Chemical Basis for the Affinity Maturation of a Camel Single Domain Antibody*

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Affinity maturation of classic antibodies supposedly proceeds through the pre-organization of the reactive germ line conformational isomer. It is less evident to foresee how this can be accomplished by cameld heavy-chain antibodies lacking light chains. Although these antibodies are subjected to somatic hypermutation, their antigen-binding fragment consists of a single domain with restricted flexibility in favor of binding energy. An antigen-binding domain derived from a dromedary heavy-chain antibody, cAb-Lys3, accumulated five amino acid substitutions in CDR1 and CDR2 upon maturation against lysozyme. Three of these residues have hydrophobic side chains, replacing serines, and participate in the hydrophobic core of the CDR1 in the mature antibody, suggesting that conformational rearrangements might occur in this loop during maturation. However, transition state analysis of the binding kinetics of mature cAb-Lys3 and germ line variants show that the maturation of this antibody relies on events late in the reaction pathway. This is reflected by a limited perturbation of $k_a$ and a significantly decreased $k_d$ upon maturation. In addition, binding reactions and the maturation event are predominantly enthalpically driven. Therefore, maturation proceeds through the increase of favorable binding interactions, or by the reduction of the enthalpic penalty for desolvation, as opposed to large entropic penalties associated with conformational changes and structural plasticity. Furthermore, the crystal structure of the mutant with a restored germ line CDR2 sequence illustrates that the matured hydrophobic core of CDR1 in cAb-Lys3 might be compensated in the germ line precursor by burying solvent molecules engaged in a stable hydrogen-bonding network with CDR1 and CDR2.

The success of the humoral immunity relies heavily on the fast generation of highly specific and tight binding antibodies against virtually any foreign target molecule. These antibodies occur concomitantly as membrane-bound receptors on B-cells or as soluble proteins. The antigen-combining sites of classic antibodies are composed of two variable domains (VH and VL) located at the N-terminal end of the heavy and light chains (1). Both domains display high sequence diversity, mainly concentrated in three complementarity-determining regions (CDRs) in each V domain. Structurally, these regions form loops, clustered at the N-terminal side of the folded domain, and provide the antigen contacts. Upon antigenic challenge, somatic hypermutation or gene conversion mechanisms further diversify the V sequences as well as the affinities of reactive B-cell receptors. A clonal selection system then enriches for those B-lymphocytes that display receptors with a higher affinity for the antigen. This process of somatic hypermutation and clonal selection is known as antibody (or B-cell receptor) affinity maturation (2–4).

It has been reported on several occasions that antibody paratopes for haptenic molecules or small peptides lose conformational plasticity upon maturation. Wedemeyer et al. (5) showed by x-ray crystallography that the anti-hapten antibody 48G7 acquired mutations that result in the pre-organization of the reactive conformation of a highly flexible germ line paratope, in the mature antibody. The observed conformational changes include large rigid body movements between VH and VL as well as backbone and side-chain rearrangements within the hypervariable CDR loops. Manivel et al. (6) observed that large entropic penalties were associated with antibodies from primary responses binding to a synthetic peptide. These penalties were significantly reduced upon maturation. In sharp contrast, a detailed analysis of the effects of the adaptations introduced during the affinity maturation of paratopes against larger antigens remains elusive. However, by comparing HyHEL10 variants at different maturation stages, Li et al. (7) indicated that the antigen maturation for this antibody results in an increased complementarity involving a fraction of the apolar buried surface area of the paratope but not by its pre-organization.

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The atomic coordinates and structure factors (code 1XFP) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: VH, variable domain of the heavy chain of an immunoglobulin; VL, variable domain of the light chain of an immunoglobulin; VHH, variable domain of a heavy chain antibody; eAb, camel single-domain antibody; CDR, complementary determining region; V, variable gene; D, diversity gene; J, joining gene; HEWL, hen egg white lysozyme; EDC, N-ethyl-N'-dimethylenaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; EDC/NHS, mixture of EDC and NHS (concentrations of 200 and 50 mM, respectively); PDEA, 2-(2-pyridydildithio)ethaneamine; PBS, phosphate-buffered saline; ASA, accessibility surface area; SASA, change of accessible surface area; $T_s$, absolute temperature; $k_p$, second order rate constant of association; $k_d$, first order rate constant of dissociation; $k_{sp}$, thermodynamic dissociation constant or affinity constant; $R$, Rydberg gas constant; $\Delta H^\circ$, $\Delta G^\circ$, and $\Delta S^\circ$ are the standard Gibbs free energies of binding, association, and dissociation, respectively; $\Delta H_{\text{ion}}$, $\Delta G_{\text{ion}}$, and $\Delta S_{\text{ion}}$ are the standard enthalpy of binding, association, and dissociation, respectively.
**Camelidae** possess, in addition to classic IgG molecules, a large fraction of naturally occurring heavy-chain antibodies that are devoid of light chains (8, 9). The variable domain of camel heavy-chain antibodies (VHH) is assembled by V-D-J gene rearrangement but is distinct from the VH of classic antibodies. Different sets of germ line VH and VHH genes encode the V-gem line repertoire in the cameldid genome, whereas the D and J genes are shared between both antibody classes. The affinity maturation process in camelid antibodies remains unexplored apart from the comparisons between cDNA and germ line V-gene segments that revealed the presence of a somatic hypermutation mechanism in camel heavy-chain antibodies (10).

