_{agg}. In fact, one can expect that these stabilizing mutations within the Fab do not affect the first denaturation step, which corresponds to denaturation of the C_H2 domain. This transition is characterized by the transition temperature T_1, which in turn very well correlates with the onset temperature T_{onset}.

The lack of an effect of the stabilizing mutations on the macroscopic aggregation furthermore indicates that the stability of the Fab is not the determining factor for IgG aggregation (Fig. 5, black circles). IgG_{WT} as well as IgG_M do not aggregate in the presence of 1 M $NaClO_4$ and 1 M arginine in the monitored temperature range, i.e., 20–90°C. On the other hand, the studied IgGs form aggregates in the presence of 1 M Na_2SO_4 over the whole temperature range. These observations are in agreement with the well-known salting-in effects of $NaClO_4$ and arginine, as well as the salting-out effect of Na_2SO_4.[53,54]

The order of additives according to the onset temperatures for IgGs (from least to most stable) is as follows (Table S3), and virtually identical for IgG_{WT} and IgG_M:

IgG_{WT}: $NaClO_4$<Arg<NaCl<PBS<Na_2SO_4<Betaine <Sarcosine< TMAO<Sorbitol<Sucrose<Trehalose

IgG_M: $NaClO_4$<Arg<NaCl<PBS<Betaine<Na_2SO_4 <Sarcosine<TMAO<Sorbitol<Sucrose<Trehalose

To analyze the effect of the additives, we also utilized a parameter derived from our recent work,[6] namely the apparent denaturation rate $k_{2,app}$, whose temperature dependence allows prediction of kinetic stability over a wide temperature range. The logarithm of this parameter can be written as $\log(k_2 \frac{K}{1+K})$, (for comparison, we used the values of the parameter at 37°C) in which k_2 is the denaturation rate constant of the second step (the Fab) and K is the denaturation equilibrium constant of the reversible first step (the C_H2 domains) (Fig. S5). In our previous work, we could show how the parameter $\log(k_2 \frac{K}{1+K})$ describes the change of the rate-determining step with temperature, and therefore helps in predicting unfolding rates at temperatures other than those examined — or notes the lack of predictability. Figure 6 shows that T_{onset} very well correlates with $\log(k_2 \frac{K}{1+K})$. The reason for this is that T_{onset} correlates with the transition temperature T_1 of the first transition, on which the equilibrium constant K depends. This causes the above-mentioned good correlation of T_{onset} with the parameter $\log(k_2 \frac{K}{1+K})$ at temperatures lower than T_2^*. The order of additives according to their effects on this parameter (from smaller to higher value) is as follows (Table S3), where both extremes are similar for IgG_{WT} and IgG_M, and also to the ranking according to T_{onset}:

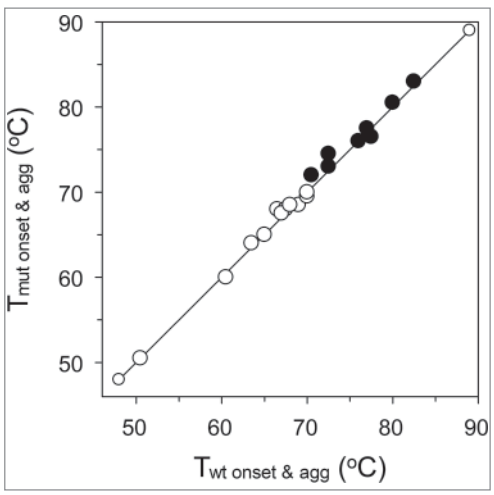

Figure 5. Correlation between onset temperatures (white circles) and aggregation temperatures (black circles) of IgG_{WT} (x-axis) and IgG_M (y-axis) in the presence of studied additives. The diagonal line in the graph shows the ideal correlation between the parameters.

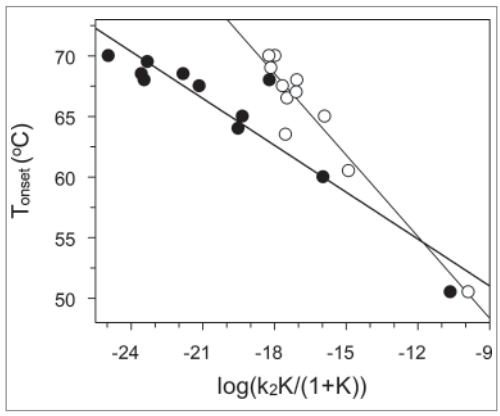

Figure 6. Correlation between onset temperatures and log $k_{2,app}$ of IgG_{WT} (white circles) and IgG_M (black circles) in the presence of studied additives. The linear fits in the graph have correlation coefficients -0.9596 and -0.9334 for IgG_{WT} and IgG_M, respectively.

