A single residue switch reveals principles of antibody domain integrity

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Despite their importance for antibody architecture and design, the principles governing antibody domain stability are still not understood in sufficient detail. Here, to address this question, we chose a domain from the invariant part of IgG, the \( C_{\text{H}2} \) domain. We found that compared with other Ig domains, the isolated \( C_{\text{H}2} \) domain is a surprisingly unstable monomer, exhibiting a melting temperature of \( \sim -44 \) °C. We further show that the presence of an additional C-terminal lysine in a \( C_{\text{H}12} \) variant substantially increases the melting temperature by \( \sim -14 \) °C relative to \( C_{\text{H}2} \) WT. To explore the molecular mechanism of this effect, we employed biophysical approaches to probe structural features of \( C_{\text{H}2} \). The results revealed that Lys\(^{101} \) is key for the formation of three secondary structure elements: the very C-terminal \( \beta \)-strand and two adjacent \( \alpha \)-helices. We also noted that a dipole interaction between Lys\(^{101} \) and the nearby \( \alpha \)-helix, is important for stabilizing the \( C_{\text{H}2} \) architecture by protecting the hydrophobic core. Interestingly, this interaction between the \( \alpha \)-helix and C-terminal charged residues is highly conserved in antibody domains, suggesting that it represents a general mechanism for maintaining their integrity. We conclude that the observed interactions involving terminal residues have practical applications for defining domain boundaries in the development of antibody therapeutics and diagnostics.

Immunoglobulin G (IgG) is a tetramer of two identical light and two identical heavy chains. These contain two and four Ig domains, respectively. The Fc region of IgGs consists of two constant heavy chain domains called constant heavy chain 2 (\( C_{\text{H}2} \))\(^2 \) and constant heavy chain 3 (\( C_{\text{H}3} \)). Both domains have been shown to play a key role in mediating effector functions and preserving antibody stability (1, 2). Understanding the elements that govern the stability of antibody domains is of key interest. So far, mainly the hydrophobic core of the \( \beta \)-barrel and the disulfide bridge have been considered in this context (3, 4). Recently, we found for the variable light chain (\( V_L \)) domain that residues at the very termini (Ile\(^2 \) and Arg\(^{108} \)) are important for the integrity of the domain (5, 6). Furthermore, the constant light chain (\( C_L \)) domain was destabilized when the N-terminal Arg\(^{108} \) was deleted (6). To test the concept of a stabilizing effect of terminal residues on the Ig-fold, we turned to the conserved \( C_{\text{H}2} \) domain of the Fc fragment. As a typical antibody domain, the \( C_{\text{H}2} \) domain exhibits the highly conserved “Ig-fold,” consisting of two \( \beta \)-sheets creating a Greek-key \( \beta \)-barrel, which is stabilized by a disulfide bond in the hydrophobic core (7). Here, we analyzed the conformational stability of a recombinantly expressed murine IgG \( C_{\text{H}2} \) domain derived from the murine mAb MAK33, whose individual domains and fragments have been the object of a number of studies (8–13). Other than the \( V_L \) and \( C_L \) domains, \( C_{\text{H}2} \) is N- and C-terminally flanked by other antibody domains, the \( C_{\text{H}1} \) and \( C_{\text{H}3} \) domains, respectively. Analysis of the folding pathway and the structure of the MAK33 \( C_{\text{H}2} \) domain showed that the isolated \( C_{\text{H}2} \) domain is a monomer with a low structural stability compared with other antibody domains (14, 15). Surprisingly, it was reported that a human IgG \( C_{\text{H}2} \) domain can be stabilized by deleting seven residues at the N terminus (16). Thus, the elements governing the stability of the \( C_{\text{H}2} \) domain are still enigmatic. Here, we investigated the impact of C-terminal amino acid extensions on the stability of the immunoglobulin-fold with a view to understand the mechanisms that govern the stability of antibody domains. This is of particular interest in the context of therapeutic and diagnostic antibodies because the \( C_{\text{H}2} \) domain is currently under development as an antibody scaffold, which easily penetrates into tissues mediating effector functions as well as target binding (17, 18). We find that lysine 101 at the C terminus, which is part of a linker connecting \( C_{\text{H}2} \) to \( C_{\text{H}3} \), plays a crucial role for the stability of the \( C_{\text{H}2} \) domain.

Results

The structural elements determining antibody domain stability are not sufficiently understood. For the \( V_L \) domain, we showed that the nature of the terminal residues is important for its integrity (5, 6). To test the impact of C-terminal residues on an antibody domain that is followed by a linker and another Ig
domain, we chose the MAK33 IgG1 C<sub>H2</sub> domain. C<sub>H2</sub> variants in this study were extended by amino acids naturally occurring C terminally of the MAK33 C<sub>H2</sub> domain, i.e., they connect it to the C<sub>H3</sub> domain (14, 19) (Fig. 1, A and B). In total, we investigated four variants, C<sub>H2</sub>-WT (henceforth referred to as “C<sub>H2</sub>”) and variants extended by one to four residues: C<sub>H2</sub>-S, C<sub>H2</sub>-SK, and C<sub>H2</sub>-SKTK.