Due to the absence of the VL domain in cameldid heavy-chain antibodies, it is evident that inter-domain movements cannot contribute to the flexibility of the paratope. In addition, the frequent occurrence of interloop disulfide bridges and the high percentage of backbone atoms involved in antigen contacts (8) may considerably reduce the paratope flexibility. Therefore, the occurrence of a rigidification process of the paratope in heavy-chain antibodies during affinity maturation, as observed for classic antibodies, remains an open question.

In this report we constructed germ line revertants of the hen egg white lysozyme (HEWL)-specific VHH, cAb-Lys3 (11, 12). The matured VHH and the germ line revertants were subsequently subjected to a transition state analysis using surface plasmon resonance technology. The enthalpic and entropic contributions in the cAb-Lys3-HEWL association were determined to evaluate the plasticity and interaction strengths of the interface residues during the course of the affinity maturation. In addition, the crystal structure of the CDR2 loop germ line revertant was solved to provide a structural understanding of the biophysical parameters of the affinity maturation process.

**MATERIALS AND METHODS**

**Reagents—**All reagents were analytical grade. HEWL was purchased from Roche Applied Science. PDEA, EDC, NHS, ethanolamine, and 1,4-dithioerythritol were obtained from Biacore AB, and cystamine was obtained from Sigma Aldrich. Restriction enzymes were purchased from Invitrogen.

**Design and Construction of the Germ Line Revertants—**We chose to evaluate the maturation event by restoring the complete or separate CDR1 and CDR2 germ line sequences of cAb-Lys3. This “cassette” reversion was chosen over the evaluation of individual mutations, because partial cloning of the mutations in the antigen maturation event cannot be traced, and some combinations of amino acid mutations might impose unacceptable loop foldings. The germ line revertants are referred to as cAb-Lys3	extsuperscript{151}, cAb-Lys3	extsuperscript{182}, and cAb-Lys3	extsuperscript{147} for the version of the CDR2, CDR1, and the complete germ line sequences, respectively. The antibody carrying the affinity matured sequence, is referred to as cAb-Lys3	extsuperscript{M}.

The cAb-Lys3	extsuperscript{12} cAb-Lys3	extsuperscript{161} and cAb-Lys3	extsuperscript{118} were all generated by PCR using mutagenic primers. The cAb-Lys3 gene cloned into a pHEN4 vector (13) was used as template for the PCR reactions. The cAb-Lys3	extsuperscript{12} mutant was generated by PCR using a mutagenic primer P1 (5'-GCA-GCA-ATT-AAT-AGT-GGT-GGT-GTG-AGC-ACT-3', an AseI restriction site is underlined) annealing at the end of the framework 2 up to codon 58 of CDR2 in the cAb-Lys3 sequence and the primer AM006 (5'-GTC-TAG-TAA-ATG-ATT-CTT-CTG-TAT-GAG-G-3') annealing in the vector sequence downstream of the cAb-Lys3 gene. An AseI generated fragment of cAb-Lys3	extsuperscript{151}-phen4 that contained the 5'-end of cAb-Lys3 and part of the pHEN4 vector sequence was annealed with the AseI-digested PCR product and further amplified with universal reverse primer and AM006. The amplification product was then digested with HindIII and BstEII, ligated into a HindIII/BstEII double digested cAb-Lys3-pHEN4 vector and transformed into Escherichia coli.