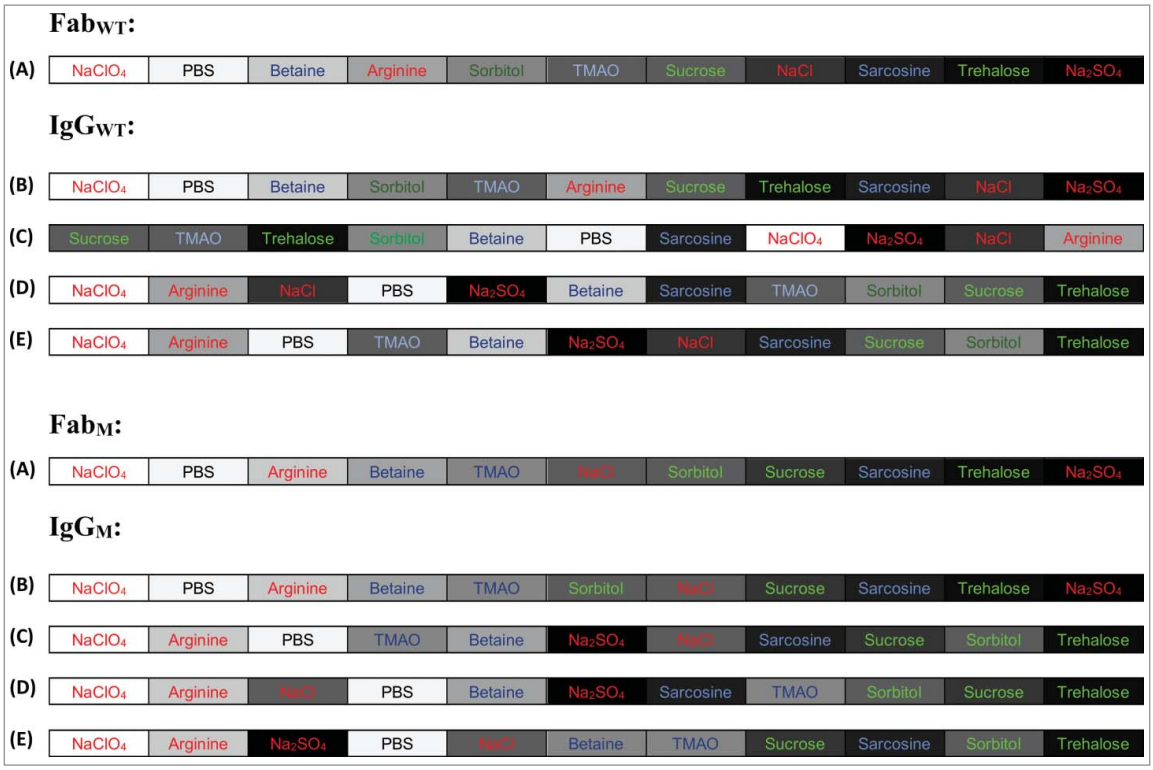

Figure 7. Comparison of different rankings for IgG_{WT} and IgG_M. The grey shades correspond to the stabilization effect of studied additives on Fab_{WT} and Fab_M fragments (rows A), as determined by thermal stability, obtained from an apparent thermal transition temperature, T_2, measured by DSC (Table S3), i.e., a darker color corresponds to a greater apparent stabilizing effect of the excipient on the given Fab. The color of the excipient names corresponds to which group it belongs, i.e., polyols (green), salts (red), methylamines (blue) and PBS (black). Colors are constant throughout the figure, grey shades throughout WT or mutant. The rows (B)-(E) correspond to different rankings (increasing "stability" from left to right) of the IgG, created according to: (B) apparent thermal transition temperature of the IgG, T_2, corresponding to the Fab part within the whole IgG, (C) the parameter $\tau_{rel,IgG} \cdot \frac{\tau_{rel,IgG}}{\tau_{rel,Fab}}$, (D) onset temperature, T_{onset}, (E) the parameter $log\left(k_2 \frac{K}{1+K}\right)$ (cf. Table S3).

IgG_{WT}: $NaClO_4$<Arg<PBS<TMAO<Betaine<Na_2SO_4 <NaCl< Sarcosine<Sucrose<Sorbitol<Trehalose

IgG_M: $NaClO_4$<Arg<Na_2SO_4<PBS<NaCl<Betaine<TMAO <Sucrose<Sarcosine<Sorbitol<Trehalose

For a better comparison, we summarized the different rankings in Figure 7. Here, grey shades correspond to de/stabilization effects of the studied additives on both the Fab_{WT} and Fab_M fragments, determined as the kinetic stability in DSC, whereas the color of the excipient name indicate to which group it belongs, i.e., polyols (green), salts (red), methylamines (blue) or PBS (black), respectively. If ranking of Fab kinetic stability corresponded perfectly to the respective IgG ranking, there should be a gradient of greyscales from left to right. From this overview it becomes obvious that while the respective rankings for IgG_M are relatively similar for all parameters, this is not the case for IgG_{WT}, as the ranking according to the parameter $\tau_{rel,IgG} \cdot \frac{\tau_{rel,IgG}}{\tau_{rel,Fab}}$ significantly differs from all others.