**Terminally extended C<sub>H2</sub> variants reveal changes in the tertiary structure**

To test whether the C<sub>H2</sub> variants are properly folded, we recorded far- and near-UV CD spectra (Fig. 1, C and D). The far-UV spectra of all C<sub>H2</sub> variants exhibit two minima at approximately 210 and 230 nm and are highly similar, indicating identical secondary structures for all variants studied (Fig. 1C). Thus, up to four additional residues at the C terminus do not lead to changes in the secondary structure of C<sub>H2</sub>. However, near-UV CD spectra show a significant change in ellipticity, particularly between 275 and 295 nm compared with the WT protein (Fig. 1D). These wavelengths are indicative of changes in the environment of aromatic residues, particularly Tyr and Trp (20, 21). Because there are no Tyr residues in C<sub>H2</sub>, the differences seem to occur in the microenvironment of the
Trp residues. There are two Trp residues present in C142, Trp40 and Trp76 (Fig. 1B). Trp40 is part of a β-strand in close proximity to the intramolecular disulfide bond between Cys24 and Cys84. Its side chain is buried in the hydrophobic core of C142. Trp76 is located in a solvent-exposed α-helix structurally close to the C terminus (14). Trp40 is highly conserved in all antibody domains, whereas Trp76 is conserved in Ig domains, which are part of the Fc fragment (7).

C142 WT is known to be almost exclusively monomeric (15). We tested whether the C142 variants were affected in their oligomeric state by size-exclusion chromatography coupled to multiangle light scattering (SEC-MALS). All investigated variants shared a similar elution profile with one major and one minor peak (Fig. 1E). MALS proved all variants to be more than 93% monomeric with a calculated molecular mass between 11.1 and 12.0 kDa for the monomers (Table 1). Because the oligomer concentration was too low, we could not determine a molecular mass of the minor peak. We found a monomer/oligomer ratio for the C142 variants between 100/0% and 94/6%. There was no correlation of the C-terminal extension and the oligomeric state, using intrinsic Trp fluorescence as a probe. After equilibrium unfolding in the presence of guanidinium chloride (GdmCl), all variants, except CH2-SKTK, showed the highest stability followed by CH2-SK. As observed in the thermal unfolding transitions, the presence of Lys101 resulted in a strong increase in conformational stability, proving that Lys101 makes a strong contribution to the integrity of the C142 domain. Additional residues of the C133 linker (Thr102 and Lys103) increase thermal and chemical resilience slightly further. In contrast, Ser100 seems to slightly decrease the conformational stability.

### Terminal extensions alter the structure and compactness of C142

To study the local environment of the Trp residues in more detail, we recorded intrinsic tryptophan fluorescence emission spectra. We detected different emission intensities as well as shifted maximum emission wavelengths between the natively folded C142 variants (Fig. 3A, solid lines). C142-S and C142-SKTK display the highest intensities, followed by C142-SK and C142. Because the fluorescence intensity is correlated to intrinsic quenching and exposure of the Trp residues, variations most likely reflect differences in the local environment. C142 and C142-S (both 338 nm) as well as C142-SK and C142-SKTK (both 331 nm) share the same maximum of the emission peak wavelength, respectively. Variants containing Lys101 (C142-SK and C142-SKTK) exhibit a pronounced blue shift of ~8 nm, which is characteristic for a more hydrophobic surrounding of Trp (23). Blue shifts and altered fluorescence intensities indicate different microenvironments of one or both Trp residues for C142 variants. Because the two Trp residues are probes for the hydrophobic core (Trp40) and for a solvent-exposed α-helix close to the C terminus (Trp76) (Fig. 1B), the structural changes can be assigned to the inner protein core and the α-helix. However, we cannot discriminate between the effects of the two Trp residues. After equilibrium unfolding in the presence of GdmCl, all C142 variants exhibit the same Trp spectra including intensities (Fig. 3A, dotted lines).

To gain further insight in conformational changes within the C142 variants, we quenched the Trp fluorescence using acrylamide (Fig. 3B). This allowed us to evaluate the accessibility of both Trp residues and therefore of the hydrophobic core and

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### Table 1

| Variant | Tm°C | D1/2 M | Molecular mass of prevalent species (Da) | Relative distribution monomer/oligomer |
|---------|------|--------|------------------------------------------|--------------------------------------|
| C142 wt | 44.4 | 0.7    | 11.1 ± 0.3                               | 95.5                                 |
| C142-S  | 43.6 | 0.4    | 11.1 ± 0.2                               | 100.0                                |
| C142-SK | 58.6 | 0.2    | 12.0 ± 1.4                               | 99.1                                 |
| C142-SKTK | 60.1 | 0.2 | 11.3 ± 0.3                              | 94.6                                 |
Stability of the C_{12} antibody domain

Figure 2. Stability of C_{12} variants. A, thermal, and B, chemical equilibrium unfolding transitions of C_{12} variants reveal a significant impact of the C-terminal Lys^{101} on the conformational stability. For chemical denaturation, aliquots of the C_{12} variants were incubated for at least 12 h with increasing concentrations of GdmCl and monitored by intrinsic tryptophan fluorescence.