The cAb-Lys3	extsuperscript{12} reversion mutant was constructed using splicing by overlap extension from two PCR fragments with a 15-nucleotide overlap in their CDR1 coding areas. One fragment resulted from a PCR with reverse primer annealing in the pHEN4 vector sequence and a mutagenic forward primer SV2 (5'-GCT-AGT-TTA-GTA-GGT-GTA-CTC-G-TG-3') annealing in the CDR1 area of cAb-Lys3	extsuperscript{M} (the Bsu36I site, introduced for screening purposes is underlined). The second fragment resulted from amplification between a universal forward primer annealing in the pHEN4 vector and a mutagenic primer SV1 (5'-AGT-TTA-GTA-GGT-GTA-CTC-G-TG-3') annealing in the CDR1 codons of cAb-Lys3. The spliced fragments were amplified with reverse primer and forward primer, digested with HindIII and BstEII, and ligated in a HindIII/BstEII double digested cAb-Lys3	extsuperscript{p}-PHEN4 vector (13) and transformed into E. coli. The strategy to construct cAb-Lys3	extsuperscript{147} is similar to that of cAb-Lys3	extsuperscript{12} except that the cAb-Lys3	extsuperscript{147}-pHEN4 was used as template.

Finally, all mutants were recloned into a cAb-Lys3-pHEN6 vector (containing a C-terminal His	extsubscript{6}-encoded tag) (14) by digestion with HindIII and BstEII, gel purification and ligation in a HindIII/BstEII double digested cAb-Lys3-pHEN6 vector and transformation into E. coli. All mutant genes were confirmed by nucleotide sequencing.

**Expression and Purification—**Expression was carried out in E. coli BL21 (DE3) supplemented with 20% glycerol. Synchrotron data for the crystals were collected with a MAR-345 imaging plate at a wavelength of 0.903 Å using a 100 kV X-ray generator. Data were processed in DENZO, scaled in Scalepack, integrated, and merged using TREK1 with 160,000 reflections in the range 0<1<25°. The Matthews coefficient was calculated as 1.99 Å	extsuperscript{3} per Daltons with 2.94 Å resolution. Data collection was frozen after transfer into the precipitant solution and flash-cooled using a liquid nitrogen stream at a temperature of 100 K.

Calculation of the Transition State Enthalpy and Entropy—The enthalpy and entropy of activation and equilibrium for the interaction of each cAb-Lys3 variant was estimated using the equations: 

\[
\Delta G^\circ = RT \ln K_d \text{ (1)}
\]

\[
\Delta S^\circ = R \ln K_d (T - T_d) \text{ (2)}
\]

\[
\Delta H^\circ = \Delta G^\circ + \Delta S^\circ \text{ (3)}
\]

\[
K_d = \frac{[E]_0 [L]_0}{[E][L]} \text{ (4)}
\]

\[
T_d = \frac{\Delta H^\circ}{R \ln K_d} \text{ (5)}
\]

where \( R \) is the Rydberg gas constant, \( T \) the absolute temperature, \( k_b \) (\( m \text{ s}^{-1} \)) and \( k_j \) (\( s^{-1} \)), respectively, the association and dissociation rate constant of binding; \( K_d \) (\( s^{-1} \)) the dissociation constant; \( \Delta G^\circ \), \( \Delta S^\circ \), and \( \Delta H^\circ \) are the standard Gibbs free energies of binding, association, and dissociation, respectively. By plotting \( \ln k_d(T) \) versus \( 1/T \) and \( \ln k_d(T) \) versus \( 1/T \), the Gibbs free energies of dissociation are obtained, respectively.

**Crystallization and Structure Solution of the cAb-Lys3	extsuperscript{147} Revertant—**Crystals of cAb-Lys3	extsuperscript{147} in complex with HEWL were grown at 4 mg/ml by hanging drop vapor diffusion with 2 mM sodium formate and 100 mM sodium HEPES, pH 7.5, as precipitant solution. The crystal used for data collection was frozen after transfer into the precipitant solution supplemented with 20% glycerol. Synchrotron data for the crystals were...
modified in the cAb-Lys3 gene. Remarkably, only two hotspots residing within the area encoding the framework regions are altered.

The alignment of the cvhhp11 and cAb-Lys3 amino acid sequences revealed seven amino acid replacements. Five of these replacements are located in the CDRs, of which three (Tyr-29 → Ile, Ser-30 → Gly, and Ser-31 → Pro) occur in the CDR1 and two (Ser-52a → Met and Ser-56 → Ile) are located in the CDR2 (Figs. 1 and 2A).