Discussion

It is of great practical importance to understand that formulations, i.e., the solvent composition, are affecting both the thermal as well as the colloidal stability of IgGs and their fragments. In fact, the stability of IgGs is critical for both their storage and usage, and therefore has been a major focus of antibody research for decades (for reviews see refs. 33,55-58 and citations within these articles). However, these studies mainly focused on the additives' effect on the *thermodynamic*, but not on the *kinetic* stability of IgGs. Since the kinetic components of the molecule's stability are of great importance for their applicability and, under almost all relevant conditions, antibodies do not denature in a fully reversible manner, we used our recent model to investigate the kinetic aspects of the three-step thermal denaturation of IgGs, as indicated by Equation 5. This approach enabled us to determine the effects of three chemically different groups of additives on various parameters of kinetic stability in depth, such as the frequency factor (*Ff*) and the activation energy (E_a) of the first and pivotal irreversible step.

In the present study, we investigated how ten selected representative additives affect the conformational kinetic stabilities of both Fabs and whole IgGs, one engineered for stability, the other not. The concentration of the additives was deliberately selected to be 1 M, i.e., high enough to be able to affect stability of the proteins we studied. In fact, we believe that more detailed analysis of the effect of increasing ionic strength on kinetic properties may reveal other important aspects of ionic excipients for IgG formulation, but this will be the objective of subsequent studies.

Mechanistic insight by comparing additives with different parameters

To analyze the influence of the additives on the conformational stability of IgGs, we used three different methods/parameters: (1) the onset temperature, (2) the parameter $k_{2,app}$ derived from the three-step model of IgG thermal denaturation, and

(3) a parameter $\tau_{rel,IgG} \cdot \frac{\tau_{rel,\ IgG}}{\tau_{rel,Fab}}$, which weighs the observed relative half-lives of the IgG by the destabilizing effect that some additives have on the Fab within the IgG.

The reason for using several different methods was to understand their inter-relationship in assessing the conformational kinetic stability of IgGs and to progress beyond a mere tabulation of half-lives. The onset temperature T_{onset}, the commonly used parameter describing a beginning denaturation, has only limited applicability in determining the conformational kinetic stability of IgGs. In fact, this temperature reflects the beginning of the conformational transition of the first step in IgG thermal denaturation, which corresponds to the reversible unfolding of the C_H2 domain. Interestingly, the onset temperatures correlate very well with the parameter $\log\left(k_2 \frac{K}{1+K}\right)$ (Fig. 6), which combines a thermodynamic parameter describing the first (reversible) step, i.e., the equilibrium constant K of C_H2 unfolding, with a kinetic parameter of the second (irreversible) step, i.e., the rate constant k_2 of Fab unfolding. The correlation between T_{onset} and $\log\left(k_2 \frac{K}{1+K}\right)$ originates from the definition of the latter one. However, the parameter $\log\left(k_2 \frac{K}{1+K}\right)$, in contrast to T_{onset}, is more universal, as it predicts the kinetic stability (and thus allows the ranking) of IgGs and conditions over a wide range of temperatures.[6] T_{onset} is typically extracted from measurements of excess heat capacity or from spectral changes, and thus cannot provide detailed information about the rate of the irreversible step constituting the unfolding of the Fab, which is usually the unique part of antibody candidates. Applying our model to Fabs in isolated form and in the context of full-length IgG enabled us to provide mechanistic insight into the effects of additives on the conformational kinetic stability of IgGs.

"Preferential binding" as a unifying concept

A generally useful framework to interpret the behavior of co-solvents or additives is the concept of "preferential binding".[59] Even though this was developed to understand protein thermodynamics, it can serve at least as a qualitative tool to understand irreversible unfolding kinetics and aggregation. The key concept is that additives will compete with water for binding to the protein surface, and some bind preferentially, while others are preferentially excluded. Importantly, the additives will do this differently for altered conformations of the protein, e.g., partially unfolded states that are aggregation-prone intermediates.

In our analyses, polyols such as sorbitol, sucrose and trehalose positively influenced the kinetic stability of all analyzed antibody variants. We saw the biggest stabilizing effects for both Fabs (Fab_{WT}, Fab_M) and the engineered IgG_M as a result of an increased activation energy (Tables S1 and S2). The destabilizing effect of an increased frequency factor for polyols (as can be seen in the insets of Figs. 1A and 2A, D) is not sufficient to counteract the effect of the increased activation energy of the irreversible thermal denaturation step. Interestingly, despite the unfavorable reductions of the activation energy of the irreversible step (inset Fig. 1D), polyols still stabilize IgG_{WT} through decreased frequency factors (compared to the stability of IgG_{WT} in PBS). Among the studied polyols, the most effective stabilizer is the disaccharide trehalose, in accordance with its well-known general stabilizing effect on proteins.[60,61] This has been attributed to preferential binding to the native state, in conjunction with a rather large hydrated volume.

A comparable effect (to that of trehalose) regarding changes in both the frequency factor and the activation energy could be found for one representative of the methylamine osmolytes, namely TMAO (insets in Figs. 1C, F and 2C, F). Nonetheless, its overall effect on the half-life of IgG_{WT} is rather small. The most stabilizing agent of this group is the monomethylamine sarcosine. Its stabilization effect in all studied proteins is due to a significant increase in the activation energy (especially for the two Fabs).

