Determinants of the assembly and function of antibody variable domains

V28

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Abstract

Antibodies are modular proteins. The Fv fragment is the most important module for antigen binding and consists of the two variable domains, V\textsubscript{L} and V\textsubscript{H}. They exhibit the conserved antibody fold beta sheet structure and comprise highly variable loops (complementarity determining regions, CDRs) for antigen binding. Despite the importance of their association to form the antigen binding site, little is known about the contributions of the framework residues and CDRs on organizing this functional unit. To address this question, we exchanged conserved interface residues as well as CDR loops and tested the effects on structure, stability, association and antigen binding.

Our results show that the affinity between the two MAK33 variable domains studied is not very high, with a K\textsubscript{D} of 0.2 µM. For the V\textsubscript{L} domain, the exchange of conserved residues had only slight effects on structure, stability and functionality, except for a conserved proline residue which was crucial for both V\textsubscript{H}-V\textsubscript{L} association and antigen binding. For the V\textsubscript{H} domain however, all the conserved residues analyzed exhibited a strong influence on the properties of this domain. The most crucial V\textsubscript{H} residue for V\textsubscript{H}-V\textsubscript{L} association and antigen binding was a conserved leucine. The CDRs modulate the domain framework to a significant extent as shown by swap experiments. This includes effects on antigen binding and domain association, which interestingly, do not always correlate. Thus, the framework region is not necessarily the determining factor for V\textsubscript{H}-V\textsubscript{L} association, the CDRs also contribute. Vice versa, antigen binding is also influenced by framework mutations. Taken together, for the architecture and function of the variable domains, both, the nature of the interface and the interplay with the CDR loops are of crucial importance.
In the humoral immune response, antigen recognition is mediated by immunoglobulins, specifically by the N-terminal variable domains of the light chain (V_L) and of the heavy chain (V_H) which associate noncovalently to form the so-called Fv-fragment. Three hyper-variable regions (complementarity determining regions or CDRs) in V_L and V_H comprise the residues interacting with antigens. They account for approximately 25% of the variable domains [1]. CDR-H3 (i.e., the third CDR of V_H) is the most diverse of these six regions concerning length and amino acid sequence [2]. Apart from the CDRs, both variable domains exhibit a conserved β-barrel framework stabilized by an internal disulfide bridge [1-4]. They are composed of two β-sheets, one with four strands (A,B,D,E) and one with six strands (A’,G,F,C,C’,C”), with the strands GFC‘C involved in the V_H-V_L interface. The interface β-sheets are additionally twisted, leading to a three-layer packing. The residues from the edge strands are the central part of the interface [1]. Several studies on the V_H-V_L packing geometry showed that residues within the framework as well as interface contributing residues of the CDRs can influence the interface [1, 3, 5-7]. 75 % of the interface residues are constituted by framework β-sheets and 25 % by the hypervariable loops (inter-strand links between GF, BC and C‘C”, respectively) [1, 3] especially the so-called proximate zone situated at the base of the antigen binding site and comprising residues that do not actively participate in the interface. [1, 3, 5-7]. As the association of V_L and V_H is crucial for antigen binding [3], understanding the underlying principles is of great importance.

For the V_H domain which is composed of about 125 residues and the V_L domain with about 110 residues, Chothia and co-workers suggested that the interface residues at positions 98, 44 and 36 in V_L and 103, 47, 45 and 37 in V_H according to Kabat numbering [8] are conserved [1, 5]. Wang and co-workers [9] aimed at identifying amino acid networks important for V_H and V_L function by covariation analysis. This multiple sequence alignment approach investigates covariations between residues at all possible positions. This allows to reveal conserved amino acids by the correlation of the presence of one particular amino acid with the presence of a second one at a particular sequence position. In their study they included more than 2000 V-class sequences of human, mouse, cow, camel, llama, macaque and chicken with a bias of human sequences (574 out of 2432). Generally, the majority of the most strongly
conserved amino acids identified in this study were positioned at the V<sub>H</sub>-V<sub>L</sub> interface [9]. For V<sub>L</sub>, amino acids Y36, Q37, P44, A43, L46 and F98 were found to be highly conserved, with all residues except Q37 directly in contact with V<sub>H</sub>. On the V<sub>H</sub> side, amino acids V37, R38, G44, L45, E46, W47 and W103 could be identified in the V<sub>H</sub>-V<sub>L</sub> interface with all residues except E46 and R38 in direct contact with V<sub>L</sub>. V<sub>H</sub> W47 seems to be the central node based on the number and strength of its covariations with other interface residues, the same holds true for Y36 and P44 for the V<sub>L</sub> domain.

Further computational analyses revealed two main modes of interaction for V<sub>H</sub> and V<sub>L</sub> which is either characterized by a proline or a medium/large hydrophobic residue at position 44 in V<sub>L</sub> [7]. Concerning V<sub>H</sub>, W47 seems to be essential as it was previously mutated for improved solubility and stability but none of the mutations (W47L, W47R [10]) were favorable. To produce a stable monomeric V<sub>H</sub>, three highly conserved hydrophobic interface residues in V<sub>L</sub> [44, 45, 47], were exchanged by hydrophilic residues as commonly found in camelid V<sub>H</sub>Hs [11-14]. Interface residues can also affect antigen binding [3, 7, 15-17] by influencing the positioning of hypervariable loops. Additional experimental studies addressed the influence of the exchange of particular conserved amino acids on the association of V<sub>H</sub> and V<sub>L</sub> via the stabilities of covalently linked scFv and Fab fragments [18-22]. While these studies lay the groundwork, we are still far from a detailed and comprehensive understanding of the organization of the Fv interface. In this context, it is important to determine the affinity of the association of V<sub>L</sub> and V<sub>H</sub> directly. Strikingly, for the other domain interactions in IgG the K<sub>D</sub> values differ by several orders of magnitude. For the C<sub>H</sub>3 dimer a K<sub>D</sub> < 10<sup>-10</sup> M was determined using SEC [23, 24] and the K<sub>D</sub> for the interaction between C<sub>H</sub>1 and C<sub>L</sub> was 6.2 µM [25]. This analysis is largely lacking for the V<sub>L</sub>/V<sub>H</sub> interaction.

Here, we chose to use the V<sub>L</sub> and V<sub>H</sub> domains of the murine monoclonal antibody MAK33 (κ/IgG1 subclass) as a well-studied model system [16, 18, 30, 32] to analyze the contribution of different factors on V<sub>L</sub> and V<sub>H</sub> structure and function. Their sequences contain all the conserved residues identified by Wang and coworkers except for an alanine at position 43 in V<sub>L</sub>, which is exchanged to serine in MAK33. Since the relative importance of the conserved residues for structure, stability, association and antigen binding is not clear, we mutated every conserved residue against alanine and analyzed the properties of the variants in a comprehensive manner. Additionally, we generated two V<sub>L</sub> double point mutations (Y36A/P44A,
Y36A/S43A) to investigate the potential synergistic nature of the mutation. Importantly, we focused in the analysis on the isolated variable domains and the direct influence of point mutations on their interaction, and not on Fab or scFv fragments as in previous studies [16, 20, 22] to draw conclusions concerning their stabilities and antigen binding properties. Furthermore, we performed CDR exchange experiments to address the contribution of these structural elements on domain architecture.