Localization of Substituted Residues in the VHH Paratope Structure—All five CDR residues that were replaced during affinity maturation of cAb-Lys3, cluster in the folded domain and are located at the periphery of the VHH-HEWL interface (Fig. 2B). The change in solvent-accessible surface area (ΔASA) of the “matured amino acids Ile-29, Gly-30, Pro-31, Met-52a, and Ile-56 upon antigen binding equals 122 Å². This represents nearly 50% of the total ΔASA of the V-gemline encoded part of the paratope. Remarkably, out of these five amino acids only Ile-29 and Ile-56 have atoms within a distance of 4.0 Å of the antigen (Fig. 2C). Ile-29 contacts the HEWL side chains of Trp-62 and Leu-75, and Ile-56 has atoms within 4 Å of Asp-48 and Thr-47.

In contrast to the Ile-29 and Ile-56, the remaining three matured amino acids do not participate in direct antigen recognition and seem to have rather a structural role. The ϕ and ψ angles adopted by Gly-30 allow Pro-31 (in a cis-configuration) to pack against Tyr-27 and Met-34, thereby forming a stable hydrophobic core (Fig. 2C). Residue Met-52a of the CDR2 loop packs against Met-34 and Pro-31 (Fig. 2C), thereby extending the hydrophobic core of the CDR1 loop.

Temperature Dependence of the VHH-HEWL Kinetics and Affinity—The kinetic rate constants $k_a$ and $k_d$ of the interactions between HEWL and the cAb-Lys3 variants were determined at four different temperatures (20, 25, 30, and 35 °C) by surface plasmon resonance (Fig. 3). All binding curves fitted well to a simple 1:1 “lock and key” binding model, consistent with recognition of two rigid molecules deprived from large conformational changes upon binding (22). These data are graphically represented in a RaPID plot (Fig. 3), which is a convenient two-dimensional display of the $k_a$ and $k_d$ values of each interacting pair. The diagonal lines of the plot reflect the iso-affinity interactions. A glance at this plot immediately reveals that the largest gain during affinity maturation originates from the improvement of $k_a$, whereas the $k_d$ constant was only moderately affected (at most by a factor 4) during maturation from germ line to cAb-Lys3m. Therefore, the observed ΔΔ$G^\ddagger$ values for the reversion to the CDR2 (cAb-Lys3d1, CDR1 (cAb-Lys3d2), and to the complete V-gemline sequence (cAb-Lys3d1&2), which is, respectively, equal to 1.1, 2.6, and 3.5 kcal/mol, are primarily determined by differences in off-rates.

The $\Delta H$ and $\Delta S$ Contributions to Activation, Dissociation, and Equilibrium Free Energies—Eyring plots derived from the kinetic association and dissociation data are linearly decreasing ($R^2$ of more than 0.95) for all cAb-Lys3 variants (Fig. 4, A and B). From the slopes and intercepts of these Eyring plots we retrieve the activation enthalpy and entropy contributions to the association free energy ($\Delta\Gamma_a$, dissociation free energy ($\Delta\Gamma_d$), and the free energy of binding ($\Delta\Gamma$) for each cAb-Lys3 variant (Fig. 4, C–E).

The formation of the VHH-HEWL transition state for each cAb-Lys3 variant, has a large entropy and enthalpy penalty (Fig. 4F). The enthalpy barriers, however, are significantly higher than those of the entropy, except for the cAb-Lys3d1-HEWL association, where both energies are equally unfavorable. The entropy barriers in the cAb-Lys3d1&2-HEWL and cAb-Lys3m-HEWL associations are equal. The slight difference
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TABLE II

| Nucleotide substitutions from the cvhhp11 germ line gene to cAb-Lys3 | A | G | C | T | Total |
|---------------------------------------------------------------|---|---|---|---|-------|
| cvhhp11                                                        |   |   |   |   |       |
| A                                                            | 2 | 3 | 1 | 6 |       |
| G                                                            | 2 | 2 | 2 | 6 |       |
| C                                                            | 0 | 1 | 1 | 2 |       |
| T                                                            | 2 | 1 | 1 | 4 |       |

in transition state energy ($\Delta G_{\text{ass}}^\circ = \Delta H_{\text{ass}}^\circ - T \Delta S_{\text{ass}}^\circ$) between both variants originates from a 0.6 kcal/mole difference in enthalpy penalty in favor of the cAb-Lys3m-HEWL transition state. Conversely, the HEWL interactions with the intermediate matured variants, cAb-Lys3gl1 and cAb-Lys3gl1&2, show significant differences in both $\Delta H_{\text{ass}}^\circ$ and $T \Delta S_{\text{ass}}^\circ$. However, the overall transition state energy ($\Delta G_{\text{ass}}^\circ = \Delta H_{\text{ass}}^\circ - T \Delta S_{\text{ass}}^\circ$) for these variants is almost equal to those of cAb-Lys3m and cAb-Lys3gl1&2.