Interestingly, all so far mentioned osmolytes (sorbitol, sucrose, trehalose, TMAO and sarcosine) fall into the class of additives that generally stabilize proteins by raising the free energy of both their native *and* denatured states.[62] Since their effect is larger on the denatured state than on the native state, their presence increases the energy gap, and thus the equilibrium stability. This stabilization effect follows mainly from differential binding to (or differential exclusion from) the native and denatured state, thereby shifting the equilibrium towards the native state. While they have unfavorable interactions with the peptide backbone, numerous other interactions (such as hydrogen bonding and dispersive interactions) are also affected by the presence of these additives, and these are in general favorable.[58] Betaine, the other trimethylamine, only has an insignificant effect on the kinetic stabilities of the studied proteins, analogous to its small influence on their thermodynamic stabilities.[62]

In contrast, the effect of ions (including arginine) is in many respects different from that of polyols and methylamines. We have investigated the influence of three different salts that were chosen based on the position of their anions in the so-called Hofmeister series of anions.[54] Perchlorate, due to its preferential binding to a protein surface, belongs to the *chaotropic*, chloride to the *neutral* and sulfate, being preferentially excluded from the protein surface, to the *kosmotropic* anions, judged by their effects on the thermodynamic stabilities of proteins.[53,54] In fact, perchlorate is the only additive in our study with a significantly destabilizing effect on the kinetic stability of all studied IgGs and Fabs alike. Although arginine is not a typical salt, we included it into this group due to its similar effect on the IgG's thermal denaturation. Arginine is known as an amino acid with the ability to suppress protein-protein interactions without significantly affecting the protein stability, and for this reason it is frequently used as an additive in refolding reactions.[63,64] It has a small negative effect on the kinetic stability of both IgGs and Fabs, even though the transition temperature is slightly higher for all constructs in its presence.

Although salts and arginine apparently influence proteins via multiple different types of interactions and mechanisms (such as hydrogen bonding, electrostatic, dispersive, π-cation or preferential interactions as well as preferential hydration[58]), the results of their interactions with IgGs and Fabs are very similar. This conclusion is based on DSC profiles of both IgG_{WT} (Fig. 1E) and IgG_M (Fig. 2E), where the C_H2 domain unfolds at lower temperatures, clearly separated from the denaturation of the Fab (in contrast to most other additives), which shows a similar curve as in isolation (Fig. 1B, Fig. 2B).

Recent studies on the effect of salt on either the whole immunoglobulin or only its light chain point to a correlation between the Hofmeister effect and the stability, as well as the aggregation propensity, of both studied formats.[65,66] In contrast, another recent study investigating the influence of the Hofmeister anions on the dynamics of IgGs showed a rather complex nature of the effects, without clear correlations between their position in the Hofmeister series and their influence on the dynamic properties of IgGs.[43] This is, in fact, in qualitative agreement with our findings, regarding the complex effect of the salts on the frequency factor. Finally, yet another publication, analyzing the perturbation of thermal unfolding and aggregation of the isolated Fc of IgG1 by Hofmeister anions, indicated strong destabilizing effects of all studied anions (even the kosmotropic sulfate) on the C_H2 domain.[68] This finding is in clear contrast to our results measured in the context of whole IgGs, showing that sulfate — in accordance with its kosmotropic properties — has a strong stabilizing influence on the C_H2 domain, both in the IgG_{WT} and the IgG_M context (as can be seen by comparing the T_1 values in Tables S1 and S2), in comparison with chloride (being a "neutral" salt from the point of view of the Hofmeister effect) or PBS buffer. This observation needs to be discussed in the context of the C_H2 domain possibly interacting with the Fab within the IgG during unfolding, which could be in principle be direct or indirect (see next section).

Domain interactions within the unfolding IgG

To address the potential interaction of the C_H2 domain and the Fab in more detail, we analyzed how the incorporation of either Fab_{WT} or Fab_M into the full-length IgG affects the stability of the respective C_H2 domain. In fact, if there was a specific interaction between the C_H2 domain and the Fabs, different Fabs with different stabilities should result in dissimilar interaction with the C_H2 domains. Consequently, parameters describing the thermal denaturation of the C_H2 domains should differ when these domains are part of either IgG_{WT} or IgG_M. Varying interactions between the different Fab and the C_H2 domain should thus be reflected in the transition temperatures of the respective IgG, and likely also in the enthalpies of the C_H2 domain's thermal transition.[68] However, the corresponding correlation plots in Figure 4 show a nearly identical dependence of the transition *temperatures* of C_H2 domains in IgG_M *vs.* IgG_{WT} on different additives (Fig. 4A), even though significant deviations from such a correlation are seen when plotting the transition *enthalpies* of the C_H2 domain in IgG_M *vs.* IgG_{WT} (Fig. 4B). Importantly, the absence of an effect of the stabilizing Fab mutations on the transition temperature of the unfolding of C_H2 domains strongly speaks, according to the model of Brandts and colleagues,[68] against such enthalpic interactions between the C_H2 domain and the Fab. Thus, the observed difference in transition enthalpies must be most likely due to an indirect effect, caused by intramolecular interactions between the denaturing Fabs (due to their high effective concentration in the full-length IgGs) that are transmitted to the C_H2 domains (Fig. 8), as discussed below.