Figure 3. Conformational differences of C_{12} variants. A, intrinsic tryptophan fluorescence of both, Trp^{40} and Trp^{76}, probes conformational changes. All folded C_{12} variants (solid lines) display different Trp fluorescence intensities at the same concentration (7 M). However, variants extended by Lys^{101} feature altered emission maxima with a significant blue shift of 8 nm compared with C_{12} WT and C_{12}-S. For comparison, all variants show identical spectra when unfolded using 3.8 M GdmCl (dotted lines). B, Stern-Volmer plots show different acrylamide quenching profiles for C_{12} variants. The introduction of the C-terminal Lys^{101} (C_{12}-SK and C_{12}-SKTK) leads to a significantly lower ability of acrylamide to quench intrinsic Trp fluorescence. Thus, Lys^{101} alters the accessibility of the Trp residues.

the α-helix close to the C terminus. C_{12}-2 and C_{12}-2-S featured a strong exponential relationship between fluorescence signal and acrylamide concentration. An acrylamide concentration of 0.1 M caused a 2.2-fold Trp fluorescence quenching. For 1 M acrylamide, we observed 37- and 40-fold lower Trp emission intensities for C_{12}-SK and C_{12}-SKTK, respectively. Accordingly, CH_{2}-SK and CH_{2}-SKTK exhibit Stern-Volmer plots, which are flatter and less exponential than for the variants without Lys^{101}. Interestingly, the presence of Lys^{101} at the C terminus significantly changed the quenching intensity, indicating an altered accessibility for both Trp residues. Exponential Stern-Volmer correlations suggest distinct accessibility of the quencher for two fluorophores within one protein (24, 25). In contrast, linear plots indicate equal accessibility of acrylamide for two tryptophans. Thus, the less pronounced the exponential growth, the more similar are the dynamics of both Trp residues. The Trp residues of C_{12}-2 variants containing Lys^{101} feature a 2-fold less quenching than C_{12}-2 variants without Lys^{101}, proving their lower accessibility for acrylamide. Most likely, this alteration is due to a more compact microenvironment of the Trp residues in the presence of Lys^{101}. However, we cannot discriminate between the impact of Trp^{40} and Trp^{76} on the fluorescence emission. In summary, C_{12}-2-SK and C_{12}-2-SKTK experience locally distinct structural differences compared with WT and C_{12}-2-S, which is attributed to the presence of Lys^{101}.

Lys^{101} shields core area and helical structure in its proximity

To reveal differences in protein dynamics and structure, we performed hydrogen/deuterium exchange (HDX) experiments followed by mass spectrometric analysis of C_{12}-2 and C_{12}-2-SKTK (Fig. 4). Along the entire primary structure, C_{12}-2 showed higher deuterium uptake than C_{12}-2-SKTK for all time points (Fig. S1). To focus on the HDX level alterations between C_{12}-2 and C_{12}-2-SKTK, we calculated the relative uptake ratio (C_{12}-2/CH_{2}-2-SKTK) and plotted it against the residue number of the C_{12} sequence (Fig. 4A). For distinct regions, particularly the α-helix and its extension (residues 70–81) we observed pronounced differences in the relative uptake level (Fig. 4, region 3). These differences are mainly localized in structural vicinity of the C-terminal region of C_{12}-2 (Fig. 4B). We observed no substantial effects for the N-terminal part, but a slightly decreased HDX for another nearby α-helix (residue 8 to 15; Fig. 4, region 1). Particularly two regions, from Pro^{70} to Glu^{81} and surprisingly Trp^{90} revealed strongly decreased HDX levels for C_{12}-2-SKTK compared with WT (Fig. 4, regions 2 and 3). For Trp^{90}, we were able to achieve pseudo-single residue resolution by HDX because we
obtained peptides differing only in the presence or absence of Trp$^{40}$ (Fig. S2). The affected α-helix (Met$^{72}$–Asn$^{78}$) in proximity to the C terminus is located between the two Ig-folds characteristic β-strands E and F, which are together with β-strands B and C define the hydrophobic core (26, 27). Thus, both β-sheets making up the β-sandwich-like Ig-fold in constant domains (CFG and ABED) experience decreased dynamics due to the presence of Lys$^{101}$. Moreover, the very C-terminal residue (Fig. 4, region 4) exhibits a decreased HDX slightly more pronounced than the first helix (residues 8–15). Among the residues that experienced altered HDX are Trp$^{40}$ and Trp$^{76}$ as indicated by acrylamide quenching.

In summary, we found the C-terminal extension of SKTK to impact the dynamics and local structure of a number of secondary structure elements. By correlating the HDX data with unfolding transitions, we can map the effect on Lys$^{101}$ because this residue is responsible for the strong increase in conformational stability. Important elements of the Ig-fold, which are part of the hydrophobic core and connected by an α-helix, are affected by the C-terminal presence of Lys$^{101}$, indicating a more compact and protected protein core.

The presence of Lys$^{101}$ leads to a more structured helix and C-terminal region

$^1$H, $^{15}$N-Heteronuclear single quantum coherence NMR experiments as well as $^1$H, $^{13}$C, $^{15}$N-triple resonance experiments were performed using WT CH2 and the variant EV-CH2-SK. For the NMR analysis, the variant EV-CH2-SK has been employed, because it gave better spectra. The N-terminal extension of the protein did not impact the overall conformation of the CH2 domain (Fig. S3–S6). Additionally, the conformational stability of EV-CH2-SK is not substantially affected by the two introduced N-terminal residues (Fig. S7).

Differences in backbone amide $^1$H and $^{15}$N chemical shifts for CH2 and the variant EV-CH2-SK are indicative for conformational differences (Fig. 5A). In contrast to substantial $^1$H, $^{15}$N-chemical shift changes for residues in the C-terminal region (residues 95–99), we observed no assignable resonances for most residues located in the α-helical regions (residues 10–14 and 72–78). This suggests that these regions change their mode of interaction upon the presence of the Lys$^{101}$. Consistent with that, HDX experiments identified all three regions to be impacted by Lys$^{101}$ (Fig. 4). Moreover, we found intense chemical shift changes for residues capping the α-helices (residues 15–16 and 71–72), which showed less intense HDX (Fig. 4B, regions 1 and 3).