High enthalpy and entropy barriers ($\Delta H_{\text{diss}}^\circ$ and $T \Delta S_{\text{diss}}^\circ$) (Fig. 4D) are also observed upon dissociation of the VHH-HEWL complexes for all variants. Here again, the enthalpy barriers are significantly higher than those of the entropy. These enthalpy barriers of dissociation increase slightly upon maturation ($\Delta H_{\text{diss}}^\circ = 1.4$ kcal/mol for cAb-Lys3gl1&2 $\rightarrow$ cAb-Lys3m). The entropy penalties ($T \Delta S_{\text{diss}}^\circ$) in the dissociation event differ between cAb-Lys3gl1&2 and cAb-Lys3m by 1.0 kcal/mol.

Enthalpy and entropy contributions to the $\Delta G^\circ$ of the equilibrium binding reaction of each mutant to HEWL are shown in Fig. 4E. For all variants, except for the cAb-Lys3gl1-HEWL interaction, which has a slightly negative $T \Delta S^\circ$, favorable enthalpy, and entropy contributions to $\Delta G^\circ$ are observed. As can be deduced from the association and dissociation phases, the enthalpy contributions are higher than those of the entropy in the VHH-HEWL equilibrium reactions for all cAb-Lys3 variants. The difference of 3.5 kcal/mol in $\Delta G^\circ$ between cAb-Lys3gl1&2 and cAb-Lys3m is the result of a 2.1 and 1.1 kcal/mole difference in $\Delta H^\circ$ and $T \Delta S^\circ$, respectively. The cAb-Lys3gl1 has the most favorable enthalpy component but is countered by an unfavorable entropy penalty. The most favorable binding entropy is observed in the cAb-Lys3gl1-HEWL equilibrium reaction. The $T \Delta S^\circ$ term differs from cAb-Lys3m by 0.7 kcal/mol for this mutant. The binding enthalpy for cAb-Lys3gl1 is, however, 1.8 kcal/mole less favorable than in the cAb-Lys3m-HEWL interaction.

In summary, the difference in binding energy ($\Delta G^\circ$) of 3.5 kcal/mol between cAb-Lys3gl1&2 and cAb-Lys3m is primarily due to an increase in favorable enthalpy (2.1 kcal/mol) (Fig. 4, E and F). This increased favorable enthalpy is essentially obtained in the stabilization phase of the transition state complex into the final VHH-HEWL hetero-dimer (1.4 kcal/mol). The difference in $T \Delta S^\circ$ between both mutants is equal to 1.1 kcal/mol originating exclusively from the stabilization phase. In the association event, the on-rate constants as well as the activation energies ($\Delta G_{\text{ass}}^\circ$) do not differ much among the variants, in contrast to the relative enthalpy and entropy activation barriers for the cAb-Lys3gl1-HEWL and cAb-Lys3gl1&2-HEWL association reactions.

Crystal Structure of the cAb-Lys3gl1&2 Variant in Complex with HEWL—Crystallizations of the maturation variants in complex with HEWL were undertaken to clarify the thermodynamic and kinetic data. The cAb-Lys3gl1 and cAb-Lys3gl1&2 variants in complex with HEWL resist crystallization, possibly because matured CDR1 sequences are critical for the crystal lattice contacts as shown for the matured cAb-Lys3 in complex with HEWL (11). The cAb-Lys3gl1&2-HEWL complex was successfully crystallized, and the superposition of the cAb-Lys3m and cAb-Lys3gl1&2 structures revealed that all backbone atoms occupy identical positions in both complexes. Furthermore, the Ca, Cβ, and Oy atoms of Ser-52a and Ser-56 in the germ line variant possess a structurally equivalent position to the Ca, Cβ, and Cy atoms of the matured-type residues Met-52a and Ile-56.
The absence of atoms due to the Met-52a → Ser substitution is compensated by the insertion of two buried water molecules between CDR1 and CDR2. The hydrogen bond network created by these two buried water molecules and the Oy of Ser-52a and the backbone carbonyl oxygen of residue Pro-31 in cAb-Lys3312 (Fig. 5A), is replaced in cAb-Lys3m by the Met-52a side-chain that contacts the CDR1 (Fig. 5B).

In contrast to the Met-52a that does not contact HEWL, the other matured amino acid of the CDR2, Ile-56, is involved in extensive interactions with HEWL (Fig. 2C). The germ line encoded Ser-56 side-chain of the cAb-Lys3312 variant is also engaged in HEWL interactions, e.g. the Oy of Ser-56 is hydrogen bonded to the Oy1 of Thr-47L (not shown).