If there was no difference in the thermal denaturation and the stability of both isolated Fabs and of the same fragments

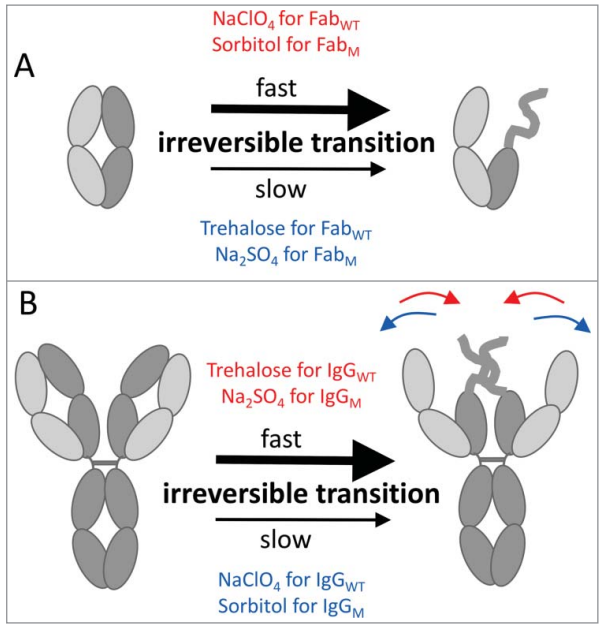

Figure 8. Schematic description of the opposite effect of preferentially excluded (stabilizing) and preferentially bound (destabilizing) excipients on the individual Fab (A) and the Fab within IgG molecule (B). The additives individually stabilizing the Fabs, e.g., trehalose for Fab_{WT} and Na_2SO_4 for Fab_M, increase the intramolecular interactions of the Fabs within IgG (indicated by the curved red arrows) but also the intermolecular interactions, leading to an increased rate constant of the first irreversible step, k_2 in Equation 5, as indicated by the thick straight arrow, and/or an increased aggregation propensity (Fig. S3, Fig. 7), respectively. On the other hand, the additives individually destabilizing the Fabs, e.g. $NaClO_4$ for Fab_{WT} and sorbitol for Fab_M, decrease both the intramolecular interactions of the Fabs within IgG (indicated by the curved blue arrows) as well as the intermolecular interactions, resulting in a decreased rate constant of the first irreversible step as indicated by the thin straight arrow, and/or an decreased aggregation propensity (Fig. S3, Fig. 7), respectively.

in the full-length IgG, the plot of the activation energy E_{a2} of the first irreversible step of IgG versus that of the irreversible step of the isolated Fab should strongly correlate. However, this is not the case, neither for IgG_{WT} nor IgG_M (Fig. 3). In these plots, a significant decrease of E_{a2} is found for the incorporation of Fab_{WT} into the IgG scaffold under all studied conditions (Fig. 3A), while the tendency for the engineered Fab is similar but smaller (Fig. 3B). These results suggest interactions between the Fabs within the IgG. In fact, if intramolecular interactions of partially unfolded Fabs are responsible for the off-diagonal points in Figure 4B, they should be closer to the diagonal for additives that weaken hydrophobic interactions. Of the studied additives, the chaotropic salt sodium perchlorate and the amino acid arginine are the only ones that suppress protein-protein interactions. Perchlorate anions decrease hydrophobic interactions by binding to exposed hydrophobic patches on proteins,[54,69] while arginine increases the native protein's solubility through favorable interactions with most amino acid side chains without significantly denaturing the protein.[63,70] Indeed, the kinetic stabilities of Fabs in isolation and as part of IgG become comparable in the presence of these two additives (i.e., there is only a 2–10 fold decrease in stability upon integration into the IgG framework for the WT constructs in the presence of arginine and perchlorate, compared to several hundred/thousand fold for, e.g., polyols and methylamines, see Figures 3, S3 and Tables S1, S2). In other

words, arginine has a more negative effect on the kinetic stability of the Fab than on that of the IgG. It has been noted before that the effect of arginine on preventing aggregation of IgG can be favorable at low temperature and neutral pH, while it promotes aggregation at high temperature and acidic pH.[71] This has been explained by the differential binding of arginine, depending on the conditions, to hydrophobic residues (preventing aggregation) and to acidic residues (promoting aggregation). A similar, but smaller effect for both Fab variants is also observable in the presence of 1 M NaCl, possibly due to suppressed electrostatic interactions (Fig. S3).

Interestingly, the effect of other additives on the kinetic stabilities of Fab_{WT} and Fab_M significantly differs. While upon its incorporation into IgG, the Fab_{WT} fragment loses its kinetic stability under all studied conditions (compared to the PBS-only sample) (Table S1), the stability of Fab_M in the IgG_M decreases only in the presence of salts (including arginine) and the methylamine betaine; in the presence of all other methylamines and all polyols, the kinetic stability of Fab_M increases in comparison with its kinetic stability in PBS. This strongly indicates that the kinetic stability of Fabs upon incorporation into the IgG framework (and thus also the kinetic stability of the full-length IgG) depends both on its intrinsic properties and solvent formulations (Figs. S3 and 8).