Analysis of the $^{13}$Cα chemical shift of the two proteins yields information on changes in the local secondary structure propensity (28, 29). Positive $\delta^{13}$Cα chemical shift differences ($\delta$(experimental) – $\delta$(random coil)) are indicative for formation of helical structure, whereas negative shift differences suggest the population of β-sheet structure. Of notice, the crystal structure of the CH2 domain is derived from the entire Fc fragment, comprising CH2 including Lys$^{101}$ and CH3. Comparing the two CH2 variants, we observe helical propensity for residues 10–13 and 73–80 in EV-CH2-SK, whereas there were no assignable resonances for the respective residues in the WT (Fig. 5B, results for alternative sets of random coil shifts are provided in Fig. S8–S10). This is in agreement with our finding that HDX is substantially decreased for these regions of CH2-SKTK (Fig. 4) and suggests that the α-helices visible in these regions in the crystal structure (PDB 3HKF) only form in presence of Lys$^{101}$. In addition, the negative secondary chemical shifts observed for the C-terminal region (residues 95–99) is indicative of the formation of a β-strand in EV-CH2-SK that is visible in the crystal structure, but not present in the WT. These additional secondary structure elements, two α-helices and β-strand, contribute to the overall stabilization of the protein observed in the presence of Lys$^{101}$ (Fig. 2).

Lys$^{101}$-helix dipole interactions induces conformational rigidity

Molecular dynamics (MD) simulations were performed on the WT, and the extended variants CH2-S and CH2-SK in
Stability of the $C_{H2}$ antibody domain

Figure 5. NMR chemical shift changes for $C_{H2}$ and EV-$C_{H2}$-SK. A. $\text{H}, \text{N}$-chemical shift differences observed for $C_{H2}$ and EV-$C_{H2}$-SK. The largest changes in chemical shifts occur in the C-terminal part of the protein, whereas the $\alpha$-helical region involving residues 10–14 and 72–78 could not be assigned for $C_{H2}$ WT. Residue numbering was according to the $C_{H2}$ WT sequence. Asterisks indicate unassigned residues. B. $\delta^{13C}$ chemical shift differences ($\delta^{13C}$ experimental) – $\delta^{13C}$ random coil). Random coil chemical shifts were taken from Wishart and Sykes (28). Positive values indicate propensity for an $\alpha$-helix, negative values for a $\beta$-strand. The top panel shows the chemical shift differences for WT $C_{H2}$, the bottom panel for EV-$C_{H2}$-SK. Blue and red bars at the bottom of the lower panel show the secondary structure as indicated in the crystal structure (PDB 3HKF), full height shaded areas highlight the major changes in secondary structure propensity. The secondary chemical shifts of assignable residues in the extended variant indicate the presence of two stable $\alpha$-helices in the regions between residues 10–14 and 73–80 in EV-$C_{H2}$-SK. These residues probably show dynamics on a time scale that makes the residues unresolvable by NMR in the WT. In addition, the C-terminal region of EV-$C_{H2}$-SK shows propensity to form a $\beta$-strand, whereas the same region in the WT appears more likely to occupy a random coil conformation. Asterisks indicate unassigned residues.

Figure 6. Mean solvent accessibility during MD simulations. The ratio in mean SASA of residues in $C_{H2}$ WT relative to $C_{H2}$-SK (green) and relative to $C_{H2}$-SKTK (blue) was calculated for each residue over a simulation period of 1 $\mu$s at 300 K. A SASA ratio >1 indicates increased mean accessibility relative to $C_{H2}$-SK or relative to $C_{H2}$-SKTK, respectively (obtained as sliding window average over 10 consecutive residues).

Figure 7. NMR chemical shift changes for $C_{H2}$ and EV-$C_{H2}$-SK. A. $\text{H}, \text{N}$-chemical shift differences observed for $C_{H2}$ and EV-$C_{H2}$-SK. The largest changes in chemical shifts occur in the C-terminal part of the protein, whereas the $\alpha$-helical region involving residues 10–14 and 72–78 could not be assigned for $C_{H2}$ WT. Residue numbering was according to the $C_{H2}$ WT sequence. Asterisks indicate unassigned residues. B. $\delta^{13C}$ chemical shift differences ($\delta^{13C}$ experimental) – $\delta^{13C}$ random coil). Random coil chemical shifts were taken from Wishart and Sykes (28). Positive values indicate propensity for an $\alpha$-helix, negative values for a $\beta$-strand. The top panel shows the chemical shift differences for WT $C_{H2}$, the bottom panel for EV-$C_{H2}$-SK. Blue and red bars at the bottom of the lower panel show the secondary structure as indicated in the crystal structure (PDB 3HKF), full height shaded areas highlight the major changes in secondary structure propensity. The secondary chemical shifts of assignable residues in the extended variant indicate the presence of two stable $\alpha$-helices in the regions between residues 10–14 and 73–80 in EV-$C_{H2}$-SK. These residues probably show dynamics on a time scale that makes the residues unresolvable by NMR in the WT. In addition, the C-terminal region of EV-$C_{H2}$-SK shows propensity to form a $\beta$-strand, whereas the same region in the WT appears more likely to occupy a random coil conformation. Asterisks indicate unassigned residues.

which corresponds to the short $\alpha$-helical segment in the vicinity of the C-terminal Lys$^{103}$ residue. The absence of this stabilizing interaction with the helix dipole in case of the $C_{H2}$ WT and $C_{H2}$-S likely influences also the conformation and flexibility of the helical segment and in turn causes an increased HDX for $C_{H2}$-S and $C_{H2}$-S. Interestingly, the simulations indicate that this is mostly due to a conformational shift and not necessarily due to increased mobility (Fig. S11).