Our results on the effects of mutations on domain structure, stability, association and antigen binding together with CDR exchange experiments reveal complex relationships between structural and functional properties within the $V_L$ and $V_H$ domains.
Results

The association of \( V_H \) and \( V_L \) is particularly sensitive to mutations in \( V_H \)

To determine the influence of specific residues on the association of \( V_L \) and \( V_H \), amino acids were selected for mutation which had been predicted to be important [9]. Based on the results of Wang and coworkers we created six single (Y36A, Q37A, S43A, P44A, L45A, and F98A) and two double point mutants for \( V_L \) (Y36AP44A and Y36AS43A), as well as seven single point mutations for \( V_H \) (V37A, R38A, R44A, L45A, E46A, W47A, and W103A). As shown in figure 1A, these residues lie in or near the interaction interfaces of \( V_L \) and \( V_H \). For R38A and E46A in \( V_H \), it turned out that the variants were unstable and aggregation-prone. Therefore, they were not considered further.

In previous studies, the association of the \( V_L \) and \( V_H \) domains was analyzed using scFvs or Fab fragments [16, 20, 22]. This is a relatively indirect approach, as the domains were either artificially linked (scFvs) or two additional covalently linked domains were present (C\(_L\) and C\(_H\)1 in the Fab). Here, we used assays that report directly on the formation of the \( V_L \) and \( V_H \) heterodimer. With sedimentation equilibrium analytical ultracentrifugation (AUC) experiments, a K\(_D\) of 0.2 ± 0.05 µM was determined for the wild type (wt) \( V_L \) and \( V_H \) domains (table 1). Hence, the variable domains interact more strongly than C\(_H\)1 and C\(_L\) [25], but have a lower affinity compared to the C\(_H\)3 homodimer [23, 24].

For all \( V_H \) mutants studied, a decrease in the binding affinity was observed. According to the K\(_D\) values determined by AUC, W47A and R44A have a 6 - 8 fold higher K\(_D\) compared to \( V_H \) wt. V37A, L45A and W103A showed a marked decrease in affinity with a K\(_D\) that was two orders of magnitude higher and for L45A the interaction with \( V_L \) was hardly detectable, resulting in a K\(_D\) of ~ 300 µM (table 1). Surprisingly, the mutation of residues in \( V_L \) had only minor effects on the affinity for \( V_H \). Only \( V_L \) P44A (figure 1; highlighted in red) with a K\(_D\) of 15.2 µM strongly decreased the interaction between \( V_L \) and \( V_H \). (table 1, figure 1 B; orange). Concerning the two double mutations (Y36A/P44A, Y36A/S43A) which were generated to investigate whether the effects observed for single mutants are additive, unexpected results were obtained. Y36A/S43A, which is the combination of two point mutations with no change in K\(_D\), leads to the expected wt like K\(_D\). But the point
mutation P44A with the highest $K_D$ for the association with $V_H$ (15.2 µM), exhibited improved binding when combined with Y36A, suggesting compensatory effects (table 1).

In conclusion, with the exception of P44, replacement of each of the conserved residues of $V_L$ does not affect dimerization with the $V_H$ domain. In contrast, most of the conserved $V_H$ residues showed a clear influence on association.

**The role of conserved residues for variable domain structure and stability**

Besides influencing the interaction between the variable domains, the conserved residues might also play a role in the structures of the individual domains. To address this, we recorded CD spectra. The characteristic shape of the $V_L$ FUV-CD spectrum [26] was observed for all the alanine exchange mutants tested (figure 2 A), with only small deviations in the amplitude observed for some variants (e.g. F98A, P44A or Y36A). Thus, the β-sheet structure of the $V_L$ domain seems to tolerate single point mutations of conserved residues. NUV-CD spectra confirm this conclusion (figure 2 C). In the case of the $V_H$ domain, point mutants exhibited more pronounced effects on the FUV-CD spectra (figure 2 A and B) including shifts in the minimum (e.g. W47A). The same holds true for the NUV-CD spectra (figure 2 C and D), suggesting major changes in the tertiary structure. This supports the notion that the structure of $V_H$ is less tolerant than $V_L$ against the exchange of conserved residues.

To determine whether the stability of the respective domain was affected by the mutation of conserved residues, denaturant-induced (GdmCl) unfolding transitions were measured (figure 3, table 1). All transitions were fitted to a two-state model. The $V_L$ domain showed a midpoint for GdmCl-induced unfolding of 1.17 ± 0.68 M GdmCl. Strikingly, $V_L$ S43A was slightly more stable than the wt against GdmCl-induced unfolding (figure 3 A and C, table 1). However, the variants $V_L$ Y36A and $V_L$ L46A exhibited decreased $D_{1/2}$ values, compared to the wt. The cooperativities of these unfolding transitions are in a comparable range for all $V_L$ variants, from 15.8 ± 8.6 to 33.7 ± 18.6 kJ mol⁻¹M⁻¹ (figure 3, table 1). For the least stable $V_L$ point mutation Y36A, two double mutations (Y36A/P44A, Y36A/S43A) were generated to investigate the potential synergistic nature of the observed effects. Indeed when incorporating the most stable mutation, S43A, the stability of Y36A/S43A increased slightly.
compared to Y36A. For Y36A/P44A, where P44A alone has wt-like stability, almost no difference in stability was visible compared to Y36A (figure 3, table 1).

For V\textsubscript{H} wt, we determined a midpoint for GdmCl-induced unfolding of 0.21 ± 0.2 M GdmCl and thus this domain is much less stable than V\textsubscript{L} (figure 3 B and D, table 1). The analysis of the V\textsubscript{H} mutants showed that the stability of this domain is very sensitive to manipulations of conserved residues. It is conspicuous that all mutants already start to unfold in the presence of low GdmCl-concentrations (figure 3 B). The point mutations V\textsubscript{H} W47A and V\textsubscript{H} V37A showed the largest decrease in stability (table 1), while V\textsubscript{H} L45A, and V\textsubscript{H} W103A were slightly less stable than the wt and V\textsubscript{H} G44A was as stable as the wt. The cooperativity of unfolding for different V\textsubscript{H} mutations was subject to variation, from 15.3 ± 10.5 to 75.2 ± 19.4 kJ mol\textsuperscript{-1}M\textsuperscript{-1} suggesting that structural changes occurred [27].