All studied additives (besides sodium chloride, sodium perchlorate and arginine) enhance the destabilization effect on the Fab in the context of IgG_{WT} compared to the destabilization of IgG_M with respect to the Fab_M fragment. This suggests that the strengthening of protein-protein interactions due to preferential hydration effects, induced by "stabilizing" additives (such as polyols, methylamines and sodium sulfate), may in fact lead to a *decrease* of the kinetic stability of IgGs in the process of thermal denaturation. This may promote the interactions of the partially unfolded Fabs within the IgG. Such interactions are significantly diminished in the case of Fab_M due to the stabilizing mutations that decrease a population of partially unfolded molecules of Fab_M, and therefore, even at the high effective concentration of Fab_M in the full-length IgG, the strengthening of the protein-protein interactions only has a minor effect on the kinetic stability of the IgG. This finding corresponds with the conclusion of Brader et al.,[24] according to which the Fab stabilization is recommended as a strategy for improving IgG stability.

Ranking the additives

The described analyses, using parameters $\tau_{rel,IgG} \cdot \frac{\tau_{rel,\ IgG}}{\tau_{rel,Fab}}$ and $\log\left(k_2 \frac{K}{1+K}\right)$, allowed us to propose beneficial additives for the formulation of the tested IgGs. According to our findings, the formulation for IgG_{WT} should contain the neutral salt NaCl, the kosmotropic salt Na_2SO_4 or the amino acid arginine, as all of them have significant effects on increasing the kinetic stability of IgG_{WT} in comparison with PBS (factor ~50-150). On the other hand, the most stabilizing additives for IgG_M are (ordered according their stabilizing effect): trehalose>sorbitol>sarcosine>sucrose. The observation that some stabilizing additives for IgG_M have strongly destabilizing effects on IgG_{WT} underlines the fact that the de/stabilizing effects of additives depend in a complex way on the intrinsic properties of a given IgG, such as stability and aggregation propensity (including the intramolecular aggregation tendency of the Fabs), and therefore on which protein domains have been engineered within these antibodies.

Next to the optimized IgG formulations, our data also allowed us to identify beneficial additives for the analyzed isolated Fabs. Based on the above-mentioned interpretations of our results, for dilute solutions of Fab_{WT} and Fab_M one might utilize any of the stabilizing additives which are, in fact, identical for both molecules (Table S1 and S2): all studied polyols, sulfate, sarcosine, and TMAO. However, the situation is different when working with highly concentrated solutions of Fabs, since destabilizing effects of intermolecular interactions between Fabs might take place, similar to the situation within the IgG. In such cases, the same additives as those recommended for the full IgGs should be used, i.e., sodium chloride, sodium perchlorate, and arginine.

This conclusion is also obvious from ranking the additives according to their effects on conformational kinetic stability of IgGs. In fact, the rankings according to the onset temperature and the parameter $\log\left(k_2 \frac{K}{1+K}\right)$ are very similar for both IgG_{WT} and IgG_M, reflecting general de/stabilization effects of the used additives on proteins. On the other hand, the ranking of the additives based on the parameter $\tau_{rel,IgG} \cdot \frac{\tau_{rel,IgG}}{\tau_{rel,Fab}}$ significantly differs for IgG_{WT} and IgG_M, thus pointing to the role of stabilizing mutations in the Fab for the conformational kinetic stability of IgG.

Importantly, our results clearly show that an increased conformational kinetic stability of the Fab has only an insignificant effect on the IgG's colloidal stability. Although there is a tendency that indicates that conformational stabilization of IgGs by additives also shifts the aggregation temperatures to higher temperatures (Fig. S6), these stabilizing mutations have no apparent effect on the colloidal stability of IgGs (Fig. 5). There is a strong dependence of aggregation temperatures of IgGs on the protein concentration, and on the other hand, we find no dependence of the conformational stability on concentration, pointing to an exclusively intramolecular phenomenon of the latter. Thus, the structure of the intermediates that are responsible for starting aggregation are likely different from those that are prevented by the stabilizing mutations.

Different scenarios depending on Fab stability

In summary, thermal denaturation of IgGs consists of three consecutive transitions, corresponding to the denaturation (in the following order) of the C_H2 domain, the Fab and finally the C_H3 domain.[39] From the position of transitions corresponding to the individual steps, there are three possible scenarios (Fig. 9):

1) The C_H2 domains and Fab unfold at similar temperatures, well separated from that of the C_H3 domain. In such case, DSC profiles of IgG will consist of two apparent peaks, with the first peak with higher amplitude (Fig. 9A). This scenario corresponds to the thermal denaturation of IgG_{WT} in this work or, for example, infliximab, a chimeric antibody with murine variable domains.[72]

2) The unfolding of the C_H2 domain proceeds at lower temperature than unfolding of the Fab and the C_H3 domain, for which transition temperatures are similar. In such a case, the