DISCUSSION

In this report we analyzed the functional and structural effects of the affinity maturation event of cAb-Lys3, the antigen-binding domain of a camelid heavy chain antibody against HEWL (11, 12). Sequence alignment with the dromedary VHH germ line genes revealed that cAb-Lys3 emerged from a recombination with the cvhhp11 germ line gene. Both gene fragments share a critical Cys codon at position 33 (Fig. 1) and a high sequence identity of 93.7%.

Somatic hypermutations in mouse antibodies are biased toward nucleotide transitions (2). Such a bias was not observed between the cvhhp11 germ line and cAb-Lys3 sequence, possibly because the mutations were under a high antigen selection pressure. However, an observed 1/1 transition/transversion bias for the mutations in other VHHs suggests that a conventional genetic mechanism is employed for in vivo somatic hypermutation of camelid heavy chain antibodies.

The replacement mutations in the CDR loops occur at the paratope periphery of the VHH-HEWL interface (Fig. 2B). The diversification at the periphery of the paratope during the affinity maturation is a characteristic feature for the somatic hypermutation and selection process (23). Due to the solvent-exposed environment of this region, mutagenesis of these residues is believed to have only a moderate and non-cooperative (additive) effect on the overall binding energy (24). Consequently, a typical 10- to 100-fold increase in binding affinity is accomplished by affinity maturation. However, larger affinity differences between germ line and mature antibodies require alternative mechanisms. For instance in the case of the antibody 48G7 and 48G7M (the germ line precursor), where a 30,000-fold difference in affinity is observed, the effects of the amino acid replacements are highly co-operative with an important influence on the structural isomerism of the antibody (5).

The construction of cAb-Lys3 variants, where the CDR1 and CDR2 were reverted simultaneously or separately to the original V germ line counterparts, allowed us to assess the effects of maturation of the CDR loop structure and on HEWL binding. All association and dissociation reactions fitted well to a simple bimolecular “lock-and-key” reaction model. The VHH-HEWL interactions for the cAb-Lys3 variants differed significantly in off-rate, whereas their on-rates were almost invariant. The 300-fold difference in affinity between the germ line precursor

4 E. De Genst, D. Saerens, S. Muyldermans, and K. Conrath, unpublished results.
FIG. 4. Panels A and B are the Eyring plots for, respectively, the association and dissociation reaction. The following symbols were used to discriminate between variants: ■, for cAb-Lys3m; ●, for the cAb-Lys3gl1 mutant; ◇, for cAb-Lys3gl2; and ▲, for cAb-Lys3gl1&2. C–E, the \( T\Delta S\) (gray bars) and \( \Delta H\) (white bars) contributions to the \( T\Delta S\) (gray bars) and \( \Delta H\) (white bars) contributions to the \( \Delta G\) of equilibrium binding (E) for each cAb-Lys3 variant (indicated below the bars) with HEWL at 35 °C and in the 1 M standard state. F, reaction pathway of the cAb-Lys3gl1&2-HEWL and cAb-Lys3m reaction. The \( \Delta G\), \( -T\Delta S\), and \( \Delta H\) along the reaction coordinate are given for the cAb-Lys3gl1&2-HEWL reaction (left) and for the cAb-Lys3m reaction (right).
and the matured form of cAb-Lys3 therefore arises primarily from a decreased $k_d$ value. In addition, no detectable co-operativity between the CDR loops upon maturation was observed. Indeed, for the antigen complexation, the $\Delta \Delta G^\circ$ values of cAb-Lys3$^{gl2}$ versus cAb-Lys3$^{m-gl2}$ equaled the sum of $\Delta \Delta G^\circ_{m-gl1}$ and $\Delta \Delta G^\circ_{m-gl2}$. These results are consistent with association reactions that lack large conformational changes upon binding. This is further corroborated by the fact that the enthalpy dominates the association, dissociation, and equilibrium energies of the VH3-HEWL interaction of all variants, whereas large entropy penalties are typical for induced fit mechanisms of the VHH-HEWL interaction of all variants, whereas high enthalpy contributions that lack large conformational changes upon binding. The absence of pronounced conformational changes in the binding of the cAb-Lys3 germ line variants to HEWL is surprising. It was anticipated from the cAb-Lys3-HEWL crystal structure that three targeted residues (Ser-30 $\rightarrow$ Gly, Ser-31 $\rightarrow$ Pro, and Ser-52a $\rightarrow$ Met) would fix an otherwise flexible CDR1 loop. In the matured antibody, Met-52a belonging to the CDR2 loop packs against Met-34 in the matured cAb-Lys3. The formation of a hydrogen bond between Met-52a and Met-34 in the mature cAb-Lys3-HEWL complex structures by the insertion of two water molecules mediating a hydrogen bond network. This intricate H-bond cluster further involves the Oy of Ser-52a and the main-chain carbonyl oxygen atoms of Pro-31, Asn-72, and Thr-78.