As there is no correlation between the cooperativity values and the structural data obtained by CD measurements (figure 2) this assumption cannot be confirmed.

In summary, the stability of the V\textsubscript{H} domain is more sensitive to mutations than that of the V\textsubscript{L} domain. Our analysis identified W47A for V\textsubscript{H} and Y36A for V\textsubscript{L} as the least stable point mutations. Interestingly, V\textsubscript{L} S43A was even more stable than the wt protein. For the double mutants, an additive effect on the stability could be observed.

**Antigen binding of V\textsubscript{H} and V\textsubscript{L} is influenced by conserved interface residues**

To test how the mutation of conserved amino acids influences antigen recognition by the Fv fragment, we set up an ELISA for the MAK33 Fv-fragment and human creatine kinase as its antigen. To this end, the respective wt domain was produced with a FLAG-tag at the C-terminus for detection with an anti-FLAG antibody. The tag does not negatively influence stability, folding and the interaction between the variable domains (data not shown). In this ELISA, a concentration-dependent increase in signal is only observed when V\textsubscript{L} and V\textsubscript{H} are combined (figure 4). As the assay involves several protein interactions, only conclusions on an apparent K\textsubscript{D} seem reasonable (figure 4, table 1). For the wt Fv with either V\textsubscript{L} or V\textsubscript{H} tagged with FLAG similar K\textsubscript{Dapp}s were determined (data not shown). Antigen binding was found to be abolished for V\textsubscript{H} W47A. Apart from that, the V\textsubscript{H} mutation L45A led to the strongest reduction in the affinity for the antigen with a 12 fold increased K\textsubscript{D} compared to wt, while V37A and W103A exhibited a less pronounced decrease with a 2 fold higher K\textsubscript{D}.
In general, for the V\textsubscript{H} point mutations a low antigen binding activity correlates with a high K\textsubscript{D} for association of the Fv-fragment (see table 1). However, W47A, showed only a small decrease in the K\textsubscript{D} but no binding to the antigen.

For the V\textsubscript{L} domain, there is in most cases no apparent correlation between the K\textsubscript{D} for association and antigen binding. The V\textsubscript{L} mutations L46A, F98A, P44A, as well as the double mutant Y36AP44A have a negative influence on antigen binding (table 1), with a 5 – 12 fold increased K\textsubscript{D}. As already observed for V\textsubscript{H}/V\textsubscript{L} association, P44A exhibited the weakest binding with a 12 fold higher K\textsubscript{D} for the antigen. Interestingly, the double mutant Y36A/P44A showed only 50% of the impairment of P44A alone, so there must be a compensating effect of the Y36A mutation. This coincides with the data for the V\textsubscript{H}/V\textsubscript{L} association (table 1), where P44A exhibited the worst K\textsubscript{D} while the double point mutation Y36A/P44A showed a 2 fold higher affinity than P44A alone. Surprisingly, V\textsubscript{L} L46A and F98A, which exhibited an affinity for the V\textsubscript{H} domain similar to the wt, were defective in antigen binding (table 1). Consequently, the analysis of V\textsubscript{L} mutants supports the assumption that the affinity between V\textsubscript{H} and V\textsubscript{L} is not necessarily correlated with the ability to bind the antigen.

The CDR regions affect domain structure, stability and association

The CDRs of the antibody variable domains are elements of natural variations. How variations in these elements affect their association, structure and stability is therefore of special interest to obtain a comprehensive picture of the factors shaping the Fv-fragment. To address this question, we switched CDRs between MAK33 and unrelated variable domains. We chose human variable domain consensus sequences [28] with CDRs of similar length. For V\textsubscript{L}, we selected the 1DH5 domain, and for V\textsubscript{H} 1DHU. These human variable domains represent a class of variable domains with a highly stable structure [29].

The K\textsubscript{D}s for the association of grafted variants with wt domains were determined by AUC. For the association of the MAK33 V\textsubscript{H} domain containing the 1DHU CDRs (1DHU_MAK V\textsubscript{H}) with MAK33 V\textsubscript{L}, a K\textsubscript{D} of 0.4 \mu M was obtained. This corresponds to the value determined for the MAK33 wt domains (table 1). Wt 1DHU V\textsubscript{H} was an insufficient binding partner for MAK33 V\textsubscript{L} - with a K\textsubscript{D} of 8.8 \mu M. Interestingly, the interaction could be improved by grafting the MAK33 CDRs into the 1DHU framework. This chimera (MAK_1DHU V\textsubscript{H}) bound to MAK33 V\textsubscript{L} with a K\textsubscript{D} of 1.0 \mu M.
These observations lead to the conclusion that while for $V_H$ the framework region is important for the interaction between MAK33 $V_L$ and $V_H$, the CDRs can exhibit a marked influence. Concerning the $V_L$ grafting constructs, the observations are different: the MAK33 $V_L$ construct containing the 1DH5 CDRs (1DH5_MAK) binds to MAK $V_H$ with a roughly 10 fold lower affinity than wt $V_L$ which is similar to the 1DH5 wt value. In contrast, grafting the MAK33 CDRs onto the $V_L$-1DH5 framework (MAK_1DH5) gave a wt-like $K_D$ of 0.3 µM. So for the $V_L$ domain, the CDRs and not the framework are the determining factor for the affinity towards $V_H$ wt.

The FUV and NUV spectra of the grafting mutants gave a similar picture. The CDR exchange (1DH5_MAK) showed a FUV-CD spectrum similar to 1DH5 $V_L$ while the spectrum of MAK_1DH5 was different from both wts (figure 5 B). So already on the secondary structure level the CDRs seem to be structurally important. The NUV-CD spectra exhibit a similar pattern (figure 5 D) but here the difference in the number of aromatic amino acids, predominantly concerning the CDRs, could also play a role.

For the $V_H$ domain, both grafting mutants showed similar FUV-CD spectra (figure 5 C). The same was observed for the NUV-CD spectra of the grafting mutants compared to the wt domains; all spectra were similar in shape with variations in amplitude (figure 5 E). As for $V_L$ the observed NUV deviations might also be due to the different numbers of aromatic amino acids. For both domains the CDRs of MAK33 and 1DHU/1DH5 differ by one tryptophan and several tyrosins. Generally for MAK $V_H$, the CDR exchange does not exhibit the same impact as for the $V_L$ domain.