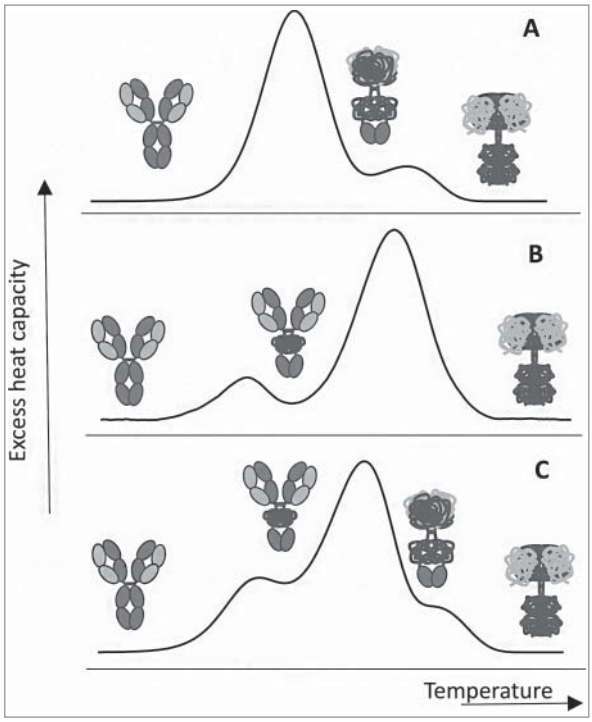

Figure 9. Schematic illustrations of different scenarios of IgG thermal denaturation: (A) scenario (1) with Fab and C_H2 domain unfolding at similar temperature, (B) scenario (2) with Fab and C_H3 domain unfolding at similar temperature, and (C) scenario (3) with C_H2, Fab and C_H3 domain unfolding one after the other at increasing temperature.

DSC profiles of IgG will consist of two apparent peaks, with the first peak having a lower amplitude (Fig. 9B), as seen, for example, in trastuzumab, a humanized antibody with engineered variable domains.[11,39]

3) All three domains (Fab, C_H2, C_H3) have different transition temperatures and the DSC profiles of such IgGs will thus consist of three peaks or two peaks with a significant shoulder on one of them (Fig. 9C). This scenario corresponds to the thermal denaturation of IgG_M in this work or, for example, etanercept.[73]

Conformational kinetic stability and/or ranking of different IgGs or IgG in the presence of different additives in scenario 2 and 3 can be analyzed by using T_{onset}, but preferentially by the parameter $\log\left(k_2 \frac{K}{1+K}\right)$ determined at the selected storage or physiological temperature.[6] In fact, the rankings of the effect of additives on IgG_M obtained by using the parameters $\log\left(k_2 \frac{K}{1+K}\right)$ and $\tau_{rel,IgG} \cdot \frac{\tau_{rel,\ IgG}}{\tau_{rel,Fab}}$ are quite similar. In these two cases, the thermal stability (described by the transition temperature T_1) of the C_H2 domain is lower than the rest of the IgG, and the instability of this domain essentially determines the kinetic stability of the IgG through the equilibrium constant, i.e., the unfolding of the C_H2 domain triggers the first irreversible step involving the unfolding of the Fab. In these two scenarios, stabilization of the C_H2 domain would lead to stabilization of the whole IgG, even though this is not part of typical IgG engineering.

In contrast, scenario 1 is more complex because the stability of the C_H2 domain and the Fab are similar and the kinetic stability of the IgG depends on specific intramolecular interactions between the Fabs during partial unfolding. In fact, Thiagarajan et al.[13] concluded that earlier unfolding of the Fab domain with the C_H2 domains led to poor stability of the whole IgG at elevated temperatures in the accelerated studies. In this scenario, a stabilization of Fab domain might be of critical importance for the stability of the whole IgG (as directly shown in the present study — IgG_{WT} and IgG_M is exactly such a case). In such IgG with limited Fab stability, where additives would be most critical, assessing the effect of additives that have relatively similar effects on protein stability by using the parameters $\log\left(k_2 \frac{K}{1+K}\right)$ may be insufficient. Part of the uncertainty comes from an extrapolation over a wide range of temperatures to low (room) temperatures, another one from partial overlap between the C_H2 and the Fab peaks. In fact, in this scenario the rankings of the effect of additives on IgG_{WT} obtained by using the parameters $\log(k_2 \frac{K}{1+K})$ and $\tau_{rel,IgG} \cdot \frac{\tau_{rel,\ IgG}}{\tau_{rel,Fab}}$ are quite dissimilar. In this case, the separate analysis of kinetic stabilities of the Fabs both in an isolated form and in the context of the full-length IgG (to be able to use the parameter $\tau_{rel,IgG} \cdot \frac{\tau_{rel,\ IgG}}{\tau_{rel,Fab}}$) may be critical and insightful. In fact, such an analysis provides direct experimental data regarding the effect of an additive on the kinetic stability and intramolecular interaction of the Fab within the whole IgG, which might be decisive for correctly ranking the effect of the additives on the conformational kinetic stability of these IgGs.

Conclusions

This work shows that the effect of additives on kinetic stability of IgGs depends, in a complex manner, both on the intrinsic properties of an IgG, particularly the stability of the Fabs with respect to the other domains, and on the nature of interactions between protein and additives. This complicated relationship has so far prevented a theoretical prediction of an efficient formulation for a given IgG. Nonetheless, we show here that the DSC profiles allow us to classify IgGs into different groups as indicated by three scenarios, and that it is possible to efficiently rank the additives according to their effect on the kinetic stability of IgGs, taking into account the relative Fab stability.

Materials and methods

Materials and additives

All chemicals and supplements for stability measurements were purchased either from Sigma-Aldrich (MO, USA) or Life Technologies (CA, USA), respectively.