In the mature cAb-Lys3, the Gly-30 peptide bond allows Pro-31 to pack tightly against Tyr-27 and Met-34, thereby forming a stable hydrophobic core that stabilizes the CDR1 loop conformation. This loop configuration, referred to as canonical loop structure type 4, has never been observed in human or mouse VHS (26). In the germ line antibody, serine residues occupy positions 30 and 31 that were expected to enforce a classic type I canonical structure (27) for the H1 loop conformation. This canonical H-bonding cluster further involves the Oy of Ser-52a and the main-chain carbonyl oxygen atoms of Pro-31, Asn-72, and Thr-78.

The absence of pronounced conformational changes in the binding of the cAb-Lys3 germ line variants to HEWL is surprising. It was anticipated from the cAb-Lys3-HEWL crystal structure that three targeted residues (Ser-30 $\rightarrow$ Gly, Ser-31 $\rightarrow$ Pro, and Ser-52a $\rightarrow$ Met) would fix an otherwise flexible CDR1 loop. In the matured antibody, Met-52a belonging to the CDR2 loop packs against Met-34 of the CDR1 loop, thereby extending the hydrophobic core of CDR1. However, the replacement of Met-52a by a more hydrophilic Ser as encoded by the V-gem sequence is neither detrimental for antigen recognition nor for the stabilization of the CDR1 and CDR2 loop structures (Figs. 4, C–E, and 5). The crystal structures proved that the interaction between Met-52a and Met-34 in the mature antibody is effectively compensated in the cAb-Lys3$^{gl2}$-HEWL structure by the insertion of two water molecules mediating a hydrogen bond network. This intricate H-bond cluster further involves the Oy of Ser-52a and the main-chain carbonyl oxygen atoms of Pro-31, Asn-72, and Thr-78.

In the mature cAb-Lys3, the Gly-30 peptide bond allows Pro-31 to pack tightly against Tyr-27 and Met-34, thereby forming a stable hydrophobic core that stabilizes the CDR1 loop conformation. This loop configuration, referred to as canonical loop structure type 4, has never been observed in human or mouse VHS (26). In the germ line antibody, serine residues occupy positions 30 and 31 that were expected to enforce a classic type I canonical structure (27) for the H1 loop conformation. This canonical H-bonding cluster further involves the Oy of Ser-52a and the main-chain carbonyl oxygen atoms of Pro-31, Asn-72, and Thr-78.

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FIG. 6. The proposed structural model of the cAb-Lys3\textsuperscript{gl1&2} mutant. Except when indicated all side-chain atoms and carbonyl oxygen of the backbone were removed for clarity. A, stereo representation of a cAb-Lys3\textsuperscript{gl1&2} structural model in which the CDR1 loop and CDR2 loop have identical backbone conformations as seen in the cAb-Lys3\textsuperscript{m} and cAb-Lys3\textsuperscript{m} crystal structures. All backbone and side-chain atoms of the CDR1 loop are shown (carbon, green; oxygen, red; nitrogen, blue; sulfur, yellow). In addition, backbone and side chain atoms of Ser-52a, Ser-56 (carbon, cyan; nitrogen, blue; oxygen, red; sulfur, yellow), and the main-chain and side-chain atoms of Tyr-99A and Tyr-100bA (magenta) are represented as sticks, and the corresponding residues are labeled. Residues Tyr-27A, Tyr-29A, Ser-31A, and Met-34A are additionally labeled. B, stereo representation of a cAb-Lys3\textsuperscript{gl1&2} structural model in which the CDR1 loop adopts a type I canonical structure. Color coding, displayed atoms, and labels are identical to those in A. Atoms of the VHH molecule within 2 Å of atoms of the CDR1 loop are represented as purple spheres.