GdmCl-induced transitions of the different $V_L$ domains showed that, in comparison, the MAK33 $V_L$ domain is least stable against chemical denaturation (figure 6 A). As expected [19], the most stable domain was 1DH5 with a $D_{1/2}$ of 2.39 ± 1.21 M. The grafted mutants showed stabilities in-between MAK33 $V_L$ and 1DH5. Interestingly, the stability of the MAK33 $V_L$ framework was increased when the CDRs were exchanged against the CDRs of 1DH5. 1DH5_MAK $V_L$ was about 20% more stable than MAK33 $V_L$ (figure 6 D). On the $V_H$ side, 1DHU showed the highest stability. The grafting mutants were again in-between (figure 6 B). When the CDRs of 1DHU $V_H$ were transplanted into MAK33, the chemical stability increased, however the transition was less cooperative compared to MAK33 $V_H$ (figure 6 B). Exchanging only CDR H3 in MAK33 $V_H$ led to a slight increase in stability compared to the wt (figure 6 B). The exchange of the CDRs of 1DHU against MAK33 CDRs decreased its stability. In
summary, the analysis of the chemical stabilities allowed the same conclusion for $V_L$ and $V_H$: the CDRs influence the stability of the variable domains decisively.

When antigen binding of the chimera was analyzed, the exchange of the MAK33 CDRs with that of the human consensus sequences led to a complete abolishment in both cases, as expected. For the grafting of MAK33 CDRs on the human variable domains, an interesting picture emerged. $V_L$ (MAK_1DH5) has a slightly higher $K_D$ of 0.3 $\mu$M for creatine kinase whereas there is almost no antigen binding detectable for the $V_H$ grafting domain MAK_1DHU (> 50 $\mu$M) (table 1). So for binding of the $V_H$ domain to the antigen not only the CDRs represent a determining factor.

As CDR-H3 (i.e. the third CDR of $V_H$) is the most flexible of these six regions concerning length and amino acid sequence [2], additionally a mutant carrying a swapped CDR-H3 loop was analyzed. Concerning the $V_H/V_L$ association the CDR-H3 mutation shows a wt-like $K_D$ of 0.4 $\mu$M. Interestingly, this construct, MAK CDR-H3 1DHU $V_H$ showed a strongly impaired binding to the antigen with a 10 fold increased $K_D$ (table 1). This demonstrates the importance of CDR-H3 for the antigen binding of MAK33.

*Molecular Dynamics Simulations reveal mutation-induced structural alterations*

For a subset of $V_H$ and $V_L$ mutations explicit solvent Molecular Dynamics (MD) simulations were performed for the heterodimer and for the individual domains. The set of simulations included point mutations that are associated with a significant reduction in $V_H/V_L$ association ($V_L$ P44A, Y36A/P44A; $V_H$ V37A, L45A and W103A) and, as a control, also substitutions that showed only modest effects on complex affinity ($V_L$ Y36A, S43A; $V_H$ R44A, W47A). Simulations were started from the geometry of the wt structure (pdb-entry:1FH5, see Methods for details). On the time scale of the simulations, none of the $V_H/V_L$ complexes dissociated and the root-mean-square deviation (RMSD) of the complexes did not exceed 0.3 nm from the start structure (figure S1). However, some of the mutations (e.g. $V_L$ P44A, $V_H$ R44A, $V_H$ L45A and $V_H$ W47A, see figure S1) resulted in overall larger final RMSDs compared to the wt indicating mutation-induced structural alterations and increased conformational freedom. Interestingly, simulations of the mutated proteins in the isolated state showed no significant differences in the RMSD (figure S2) except for $V_H$ W103A (see below). The structural distortion of the $V_L/V_H$ complexes due to some
of the mutations is also reflected in overall larger root mean square fluctuations of heavy atoms (RMSF) with respect to the mean structure. For the isolated mutated protein partners no significant difference to the wt was observed (figure S3), again with the exception of W103A.

However, in the complex the mutations with reduced binding affinity (e.g. \( V_\text{L} \) P44A, \( V_\text{H} \) R44A,V37A,L45A,W47A) showed increased fluctuations in regions at and near the binding interface but also in loops involved in antigen binding (figure S4, S5). Interestingly, especially for the substitutions that caused the largest drop in affinity between the \( V_\text{L} \) and the \( V_\text{H} \) domains an increased solvation at the interface (diffusion of water molecules into the space created by the introduction of a small Ala residue) was observed (illustrated in figure 7, table 2). Especially for \( V_\text{L} \) P44A, \( V_\text{H} \) L45A and \( V_\text{H} \) W47A, the average number of water molecules increased near the mutation site (table 2). The mutation \( V_\text{H} \) W103A resulted in significant changes of the backbone conformation around the mutation site, specifically the loop formed by residues 93-107 (figure 7), explaining the larger RMSD and RMSF observed for the isolated \( V_\text{H} \) domain in this case (figure S2 and S3).

Besides of the effect of the mutations on the binding interface, it is interesting to investigate the changes in mobility of the CDR loops involved in antigen binding. We compared the fluctuation pattern observed in the complexes and in the individual (isolated) \( V_\text{L} \) and \( V_\text{H} \) partner domains. Even in case of the wt, the RMSF pattern changes significantly in several regions that include regions involved directly in binding the partner domain but also regions involved in antigen binding (figure S3, S4, S5). For example, the antigen binding loop \( V_\text{H} \): 93-107 shows large fluctuations in the absence of the \( V_\text{L} \) binding partner (figure S3) which drop significantly in the complex (figure S4). Hence, complex formation of the \( V_\text{L} \) and \( V_\text{H} \) domains appears to lock some of the antigen binding loops into distinct conformations. This effect is qualitatively also observed for several mutants, however, for some loop regions the reduction of CDR loop mobility upon binding is smaller compared to the wt. This is especially seen for the \( V_\text{H} \): 93-107 region.

In addition to simulations of point mutations, we also studied a subset of the loop exchange constructs (MAK_1DH5, 1DH5_MAK, MAK_1DHU and 1DHU_MAK). In experiments these variants affected the binding affinity between \( V_\text{H} \) and \( V_\text{L} \) domains much less than some of the interface point mutations (see above). During simulations
on the time scale of 100 ns, these variants did not show significant differences of the calculated RMSF compared to the wt (figure S6).
Discussion

The relationship between structure, stability and binding affinity of $V_H$ and $V_L$ is still enigmatic. This is an important aspect for understanding antibody architecture both as the basis of our immune system and also in the context of the engineering of antibodies for therapeutic purposes. In this context, it was found that in mutants an increase in affinity is often accompanied by a decrease in stability and *vice versa* - and these consequences are difficult to predict [30-36]. In our study we analyzed the association of the variable domains for the first time directly. This allowed us to specifically determine the contribution of framework and CDR mutations on the interaction of $V_H$ and $V_L$. For the wt domains, a $K_D$ of 0.2 $\mu$M was determined. Consequently, the two domains interact more efficiently than $C_H1$ and $C_L$, but not as efficient as the $C_H3$ homodimer [23, 25]. This relative weak interaction (in the absence of the $C_H1$ and $C_L$ domains) necessitates the covalent linkage of Fv fragments via a peptide and thus creating a pseudo-monomeric fusion protein (scFv) which can be used as a therapeutic agent [37, 38]. Simulations of scFvs have shown that the stability of the interface between the two variable domains plays a critical role for the overall stability of an antibody (or fragment) as dissociation proceeds unfolding [39].