To explain the reduced stability of the $C_{H2}$ WT and $C_{H2}$-S compared with the $C_{H2}$-SK variant, we performed umbrella sampling (US) free energy simulations along the reaction coordinate that results in dissociation of the C-terminal segment from the folded $\beta$-strand to form a fully solvated conformation (see “Experimental procedures”). Such a transition indicates the free energy contribution of the C-terminal region to stabilize the folded structure. The difference between the calculated free energy for the variants versus the WT is a measure of the loss in stabilization due to the presence of the folded C-terminal strand.

The simulations predict a similar reduced stabilization of $C_{H2}$ and $C_{H2}$-S relative to $C_{H2}$-SK and $C_{H2}$-SKTK, respectively, by ~4 kcal mol$^{-1}$ (compare the difference in plateau-free energy values reached in case of $C_{H2}$ and $C_{H2}$-S versus $C_{H2}$-SK and $C_{H2}$-SKTK variants upon dissociation of the C-terminal strand in Fig. 7). The significant free energy difference in favor of the $C_{H2}$-SK and $C_{H2}$-SKTK variants agrees qualitatively well with the observed differences in melting temperature of the two variants versus WT. Inspection of snapshots from different US windows indicates that the origin of the free energy difference seems to be indeed the interaction of the Lys$^{103}$ side chain with the adjacent helical segment (residues 72–81). This interaction is still visible in the intermediate US windows that result in the largest free energy increase (Fig. 7). At the same US intervals, the C terminus of the WT or the $C_{H2}$-S variant are already dissociated. Hence, the presence of the Lys$^{103}$-helix dipole interaction results in an
Stability of the C_{\varepsilon2} antibody domain

increase of the potential of mean force (PMF) and stabilizes the folded state significantly.

Discussion

Here, we determined the impact of residues located C-terminally of the C_{\varepsilon2} domain in IgG1 on its conformational stability and protein dynamics. Importantly, the investigated terminal residues (SKTK) are not part of defined secondary structure elements as shown in the crystal structure (Fig. 1B, PDB 3HKF). The presence of these residues resulted in a pronounced stability increase of $\sim 14^\circ C$. By assessing C_{\varepsilon12} variants extended by one to four residues, we were able to attribute this strong stabilizing effect to a single residue: Lys$^{101}$. This was rather unexpected, as residues of the $\beta$-sandwich would have been the most likely suspects for determining the stability of the domain. Our analysis revealed a complex interaction pattern that maintains the integrity of the domain. HDX experiments showed significantly decreased dynamics for the $\alpha$-helix connecting the $\beta$-strands E and F. This $\alpha$-helix links both $\beta$-sheets, which make up the characteristic Ig-fold $\beta$-barrel. As a consequence, the hydrophobic core is more compact and this results in an overall higher structural integrity as also indicated by the different microenvironments of the two Trp residues. In this context, our NMR experiments demonstrated that this important $\alpha$-helix is not stably formed in the absence of Lys$^{101}$, which is further supported by increased HDX and surface solvent accessibility (SASA) in this region. In addition, our HDX and NMR data show decreased dynamics for the second $\alpha$-helix connecting $\beta$-strands A and B. Interestingly, this $\alpha$-helix is in close structural proximity to the aforementioned $\alpha$-helix. Thus, the polar interaction between the $\alpha$-helix dipole and Lys$^{101}$ is required to initiate and maintain stable $\alpha$-helices. Moreover, for C_{\varepsilon12}-SK, the secondary structure propensity analysis indicates the formation of the very C-terminal $\beta$-strand G only in the presence of Lys$^{101}$. As the three secondary structure elements are all dependent on Lys$^{101}$, this residue is a key determinant for the overall conformational stability. In agreement with the experimental data, our simulations indicate that the Lys$^{101}$ interaction with the nearby $\alpha$-helix makes a significant free energy contribution in favor of the folded state. Feige et al. (30) showed that the rapid folding of the $\alpha$-helical structures in antibody domains orients the two $\beta$-sheets in a favorable way. In this context, the interaction of Lys$^{101}$ with the $\alpha$-helix and the formation of the second $\alpha$-helix in C_{\varepsilon2} initiate the native folding pathway by assuring the proper orientation of the residues forming the hydrophobic core and the $\beta$-sheets, specifically $\beta$-strands E and F. The conserved polar interaction between Lys$^{101}$ and the $\alpha$-helix dipole indicates a stabilizing effect of Lys$^{101}$ on the Ig-fold, mediated by the $\alpha$-helix between $\beta$-strands E and F (7). Thus, in addition to the intramolecular disulfide bonding, the $\alpha$-helix between strands E and F, stabilized by Lys$^{101}$, makes an important contribution. By sequential and structural alignment, we found that Lys$^{101}$, or alternatively another positively charged residue like arginine, and its interaction with the adjacent $\alpha$-helix is conserved among constant Ig domains except C_L and C_{\varepsilon1} of which the latter one is natively unfolded (Fig. S12) (31). Interestingly, C-terminal charged residues of C\varepsilon1 interact with an $\alpha$-helix of C_L when associated in a Fab fragment (PDB 2QSC and 3DZ9), potentially affecting the conformational stability of both domains. Moreover, C\varepsilon1 comprises a C-terminal Glu followed by a highly conserved Cys, which connects the light chain with the heavy chain by linking C_L and C_{\varepsilon1} (PDB 2FB4). Thus, except for C_L and C_{\varepsilon1}, the structural feature identified in this study seems to be evolutionarily favored to assure the proper initiation of folding and conformational stability of other constant Ig domains. Traditionally, the formation of a hydrophobic core is seen as the main stabilizing interaction that determines the folding and stability of a globular protein (32–34). However, the conserved polar interaction between a positively charged side chain and an $\alpha$-helix (dipole) at the surface of the C_{\varepsilon12} domain also make a significant contribution to stability as shown by our free energy simulations and unfolding studies. A solvent-protected core, optimally oriented $\beta$-sheets, and proper folding of secondary structure elements like the interacting $\alpha$-helix and a C-terminal $\beta$-strand are thus important factors for structural integrity and explain the strong increase in conformational stability. A strong stabilization by an interaction