Production and purification of IgGs

Full-length IgG antibodies and Fabs of the biophysically well-characterized clone 6B3, which bind myoglobin and were selected from the Human Combinatorial Antibody Library (HuCAL) (Fig. S1) were expressed and purified from mammalian cell culture supernatants, as described previously.[74]

Preparation of samples

All samples consisted of PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, adjusted to pH 7.4) as a

basis. The samples containing 1 M salts were prepared by appropriate dilution of the corresponding stock solution, usually prepared as 2 M stock in PBS, with PBS as diluent, and the pH was adjusted to pH 7.4 if necessary. All solvents were filtered through 0.22 mm filters (Millipore) and stored at 4°C.

DSC measurements

All presented DSC measurements were performed at a scan rate of 1 K/min as described previously.[5,6] We also analyzed effects of scan rate (0.25 K/min) and IgG concentration (0.25 mg/ml) on DSC profiles of IgGs in the presence of studied additives. The pH of all samples was determined before and after the measurements and only those measurements, at which the pH of solvent was in the range of pH 7.3-7.5, were further analyzed. These analyses confirmed our previous findings,[5,6] i.e., a dependence of DSC scans on the scan rate, but without an influence on apparent denaturation mechanism and the independence of DSC scans on IgG concentration in the presence of the additives (data not shown).

Analysis of DSC scans of Fabs

The thermal transition of Fabs proceeds in an apparent one-step irreversible transition. This process can be described by the following equation:

$$N \xrightarrow{k_2} F \tag{3}$$

where N is the native state and F is the final (denatured) state of the Fab; k_2 is the first-order rate constant of this irreversible reaction. The subscript "2" is used to highlight that the description of the thermal denaturation of the isolated Fabs was in agreement with the description of the same fragment within the whole IgG molecule (see Eq. 5 below). Equation 3 is a simplified case of the Lumry-Eyring model[75] for irreversible protein unfolding: $N \leftrightarrow U \rightarrow F$, where it is assumed that the equilibrium between the native (N) and the unfolded (U) states is always established and that the amount of the unfolded state remains low, with F being the final (unfolded) state in this transition. For fitting the thermal transition of Fabs described by Equation 3, the following equation was used[75,76]:

$$c_p^{excess} = \frac{\Delta H_2 k_2}{\nu} \exp\left(-\frac{1}{\nu} \int k_2 dT \right) \tag{4}$$

Analysis of DSC scans of IgGs

The thermal denaturation of full-length IgG molecules can be described by the following equation:

$$N \xleftrightarrow{K} U \xrightarrow{k_2} D \xrightarrow{k_3} F \tag{5}$$

where N is the native state, U and D are intermediate states of the thermal denaturation and F is the final (denatured) state of the IgGs; K is the equilibrium constant of the first reversible transition while k_2 and k_3 are the rate constants of the subsequent irreversible reactions.

A detailed description of the derivation of the equation for the excess heat capacity leading to the model in Equation 5 is provided in our recent work.[5] Briefly, the excess heat capacity – the parameter measured in DSC experiments – is expressed by the general equation satisfying the above-mentioned model:

$$c_p^{excess}(T) = -\Delta H_1 \frac{dn_N}{dT} + \frac{\Delta H_2 k_2}{\nu} n_U + \frac{\Delta H_3 k_3}{\nu} n_D \tag{6}$$

where ΔH_1, ΔH_2 and ΔH_3 are the molar enthalpy changes for the first, second and third step, respectively; n_N, n_U and n_D are the molar fractions of the corresponding states of the protein and ν is the scan rate in K/min. Substitution of the molar fractions by derived equations leads to the following equation:

$$c_p^{excess}(T) = \Delta H_1 \frac{K}{(K+1)^2} \left(\frac{k_2}{\nu} + \frac{\Delta H_1}{RT^2} \right) \varepsilon_K + \Delta H_2 \frac{K}{K+1} \frac{k_2}{\nu} \varepsilon_K + \Delta H_3 \frac{k_3}{\nu^2} \varepsilon_3 \int \left(\frac{k_2 K}{K+1} \frac{\varepsilon_K}{\varepsilon_3} \right) dT \tag{7}$$

where

$$K = \frac{k_1}{k_{-1}} = \exp\left[-\frac{\Delta H_1}{R} \left(\frac{1}{T} - \frac{1}{T_{1/2}} \right) \right]$$

$$k_x = \exp\left[-\frac{E_x}{R} \left(\frac{1}{T} - \frac{1}{T_x^*} \right) \right], \quad for\ x = 2\ or\ 3$$

$$\varepsilon_K = \exp\left(-\frac{1}{\nu} \int \frac{k_2 K}{K+1} dT \right)$$

$$\varepsilon_3 = \exp\left(-\frac{1}{\nu} \int k_3 dT \right)$$

Abbreviations used

DSC	differential scanning calorimetry
M	mutant
PBS	phosphate-buffered saline
TMAO	trimethylamine N-oxide
WT	wild type

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Erik Sedlák http://orcid.org/0000-0003-1290-5774
Andreas Plückthun http://orcid.org/0000-0003-4191-5306

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