Of special interest is the nature of the domain interface. It has to support the association of the two domains but also allow accommodating different CDRs and their repositioning in the context of antigen binding. Thus, an individual interface residue may be involved in one or more of these processes: 1) formation of the immunoglobulin fold, 2) domain stability, 3) interaction between the variable domains, or 4) antigen binding. Our alanine scanning study of conserved residues allowed us to address each of these possibilities and differentiate between them. Consistent with the results of *in silico* analysis which showed that only very few residues (< 10) are important for adopting an immunoglobulin fold [40], the alanine mutants in the $V_L$ domain had no considerable influence on its secondary or tertiary structure. In contrast, $V_H$ is very sensitive to the exchange of conserved residues in the interface. Two $V_H$ interface residues, E46 and R38, were identified to be essential for the folding of the $V_H$ domain. In the covariation analysis there was a very high $\phi$-value for these two residues. In the structure, a salt bridge is formed between them. R38 is buried and E46 does not interact with other $V_H$ interface residues, but might
electrostatically affect $V_L$ binding [9]. Camelids and cartilaginous fish possess naturally occurring heavy chain antibodies lacking the light chain [41-43]. Interestingly a sequence alignment of MAK33 $V_H$ with the variable domain ($V_{\text{HH}}$) of the cameld $V_{HH}$ (PDB entry 2XT1) and the variable domain of “monomeric” shark IgNAR ($V_{\text{NAR}}$) (PDB entry 2I24) shows a match for the residues E46 and R38. Concerning the shark IgNAR these two residues are actually the only ones from the conserved network investigated in this study that can also be identified at corresponding positions of this otherwise highly divergent sequence. For camelid $V_{HH}$s a mutation of the hydrophobic $V_H/V_L$ interface residues (including the tetrad: V37, G44, L45, W47) in favor of hydrophilic ones was discovered [9, 43]. The increase of hydrophilic residues in the framework also holds true for the $V_{\text{NAR}}$ which probably evolved from a cell surface receptor [42, 44]. Both antibodies show high biophysical stability and their distinct structural patterns have by now been successfully applied to generate monomeric human $V_H$ domains [13, 45, 46].

Mutations of conserved $V_L$ residues had predominantly less impact than observed for $V_H$ residues. As expected, S43A behaved similar to the wt. This is the only amino acid position of the MAK33 antibody which does not fit to the conserved amino acid network identified by Wang and coworkers [16]. In their covariation analysis an alanine is the conserved amino acid at position 43.

Generally, a change in stability did not necessarily coincide with a change in functionality. For $V_L$, the proline residue at position 44 showed the most prominent effects with an impaired $V_H/V_L$ association as well as antigen binding. Surprisingly, $V_L$ L46A and F98A exhibited an affinity for the $V_H$ domain similar to the wt but they are clearly less efficient in binding the antigen (table 1). The double point mutations Y36A/P44A and Y36A/S43A indicate that the analyzed mutations do not necessarily act in an additive manner. In terms of stability, the stable mutation S43A is indeed able to improve the low stability of Y36A but, unexpectedly, for the $V_H$-$V_L$ association, Y36A in combination with the worst binder P44A doubles the affinity for $V_H$ compared to P44A alone. This coincides with the fact that these two residues are supposed to interact [7] and are the most important $V_L$ interface residues in the covariation analysis based on number and strength of covariations with other interface residues [8].
The biophysical properties of isolated V\textsubscript{H} domains are in general more affected by mutations compared to V\textsubscript{L} [29]. The MAK33 V\textsubscript{H} domain shows a low stability with a D\textsubscript{1/2} value of 0.21 M GdmCl compared to 1.17 M GdmCl for MAK 33 V\textsubscript{L}. So already small changes in stability can shift the balance for the isolated V\textsubscript{H} domain. For almost all of the V\textsubscript{H} point mutations, an increased K\textsubscript{D} for association with the V\textsubscript{L} domain could be detected, which correlated in most cases with an impaired binding of the antigen. But V\textsubscript{H} W47A, for example, showed no antigen binding while the V\textsubscript{H}/V\textsubscript{L} association was only slightly decreased. In terms of antigen binding, it has to be kept in mind that additionally to the stabilizing effect of the V\textsubscript{L} domain, antigen binding itself will stabilize the heterodimer. So there is an additional layer of stabilization. To conclude, for the MAK33 V\textsubscript{H} domain, in isolation some of the conserved V\textsubscript{H}/V\textsubscript{L} interface residues are critical for structure formation and stability. This might cause the impaired association with the V\textsubscript{L} domain. In general, association and antigen binding do not necessarily correlate.

It seems that during antibody biogenesis the effect of CDRs on the stability of V\textsubscript{H} domains is a decisive, so far underappreciated factor. Especially concerning the observed MAK33 V\textsubscript{H} instability the outcome of grafting experiments with stable human consensus sequences was interesting. The grafting constructs revealed that CDRs, in addition to antigen binding, affect variable domain structure strongly. This is especially true for CDR-H3 (table 1). Comparing the MAK33 and 1DHU/1DH5 CDRs, CDR-H3 differed most, in terms of length (14 amino acids for MAK33 versus 11 for 1DHU) as well as charge. According to Morea and coworkers, CDR H3 conformation does not only depend on the environment [2] but both CDRs can additionally be assigned to different conformation types. Since MAK33 possesses a lysine at position 94 and an aspartate at position 101, which can form a salt bridge it is assumed to have a bulged conformation in contrast to 1DHU. As the CDR-H3 also contributes to the interface and interacts with the V\textsubscript{L} domain this conformational difference might not only affect antigen binding. In our case, the exchange of the MAK33 CDR-H3 impairs antigen binding (10 fold increased K\textsubscript{D}) and slightly increases domain stability. The data for the grafting constructs shows a very different picture for the V\textsubscript{H} and V\textsubscript{L} domain. For V\textsubscript{L}, the CDRs seem to be important for structure and V\textsubscript{H}/V\textsubscript{L} association but concerning antigen binding also within the 1DH5 framework the affinity is wt like. The V\textsubscript{H} domain, though, did not exhibit such a strong CDR
dependence, for $V_H/V_L$ association, only the framework was the determining factor. However, antigen binding was almost not detectable when grafting the CDRs to the 1DHU framework. Since exchanging only CDR-H3 of the $V_H$ MAK33 domain leads to a severe impairment of the antigen binding, this indicates a crucial role of the $V_H$ domain and especially the CDR-H3 in the binding process. An explanation for these observations could be the mentioned differences between MAK33 and 1DHU $V_H$ CDR H3. Interestingly, when grafting either the CDRs or framework from the human consensus sequences on MAK33 $V_L$ or $V_H$, always led to an increase in stability. This might be an important aspect for CDR selection and the interplay with domain stability. Previous studies [15, 33] applying CDR graftings for antibody humanization approaches showed the importance and complexity of the influence of specific framework residues in the context of antigen binding and stability improvement. But they focused on stabilization by framework exchange. Here we could show that vice versa, CDRs themselves can be considered as a crucial determinant of stability.