Figure 7. Calculated free energy change upon dissociation of the C-terminal $\beta$-strand from its placement in the folded structure to a solvated unfolded state. The free energy change (PMF) was calculated along a reaction coordinate that corresponds to the center of mass distance of backbone atoms of the C-terminal segment (delimited by residues 94 to 98) and the backbone atoms of the protein (delimited by residues 1 to 91). Representative snapshots taken in the folded, intermediate, and dissociated states of the C-terminal segment are indicated (colorcode of the cartoon representation corresponds to the line color in the plot). The C-terminal Lys residue in C_{\varepsilon2}-SK is indicated as van der Waals sphere representation and still interacts with the helical segment (residues 72–81) in the intermediate state (at which the C terminus of the C_{\varepsilon2} WT is already fully dissociated).
Stability of the C\(_{\mu}2\) antibody domain

between an \(\alpha\)-helix dipole and a residue, which is not part of this \(\alpha\)-helix has not been observed before. However, a stabilizing effect of \(\alpha\)-helices by charged residues within the same \(\alpha\)-helix, particularly Glu capping the \(\alpha\)-helix, has been reported (35–37).

Our results also raise the question how to determine immunoglobulin domain boundaries correctly. When the first Ig domain sequences were described, various individual domains were aligned, e.g. murine C\(_{\mu}1\), C\(_{\mu}2\), C\(_{\mu}3\), and C\(_{\mu}4\), derived from different Ig isoforms (IgM, IgD, IgG, etc.) (38, 39). This method revealed significant homology between the different domains such as conserved cysteines and tryptophanes along with other residues (40). In parallel, it was found that every constant domain is encoded by an individual exon, which raised evidence for a separation of the domains already on the genetic level (41). Hence, the first and last residues of constant domains are determined by RNA splicing (42). This strategy of defining Ig domain boundaries was supported by structural data (43). In contrast to the constant domains, the structures of the V\(_{\lambda}1\) and V\(_{\lambda}1\) domains have been described in detail to systematically assign framework regions and complementary determining regions, of which the latter ones facilitate antigen binding (44, 45). As antibody folding and stability were primarily attributed to the hydrophobic core, the conserved disulfide bond, and the characteristic \(\beta\)-strands, domain boundaries lost relevance (3, 7, 22, 26, 46). However, as we showed for a murine V\(_{\lambda}1\), the N- and C-terminal residues, i.e. \(\text{Ile}^2\) and \(\text{Arg}^{108}\), play a major role for the integrity of the domain (5, 6). Together with other recent data (16), this renews the question of domain boundaries, especially in the context of the increased use of antibody fragments as diagnostics and therapeutics. Historically, during the early development of single chain variable fragments, the boundaries of V\(_{\lambda}1\), V\(_{\lambda}1\) Fc, and C\(_{\mu}3\) were randomly set by optimizing the position of the linker between both domains with respect to the orientation of the two domains within the heterodimer (47, 48). Biophysically optimized rather than genetically determined N and C termini might serve as a complementary method to increase the conformational stability of antibody fragments by terminal residue-based \(\alpha\)-helix stabilization resulting in lower aggregation propensity, improved pharmacokinetics, and shelf life. This approach might thus contribute to more stable, reliable therapeutics and diagnostics and is potentially applicable for engineering of other constant antibody domains. Therefore, we propose to consider the impact of N- and C-terminal linker residues for defining Ig domain boundaries. Taken together, the Ig-fold is not only maintained by intramolecular disulfides and the packing of the hydrophobic core. Ionic interactions of surface-exposed residues play an important role to facilitate and maintain proper folding of the conserved \(\alpha\)-helix and in turn the \(\beta\)-barrel structure, thereby protecting the hydrophobic core and assuring conformational stability.

Experimental procedures

Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma. Unless otherwise stated all measurements were carried out in PBS buffer (pH 7.4) at 25 °C.

Cloning, mutagenesis, expression, and purification

The C\(_{\mu}2\) encoding plasmid was previously described (15). Subcloning of the C\(_{\mu}2\) WT gene into the pET28b expression vector (Merck/Novagen, Darmstadt, Germany) was performed using sequence- and ligation-independent cloning (SLIC) (49) and transformed into Escherichia coli BL21 (DE3)-star cells (Invitrogen). C\(_{\mu}2\) variants were generated utilizing overhang primers with SLIC (50). For modifications, we used PCR primers with an appropriate 5’ overhang in the reverse primer. Success of subcloning and mutagenesis was verified by sequencing. Added residues are indicated by the one letter code behind the WT domain name, e.g. CH2-SKTK.