Pro-31 in the hydrophobic core in the CDR1 loop of cAb-Lys3\textsuperscript{m}. However, a difference of 1 kcal/mole for the dissociation entropy between cAb-Lys3\textsuperscript{m} and cAb-Lys3\textsuperscript{gl1&2} suggests that the CDR loops of the germ line variant exhibit a minor difference in flexibility. This structural model for cAb-Lys3\textsuperscript{gl1&2} is compatible with the enthalpy and entropy contributions in the association phase of both single loop mutants (Fig. 4C). In the cAb-Lys3\textsuperscript{gl1} mutant Ser-31 would be sandwiched between residues Met-92a and Tyr-27. This might destabilize the CDR1 loop by introduction of a hydrophilic residue in the hydrophobic core region, which is not compensated by a favorable hydrogen bond network as observed in the cAb-Lys3\textsuperscript{gl1&2}-HEWL complex. Such a destabilization might therefore cause flexible transitions of the loop conformation, which become fixed upon HEWL binding. A higher entropy barrier during transition state formation compared with the other cAb-Lys3 variants should reflect this event. The cAb-Lys3\textsuperscript{gl1&2}-HEWL variant displays the lowest transition state entropy barrier of all the cAb-Lys3 variants. This suggests that the introduced mutations have a stabilizing effect on the reactive paratope conformation. The observed hydrogen bonding network between the CDR1 and CDR2 loop might account for this effect. In both mutants, the differences in enthalpy barriers may originate from differences in solvation potential (28).

Our results show that the affinity maturation in the CDR1 and CDR2 loop of the VHH focuses on the annealing step of the initial encounter complex. These results are opposite to those made by Manivel et al. (6) for panels of antibodies from primary and secondary responses to a peptide antigen. These authors showed that the antibody maturation influences primordially the antigen association phase. In their analysis, the germ line antibodies displayed large entropic penalties in the association reaction, which were eliminated upon maturation. Such results are consistent with a rigidification process of the germ line paratope upon maturation. In the case of the anti-hapten antibody 48G7, maturation was achieved through the reduction of the off-rate (5). Furthermore, the germ line precursor of this antibody had to overcome a 30,000-fold difference in affinity, which was obtained by energetically highly co-operative mutations (29). These mutations resulted in a pre-organization of the reactive conformation of a flexible germ line paratope in the mature antibody. Although the affinity maturation of this antibody is essentially accomplished by reducing the off-rate, it has been suggested that the dissociation event should reveal a large entropic penalty difference between germ line precursor and mature antibody (30).

Two explanations can be proposed for the lack of congruence between the maturation mechanism of cAb-Lys3 and that of conventional antibodies. In a first possibility it can be argued that the maturation mechanism of antibodies binding to large, proteinaceous antigens may deviate fundamentally from those that are specific for small haptens or peptides. To our knowledge, this is the first time that a transition state analysis of antibody maturation was performed for an antibody specific for a protein antigen. However, Li et al. (7) showed for HyHEL10 variants, representing different maturation stages, that affinity maturation of these antibodies binding to HEWL proceeded through the burial of a higher proportion of apolar paratope surface area and by increasing epitope-paratope complementarity. Comparison of the antibody complexes to the free structures of HyHEL63 crystallized in different space groups, showed minor conformational differences. Furthermore, no correlation was found between the amount of buried surface area or number of contacts and the observed affinity difference, albeit that such a correlation has been observed in the comparison of the structures of the antibodies D.44.1 and the more matured F10.6.6 in complex with HEWL (31). However, one should note that all these antibodies were already considerably matured and therefore might not represent early events in the maturation pathway.

A second explanation for the different maturation mechanism between camelid heavy chain and conventional antibodies is based on the absence of a VL partner in the paratope of the former immunoglobulins. An antigen binding entity consisting of a single domain only will obviously lack the VH-VL flexibil-
ity, which will inevitably reduce the plasticity of the germ line paratope. A germ line-encoded cysteine forming a disulfide bond with a cysteine in the CDR3 loop might add considerable rigidity to the CDR loops. The CDR3 loop of VHs, which is on average longer than those found in VHs, often folds back onto the framework 2 region of the β-barrel where it is stabilized by the formation of a hydrophobic core (32). Furthermore, a high proportion of VHH main-chain atoms are replaced by hydrophobic residues in the apolar surface area is evident, as the polar residues in the germ line antibody are replaced by hydrophobic residues in the matured antibody. However, an increase in the fraction of apolar buried surface area. As can be calculated from the proposed structure of cAb-Lys311&2 in complex with HEWL, the somatic hypermutations do not change the total amount of ΔASA (219 Å2 compared with 221 Å2 in the matured antibody). However, an increase in the fraction of apolar surface area is evident, as the polar residues in the germ line antibody are replaced by hydrophobic residues in the matured antibody. This observation is consistent with the results of Li et al. (7) for the affinity maturation variants of the HEWL-specific HyHEL8 antibody.

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