Our MD simulations indicate that the mutations altered conformational fluctuations of the isolated mutated domains which cause structural and mobility changes at the binding interface. The non-optimal packing at the protein-protein interface leads to increased fluctuations at the interface which is also manifested in a reduction of interactions (reduced binding affinity) and also to fluctuations in the antigen binding loop regions which can reduce the binding affinity for antigens. This is in line with previous findings where it was shown that subtle changes in the interface can affect the affinity for antigen [47].

In some cases also increased solvation at the interface was observed. This was especially the case for the mutations with the most impaired $V_H/V_L$ association ($V_L$ P44A, $V_H$ L45A). The presence of water molecules at the interface reduces intermolecular contacts between protein partners by giving the interface an increasing non-specific character. Thus, the experimentally observed changes in binding affinity and stability of the mutations are due to a combination of effects.

A quantitative correlation with the experimentally observed change in antigen binding affinity due to the mutation is, however, not observed. It should be emphasized that this is also not expected since binding to the antigen is affected by each CRD loop differently and increased or decreased loop mobility can in principle cause not only
reduction but possibly also an increase in antigen binding affinity (greater adaptability to the antigen) in an unpredictable way.

Taken together our data indicate that multiple determinants regulate the $V_H$-$V_L$ association and the affinity for the antigen. The interplay between interface interactions and CDRs turned out to be complex and in consequence $V_H$/$V_L$ association and antigen binding do not necessarily correlate.

**Acknowledgements**

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Material and Methods

Unless otherwise stated, all experiments were carried out at 25°C. Measurements were performed in 50 mM sodium phosphate buffer at pH 7.5. **Cloning and protein expression:**

\( \text{V}_\text{H} \) and \( \text{V}_\text{L} \) were cloned into pET28 A (Novagen, Darmstadt, Germany) with Ncol and HindIII (NEB, Hitchin, UK) and expressed in E. coli BL21 star (Invitrogen, Carlsbad, USA). The transformed cells were grown in LB medium containing kanamycin at 37°C until an \( \text{OD}_{600} \) of 0.6-0.8 was reached. The expression was induced by the addition of 1 mM isopropyl \( \beta \)-thiogalactopyranoside (IPTG). After 12 h, cells were harvested, and preparation of inclusion bodies was carried out as described previously[48]. The purification was performed according to the procedure described for the \( \text{V}_\text{L} \) domain[26].

Single point mutations were introduced by a quick change PCR approach using the QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, USA) according to the manufacturer’s recommendations. Primers were ordered from MWG Operon (Ebersberg, Germany).

Intact protein was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

**CD and Fluorescence spectroscopy:**

CD measurements were carried out using a Jasco J-720 spectropolarimeter (Jasco, Grossumstadt, Germany) equipped with a Peltier element. Far-UV CD spectra were measured using 10 \( \mu \text{M} \) protein in 1.0-mm quartz cuvettes between 260 nm and 198 nm and near-UV CD spectra between 320 nm and 260 nm using 50 \( \mu \text{M} \) protein in 5-mm quartz cuvettes. The spectra were accumulated 16 times and buffer corrected.

For denaturant-induced unfolding transitions, structural changes were monitored by fluorescence spectroscopy at 355 nm. Excitation wavelength was 280 nm and slit widths were 1 nm (excitation) and 3 nm (emission) for \( \text{V}_\text{H} \) and 2 nm and 5 nm for \( \text{V}_\text{L} \), respectively. All measurements were performed with 1 \( \mu \text{M} \) protein in a 1-cm quartz cuvette. The samples were incubated overnight at 20°C at the different GdmCl concentrations prior to measurements.
Data evaluation was performed with Origin 8G (OriginLab, Northampton, USA); for GdmCl-transitions a two-state model was applied[49].

Analytical ultracentrifugation:

Analytical ultracentrifugation was carried out with a ProteomLab XL-I (Beckman, Krefeld, Germany) equipped with absorbance and interference optics. The measurements for sedimentation velocity experiments were performed as described previously [50].

For sedimentation equilibrium experiments samples of V_H or V_L wild type together with the binding partner were prepared in a 1:1 ratio within a concentration range of 0.5 µM - 7.5 µM and incubated on ice during AUC cell preparation. Afterwards samples were loaded into six channel epon centerpieces (12 mm window) and the equilibrium was measured at different velocities (25.000, 30.000, 34.000 or 42.000 rpm). Scans were performed at 280 nm, 250 nm or 230 nm to be in absorption range within 0.1-0.8. 25 replicates were recorded. Data was globally fitted to determine the association constant with a heterogeneous interaction model (1:1 complex, see Equation 1) which is based on the mass action law $C_{AB} = K_a C_A C_B$ with the programme Origin 8.6 (OriginLab, Northampton, MA). Three different velocities and concentrations were included in the fit.

Equation 1:

$$a(r) = C_A(r_0)\varepsilon_A d \exp \left[ M_{b,A} \frac{\omega^2}{2RT} (r_2^2 - r_0^2) \right] + C_B(r_0)\varepsilon_B d \exp \left[ M_{b,B} \frac{\omega^2}{2RT} (r_2^2 - r_0^2) \right] + K_a C_A(r_0) C_B(r_0) (\varepsilon_A + \varepsilon_B) d \exp \left[ (M_{b,A} + M_{b,B}) \frac{\omega^2}{2RT} (r_2^2 - r_0^2) \right]$$

ELISA:

Binding of the variable domains of MAK33 to the antigen creatine kinase was analyzed by ELISA. Assay components and microwell plates were from Roche (Mannheim, Germany). Samples were prepared in 10 µl volume. Different mutants were tested within a concentration range of 100 nM up to 50 µM against wild type V_L or V_H with a C-terminal Flag-tag for detection. After the addition of 90 µl reaction mix I, the sample was incubated in a streptavidine-coated microwell plate to immobilize human biotinylated creatine kinase. Incubation was performed for 45 minutes with constant agitation at 20°C or 10°C for V_L and V_H mutants, respectively. Afterwards,
the samples were washed with sterile pure water for three times. Then 100 µl/well of reaction mix II were added together with the detection antibody for the Flag-tag coupled to horseradish peroxidase in a 1:15.000 dilution. Afterwards, the samples were washed three times with water again and then 100 µl of reaction mix III was added. The product of the enzymatic reaction was monitored at 405 nm in a GENios plate reader (Tecan, Männedorf, Switzerland) for 0.5 - 3 hours until a plateau was reached.