All variants were expressed and purified as previously described for antibody V\(_{\lambda}\) domains (5). Briefly, cells were grown in LB\(_{\text{kan}}\) medium to an \(A_{600}\) of 0.6–0.8 at 37 °C. Overnight protein expression was induced by 1 mM isopropyl 1-thio-\(\beta\)-d-galactopyranoside. For NMR experiments, expression of \(^{15}\text{N}\)-labeled or \(^{13}\text{C},^{15}\text{N}\)-labeled proteins was performed in M9 minimal medium containing \(^{15}\text{NH}_4\)Cl and glucose or \(^{13}\text{C}\)glucose (Cambridge Isotope Laboratories Inc., Andover, MA), respectively. Cells were harvested the next day and inclusion bodies were prepared as previously described (51). Inclusion bodies were solubilized and denatured in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 8 mM urea, 0.1% 2-mercaptoethanol and subsequently purified using a Q-Sepharose column (GE Healthcare, Uppsala, Sweden), followed by a Superdex 75 16/60 gel filtration column (GE Healthcare). Between the two purification steps, the proteins at a concentration of approximately 1 mg/ml were refolded overnight by dialysis against 250 mM Tris-HCl (pH 8.0), 100 mM L-Arg, 5 mM EDTA, 1 mM oxidized GSH, and 0.5 mM reduced GSH at 4 °C. Protein purity and identity were verified by SDS-PAGE.

Circular dichroism spectroscopy

CD measurements were performed using a Jasco J-715 spectropolarimeter (Jasco, Grossumstadt, Germany) equipped with a Peltier element. Near- and far-UV CD spectra were measured using 50 or 10 \(\mu\)M protein in a 2- or 1-mm path length quartz cuvette between 320 and 260 nm or 260 and 300 nm wavelengths, respectively. Spectra were accumulated 16 times, buffer-corrected, and normalized for mean residue molar ellipticity. Thermal transitions were recorded using 10 \(\mu\)M protein in a 1-mm path length quartz cuvette at 215 nm wavelength with a heating rate of 30 °C/h. Transitions were fitted using a Boltzmann function.

SEC-MALS

The oligomeric state of C\(_{\mu}2\) variants was determined using a GE Superdex 75 10/300 GL column connected to a Shimadzu HPLC system (Shimadzu, Munich, Germany) equipped with Shimadzu Refractive Index- and UV-detectors as well as a Wyatt Dawn Helios II multiangle light scattering detector (Wyatt Technology, Dernbach, Germany). The column was equilibrated for at least 24 h to obtain a
stable MALS detector baseline prior to data collection. Inter-detector delay volumes, band broadening, light-scattering detector normalization, and the instrument calibration coefficient were set using 1 mg/ml of BSA (Sigma, Munich, Germany) in PBS according to standard protocols. 50 μl of 50 μM protein samples was loaded on the column. All experiments were performed at room temperature at a flow rate of 0.45 ml/min in PBS (pH 7.4), 0.05% NaN₃. The molecular mass and mass distribution of C₄₂ were determined using the ASTRA 5 software (Wyatt Technology, Dernbach, Germany).

**Intrinsic tryptophan fluorescence spectroscopy**

Intrinsic tryptophan fluorescence spectra were measured with 7 μM protein in a 1-cm quartz cuvette at 22 °C using a Jasco FP-6500 spectrofluorometer (Jasco, Grossumstadt, Germany). Fluorescence emission spectra were recorded between 300 and 400 nm with an excitation wavelength of 280 nm. Slits were set to 5 nm for both excitation and emission. All spectra were recorded two times, averaged and normalized.

For chemical equilibrium unfolding transitions, we incubated 5 μM protein with increasing concentrations of GdmCl for at least 12 h at room temperature in black 96-well microplates (number 655809, Greiner Bio-One). Tryptophan fluorescence spectra of protein samples incubated with either 0 or 4 M GdmCl were recorded the next day. By subtracting the two spectra we determined the maximal differential emission wavelength (357 nm), which was set constant for measuring the fluorescence intensity of all samples at 22 °C. Fixed wavelength measurements were performed using an Infinite M Nano microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Equilibrium unfolding transitions were fit as described before (52).

For acrylamide quenching, increasing concentrations of acrylamide were added to 5 μM protein and incubated overnight at room temperature to assure quenching equilibrium. Spectra were measured as described above. Fixed wavelength measurements were carried out using 280 and 338 nm for excitation and emission, respectively. F₀ represents the fluorescence intensity without any acrylamide. The calculated Stern-Volmer quotient F/F₀, where F is the fluorescence intensity at the respective acrylamide concentration shows the accessibility of a C₄₂ variant for acrylamide (24, 25).

**HDX-mass spectrometry**

HDX-MS experiments were performed using a fully automated system equipped with a Leap PAL RTC (LEAP Technologies, NC), a Waters ACQUITY M-Class UPLC, a HDX manager, and the Synapt G2-S electrospray ionization TOF mass spectrometer (Waters Corp.), as described elsewhere (53). Protein samples with a concentration of 30 μM were diluted 1:20 with deuterium oxide in PBS (pH 7.4) and incubated at 20 °C for 10 s, 1 min, 10 min, 30 min, and 2 h. To stop the labeling reaction and denature the sample, the labeled protein was diluted 1:1 in quenching buffer (200 mM Na₂HPO₄ × 2H₂O, 200 mM NaH₂PO₄ × 2H₂O; 250 mM tris(2-carboxyethyl)phosphine, 3 M GdmCl, pH 2.2) at 1 °C. Digestion was performed on a Waters Enzymate BEH Pepsin Column (2.1 × 30 mm) at 20 °C. Peptides were trapped and subsequently separated on a Waters ACQUITY UPLC BEH C18 column (1.7 μM, 1.0 × 100 mm) with acetonitrile plus 0.1% formic acid (v/v) and H₂O plus 0.1% formic acid (v/v) gradients. Trapping and chromatographic separation were carried out at 0 °C to minimize back-exchange. Eluting peptides were directly subjected to the TOF mass spectrometer by electrospray ionization. Before fragmentation by MS² and mass detection in resolution mode, the peptide ions were additionally separated by drift time within the mobility cell. Data processing was performed using the Waters Protein Lynx Global Server PLGS (version 3.0.3) and DynamX (version 3.0).

**NMR spectroscopy**

¹H,¹⁵N-Heteronuclear single quantum coherence NMR spectra, as well as ¹H,¹³C,¹⁵N-triple resonance spectra were acquired on a Bruker AVIII 500 or 600 MHz spectrometer with a cryogenic triple resonance gradient probe. The respective ¹⁵N- or ¹³C,¹⁵N-labeled proteins were dissolved in PBS buffer supplemented with 10% D₂O. Backbone assignments were based on three-dimensional HNCA (54–56), HNCAB (57, 58), and HN(CO)CACB (58, 59) spectra. All spectra were processed using Bruker TopSpin, and analyzed using CcpNmr (60). The protein concentrations were in the range of 60–1200 μM for two-dimensional experiments and in the range of 600–1200 μM for three-dimensional experiments.

¹H,¹⁵N-Chemical shift perturbations (Δδ) were calculated as (61),

\[
\Delta \delta^{1H,15N} = \sqrt{\frac{1}{2} (\Delta \delta_{1H}^2 + (0.14 \Delta \delta_{15N}^2))}
\]

with \(\Delta \delta_{\text{Nuc}} = \delta_{\text{Variant} 1} - \delta_{\text{Variant} 2}\). C-Chemical shift perturbations relative to a random coil peptide (secondary chemical shifts) were calculated using the difference between the observed chemical shift and the random coil chemical shift for the respective residue type (28).

**MD simulations**

MD simulations were carried out and analyzed using the Amber16 simulation package (67). Free unrestrained MD simulations and US simulations were performed to analyze the stability of the C₄₂ antibody domain employing the pmemd.cuda module of Amber16 (62). Simulations were performed on the WT C₁₂ as well as variants C₁₂-S and C₁₂-2SK (all based on the crystal structure PDB 3HKF). Each protein was solvated in TIP3P water (63) in a periodic octahedron box with a minimum distance of protein atoms to the box boundary of 10 Å. The ff14SB force field was employed and Na⁺ and Cl⁻ ions were added to neutralize the system and reach an ion concentration of 0.15 M. Energy minimization of each system was performed with the sander module of Amber16 (1500 minimization cycles). The systems were heated in steps of 100 K (10 ps per step) to a final temperature of 300 K with the solute nonhydrogen atoms harmonically restraints to the start structure.
Stability of the C\textsubscript{µ}2 antibody domain

All bonds involving hydrogen atoms were kept at optimal length. In additional 6 steps the harmonic restraints were removed stepwise. For the production simulations (i.e. started from structures at equilibrium gained from the previous equilibration phase) hydrogen mass repartitioning was employed allowing a time step of 4 fs (instead of 2 fs used during heating and equilibration). Unrestrained production simulations were extended to 1 µs for each system. Coordinates were saved every 2 ps. The root mean square deviation from the experimental structure and SASA, using the LCPO algorithm (64), were calculated using the Amber cpptraj module. The change in mean SASA per residue was calculated as difference of average SASA (per residue) of each variant versus WT. It was smoothed by averaging the results over a window of 10 consecutive residues along the whole sequence.

By using the US method (65), it is possible to efficiently extract free energy changes along a coordinate of interest by forcing it to overcome possible energy barriers. To estimate the influence of the C-terminal β-strand segment (residues 94–98) on the folding stability of the protein, we defined the reaction coordinate as the distance d between the centers of mass of the C terminus (delimited by residues 94 to 98) and the rest of the protein (delimited by residues 1 to 91). The C terminus of the protein can be dissociated gradually by applying a harmonic penalty potential with the force constant K around a reference distance d\textsubscript{ref}. A total of 25 umbrella windows with different values for K and d\textsubscript{ref} were simulated for each biomolecule to obtain overlapping histograms in d for reliable calculation of the potential of mean force (PMF: free energy change along d).

The three sets of generated umbrella windows were: (i) 11 consecutive simulations with d\textsubscript{ref} varying between 10.0 and 20.0 Å with a step of 1.0 Å and a force constant of K = 2.5 kcal/Å\textsuperscript{2} mol, (ii) 11 consecutive simulations with d\textsubscript{ref} varying between 12.0 and 17.0 Å with a step of 0.5 Å and a force constant of K = 3.5 kcal/Å\textsuperscript{2} mol, (iii) 3 consecutive simulations with d\textsubscript{ref} varying between 12.5 and 13.5 Å with a step of 0.5 Å and a force constant of K = 5.0 kcal/Å\textsuperscript{2} mol. In each umbrella window, simulations of 50 ns at 300 K were performed (time step 2 fs), whereby positional restraints with a force constant of K\textsubscript{pos} = 0.02 kcal/Å\textsuperscript{2} mol were applied to nonhydrogen atoms in residues 1–91. A PMF along the reaction coordinate d was calculated using the WHAM algorithm (66).

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