*Molecular dynamics simulations:*

Start structures of MAK33 V<sub>L</sub>/V<sub>H</sub> complexes as well as individual V<sub>L</sub> and V<sub>H</sub> domains were obtained by extracting the corresponding coordinates from the crystal structures 1FH5 [51]. Missing residues were added/corrected using the program PyMol [51] with a final sequence corresponding exactly to the wild type sequence used in the experiments. Model start structures of all mutants were generated based on the wild type structure with residue substitutions generated *in silico*. All Molecular Dynamics (MD) simulations as well as the analysis of root-mean square deviation (RMSD) and fluctuations (RMSF) were performed using the Gromacs4.6 package [52, 53] in combination with the AmberSB99_ILDN force field [54]. Proteins were solvated in dodecahedral boxes including explicit ions (Na<sup>+</sup> and Cl<sup>-</sup>) and explicit (TIP3P) water molecules [55]. The simulation systems were first energy-minimized (until the maximum force was smaller than 500 kJ/mol) followed by heating up to 300 K at a constant volume with position restraints on the protein. Subsequently, a pressure equilibration at 1 bar with position restraints on the protein was carried out. All production simulations were performed at a temperature of 300 K and a pressure of 1 bar and extended to 100 ns. Root mean square deviation (RMSD) and root mean square fluctuations (RMSF) with respect to the mean structure were calculated with g_rms and g_rmsf modules of Gromacs. Snapshots were created using VMD [56].

**Table and figure legends**

Table 1: Characteristics of MAK33 V<sub>H</sub> and V<sub>L</sub> point mutants and grafting mutants.

K<sub>D</sub> values were determined by AUC sedimentation equilibrium experiments. Three concentrations in the range between 0.5 µM and 7.5 µM with V<sub>H</sub> and V<sub>L</sub> in a 1:1 ratio were measured at three velocities and globally fitted to a heterogeneous interaction
model between two components. All mutants were tested with the wild type V_H or V_L domain. Experiments were performed at 20°C; asterisks indicate that samples were measured at 15°C due to low thermal stability. Errors of the fits for AUC data were in a range between 6% - 50%. Stabilities against the chemical denaturation (GdmCl) of the V_L and V_H mutants. Even though most of the GdmCl-induced unfolding transitions were not reversible, data were evaluated according to a two-state equilibrium unfolding model to derive the midpoint of transitions (D_{1/2}), as well as the cooperativity parameter (m), for a qualitative comparison of the data. To determine the functionality of the mutants, an ELISA with isolated variable domains and the antigen creatine kinase was performed. Detection of the V_H and V_L wild type domains was possible via an introduced FLAG-tag at the C-termini. As a reference, the signals of the wild type domains vs. FLAG-tagged domains after 25 minutes of incubation were chosen. Samples were corrected for the signal of the single FLAG-tagged variable domains. Apparent K_D values were obtained by a Boltzmann fit. Experiments were performed at 20°C. NB indicates that no binding could be observed.

Table 2: Average number of water molecules around a point mutation

Water molecules within 7 Å of the C-beta atom of a mutated amino acid were determined during a 100 ns MD simulation. The standard deviation of the number of waters is given in parenthesis.

Figure 1: Conserved residues within the interface between V_L and V_H.

In (A) the positions of conserved amino acids in the Fv fragment of MAK33 are shown. The V_L domain is depicted in light green with the three CDRs highlighted in dark green. V_H is shown in light blue and the CDRs are in dark blue. Conserved residues within the interface are illustrated as spheres and color-coded. On the right, the region within the rectangle is enlarged. For a better orientation CDR-H2 is indicated. The labelled residues were selected for an alanine-exchange mutational analysis. In (B) the top views of the interacting residues of the V_L (left) and V_H (right) domain of MAK33 are shown. Six residues were selected for V_L (Y36, Q37, S43, P44, L46, and F98) and seven for V_H (V37, R38, R44, L45, E46, W47, and W103). Structures are modified from PDB ID 1FH5. In (C), the top-views for V_L and V_H are shown and the residues labelled according to their influence on association. The numbering of the strands (a, b, c, c’, c”, d, e, f, g) is also shown. Color-code: orange
= medium influence, red = strong influence; dark green and dark blue = no influence for V_L or V_H mutants, respectively. The grey dotted line indicates CDR-H3 which is not resolved in PDB ID 1FH5.

Figure 2: Secondary and tertiary structure of V_L and V_H alanine exchange mutants.

In (A) and (B), FUV-CD spectra of V_L mutants (A) and V_H mutants (B) are shown. Color code for V_L: Y36A is red, Q37A dark cyan, S43A blue, P44A yellow, L45A purple, F98A black, Y36AS43A orange, Y36AP44A pink and V_L wild type is royal blue. Colour code for V_H: V37A is black, R44A blue, L45A dark cyan, W47A purple, W103A pink and V_H wild type marine blue. In (C) and (D) the NUV-CD spectra of the V_L (C) and V_H (D) point mutants are shown. Color code for (C) and (D) is analogue to (A) and (B), respectively. For the spectra 15 accumulations each were recorded and buffer-corrected (PBS). All measurements were performed at a protein concentration of 20 µM (FUV-CD) and 50 µM (NUV-CD) in 0.5-mm (FUV) or 5-mm (NUV) quartz cuvette at 20°C.

Figure 3: Influence of conserved residues on protein stability.

The stability of the V_H and V_L alanine point mutants towards GdmCl-induced (A and B). (A) V_L mutants: Y36A is red, Q37A dark cyan, S43A blue, P44A yellow, L45A purple, F98A black, Y36AS43A orange, Y36AP44A pink (all closed circles/lines) and the wild type marine blue (open circles/line). (B) V_H mutants: V37A is black, R44A blue, L45A dark cyan, W47A purple, W103A pink (all closed circles/lines) and the wild type royal blue (open circles/broken line).

Figure 4: Influence of conserved residues on antigen binding.

To determine the functionality of the mutants an ELISA with Fv fragments was performed. For V_L (left) and V_H (right), titrations of Fvs containing either the wild type, a representative of an inactive, a low active or a mutant with similar activity as the wild type is shown. The absorption at 405 nm at the signal maximum was corrected for the signal of the variable labelled domains alone. Experiments were performed at 20°C.

Figure 5: CDR-grafted mutants.
(A) Schematic representation of CDR-grafted mutants. The framework for $V_L$ grafting mutants (left) was 1DH5, which is the most stable human consensus sequence $V_L$ domain (from HuCAL [28]). The framework for $V_H$ grafting mutants (right) was 1DHU, which is the most stable human consensus sequence for $V_H$ domains. In (B and C), FUV-CD spectra of $V_L$ (left) and $V_H$ (right) CDR-grafted mutants are shown. The color code for $V_L$ is: MAK_1DH5 in red, 1DH5_MAK in blue; wild type 1DH5 is shown in green MAK33 in black and MAK33 CDR3 1DHU in brown. The color code for $V_H$ is: MAK_1DHU in red, 1DHU_MAK in blue and the wild type 1DHU in green and MAK33 in black. In (D and E) NUV-CD spectra of the $V_L$ (left) and $V_H$ (right) point mutants are shown.

Figure 6: The influence of the CDRs on the stability of the variable domains.

To assess the stability of CDR-grafted mutants, GdmCl-induced unfolding experiments were performed. Data for denaturant-induced transitions for $V_L$ (A) and $V_H$ (B) variants are shown. Data were evaluated according to a two-state unfolding model to obtain midpoints and cooperativity parameters of the transitions. Measurements were performed at 20°C at a protein concentration of 1 µM.

Figure 7: Interface hydration near mutation as determined by MD simulations.

The snapshots illustrate the water distribution within 7 Å of the mutation site. Protein chains are shown as cartoon ($V_L$:green, $V_H$:blue). Atoms within 7 Å of the mutation site are indicated as van der Waals spheres using atom color code for water molecules (and grey for protein atoms). For comparison the same regions are also shown for the wild type case (left panels). In case of the $V_H$ W103 mutation the average shift in backbone structure is illustrated (green cartoon) and compared to the wild type case (light blue).
Table 1: Characteristics of MAK33 $V_H$ and $V_L$ mutants.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Mutation</th>
<th>Association $K_D$ [µM]</th>
<th>Stability GdmCl $D_{1/2}$ [M]</th>
<th>Cooperativity [kJ mol$^{-1}$ M$^{-1}$]</th>
<th>Antigen binding app. $K_D$ [µM]</th>
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<tbody>
<tr>
<td>$V_L$</td>
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<td>$16.1 \pm 2.3$</td>
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<td>68.6 ± 23.3</td>
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<tr>
<td>1DHU_MAK</td>
<td>0.4 ± 0.1</td>
<td>1.26 ± 0.2</td>
<td>10.7 ± 0.9</td>
<td>NB</td>
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</tr>
<tr>
<td>1DHU_1DHU</td>
<td>8.8 ± 1.6</td>
<td>2.48 ± 1.9</td>
<td>6.3 ± 3.6</td>
<td>NB</td>
<td></td>
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<tr>
<td>MAK_MAK</td>
<td>0.4 ± 0.2</td>
<td>0.27 ± 1.2</td>
<td>7.9 ± 1.4</td>
<td>14.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>CDR3 1DHU</td>
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<td></td>
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<td></td>
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</tr>
</tbody>
</table>

* Denotes significant differences from wild type.
Table 2: Average number of water molecules around a point mutation

<table>
<thead>
<tr>
<th>Mutation</th>
<th>&lt;water&gt; in mutation</th>
<th>&lt;water&gt; in wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt; P44A</td>
<td>6.8 (2)</td>
<td>4.3 (1)</td>
</tr>
<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt; S43A</td>
<td>14 (4)</td>
<td>15 (3)</td>
</tr>
<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt; Y36A</td>
<td>1.1 (0.3)</td>
<td>0.8 (0.3)</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt; V37A</td>
<td>1.1 (0.4)</td>
<td>0.9 (0.3)</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt; R44A</td>
<td>16 (3)</td>
<td>15 (3)</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt; L45A</td>
<td>9 (3)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt; W47A</td>
<td>8 (3)</td>
<td>5 (1)</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt; W103</td>
<td>14 (3)</td>
<td>15 (3)</td>
</tr>
</tbody>
</table>
Figures

Figure 1

A

B

C

CDR-L1  CDR-H1  CDR-H3  V_L  V_H

CDR-L2  CDR-H2  W47  W47

CDR-L3  F96  L45  W103

V_L  V_H
Figure 2

A

B

C

D
Figure 3
Figure 4

A

B

Absorption @ 405 nm vs. $V_c$ (µM) for S43A, R44A, L45A, and V$_{wt}$

Absorption @ 405 nm vs. $V_n$ (µM) for V37A, R44A, and V$_{wt}$
Figure 5

A

B

C

D

E
Figure 6

A

B
Figure 7

VL: P44A
wild type

VH: V37A
wild type

VH: L45A
wild type

VH: W103A/wild type
References


51. The PyMOL Molecular Graphics System, Version 1.6. x Schrödinger, LLC.
Supplementary figures

Figure S1. Root-mean-square deviation (RMSD) of the protein backbone (V_{L}/V_{H} complex) from the experimental start structure vs. simulation time. The RMSD recorded for the wild type complex is indicated in black (the RMSD of each mutation is shown in red).
Figure S2. Root-mean-square deviation (RMSD) of the protein backbone of isolated $V_L$ or $V_H$ protein partners from the experimental start structure vs. simulation time. The RMSD recorded for the wild type protein domain is indicated in black (the RMSD of each mutation is shown in red).
Figure S3. Root-mean-square fluctuation (RMSF) with respect to the mean protein structure during each 100 ns simulation. The RMSF was calculated for all atoms of a residue and plotted vs. residue number. The RMSF obtained for the wild type V\textsubscript{L} or
\( V_H \) domain is indicated in black (the RMSF of each isolated domain mutation is shown in red).

Figure S4. Root-mean-square fluctuation (RMSF) with respect to the mean \( V_L/V_H \) complex structure during each 100 ns simulation. The RMSF was calculated for all atoms of a residue and plotted vs. residue number of the \( V_L \) domain. The RMSF
obtained for the wild type V_{L} domain (in complex with the V_{H}) is indicated in black (the RMSF of each isolated domain mutation is shown in red).
atoms of a residue and plotted vs. residue number of the V\textsubscript{H} domain. The RMSF obtained for the wild type V\textsubscript{H} domain (in complex with the V\textsubscript{H}) is indicated in black (the RMSF of each isolated domain mutation is shown in red).

Figure S6. Root-mean-square fluctuation (RMSF) with respect to the mean V\textsubscript{L}/V\textsubscript{H} complex structure during each 100 ns simulation. The RMSF was calculated for all atoms of a residue and plotted vs. residue number of the V\textsubscript{H} domain (upper four
panels) and for the $V_L$ domain (lower four panels). The RMSF obtained for the wild type case is indicated in black and for the loop exchange mutations in red.