Repertoire analysis of antibody CDR-H3 loops suggests affinity maturation does not typically result in rigidification

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1 Supplementary Data

1.1 Supplementary Command Lines

Rosetta version 2017.26-dev59567 was used for all simulations.

1.1.1 Rosetta FastRelax

Antibody Fv regions were relaxed with the following command and options:

```
/path/to/relax.linuxgccrelease -l pdb.list \
-ex1 \
-ex2 \n-use_input_sc \n-beta \n-nstruct 10
```

1.1.2 Rosetta KIC

Antibody Fv regions had their CDR-H3 loop remodeled and relative VH–VL orientation resampled with the following command and options:

```
/path/to/antibody_H3.linuxgccrelease -l pdb.list \
-ex1 \
-ex2 
-nstruct 10 @abH3.flags
```

where, abH3.flags is a file containing the following additional options:

```
-antibody::remodel       perturb_kic
-antibody::snugfit       true
-antibody::refine        refine_kic
-antibody::cter_insert   false
-antibody::flank_residue_min true
-antibody::bad_nter      false
-antibody::h3_filter     false
-antibody::h3_filter_tolerance 5
-extrachi_cutoff 0
-loops:legacy_kic false
-loops:kic_min_after_repack true
-loops:kic_omega_sampling
-loops:allow_omega_move true
-kic_bump_overlap_factor 0.36
-loops:ramp_fa_rep
-loops:ramp_rama
-loops:refine_outer_cycles 2
-loops:max_inner_cycles 20
```
1.1.3 RosettaAntibody Homology Modeling

Antibody Fv homology models were generated with RosettaAntibody in three steps: (1) assembly of the homologous components, (2) FastRelax of the grafted model, (3) CDR-H3 loop modeling and VH–VL docking.

Homologous components were selected and assembled with the following command and options:

```
/path/to/antibody.macosclangrelease -fasta pdb.fasta \
-antibody:n_multi_templates 1 \
-antibody:no_relax
```

The resulting “model-0.pdb” was relaxed with constraints by:

```
relax.macosclangrelease -s model-0.pdb \
-flip_HNQ \ 
-no_optH false \ 
-fast \ 
-constrain_relax_to_start_coords \ 
-ramp_constraints false \ 
-use_input_sc \ 
-ex1 \ 
-ex2 \ 
-struct 1
```

Finally, CDR-H3 loop modeling and docking of VH–VL was done by:

```
/path/to/antibody_H3.linuxgccrelease -s grafting/model-0_0001.pdb \
-struct 1000 @abH3.flags
```

with the following abH3.flags:

```
-antibody::remodel perturb_kic
-antibody::snugfit true
-antibody::refine refine_kic
-antibody::cter_insert false
-antibody::flank_residue_min true
-antibody::bad_nter false
-antibody::h3_filter false
-antibody::h3_filter_tolerance 5
-antibody::constrain_vlvh_qq

-ex1
-ex2
-extrachi_cutoff 0

-loops:legacy_kic false
-loops:kic_min_after_repack true
-loops:kic_omega_sampling
```
Supplementary Material

- loops:allow_omega_move true
- kic_bump_overlap_factor 0.36
- loops:ramp_fa_rep
- loops:ramp_rama
- loops:refine_outer_cycles 5

1.2 Sequences Used to Model Naïve-Reverted Antibodies

Mature sequences were aligned to germline V-genes as described in the methods. Additionally, sequences were aligned to germline J-genes using IMGT/DomainGapAlign, which yields germline alignments for both V- and J-genes. For example, the alignment of the variable region of 1T2Q can be extracted from: [http://www.imgt.org/3Dstructure-DB/cgi/details.cgi?pdbcode=1t2q](http://www.imgt.org/3Dstructure-DB/cgi/details.cgi?pdbcode=1t2q). The germline sequence was used when possible. The alignments are shown below with the mature sequence above and naïve below.

**1A4J Heavy**

| V region of 1T2Q | Germline sequence of 1A4J Heavy |
|-----------------|--------------------------------|
| QVQLVESGPHELKKPGETVKISCKASGYTFTNYGNNVKQAPKGLKWMGWINTYTGEPTYADDFAKGFAFSLETASTAY | QIQLVESGPHELKKPGETVKISCKASGYTFTNYGNNVKQAPKGLKWMGWINTYTGEPTYADDFAKGFAFSLETASTAY |
| LQINNLKNEDTATYFCVQAERLRTFDYWAGGTTTVS | LQINNLKNEDTATYFCVQAERLRTFDYWAGGTTTVS |

**1A4J Light**

| V region of 1T2Q | Germline sequence of 1A4J Light |
|-----------------|--------------------------------|
| ELVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYHLWYLQKPGQSKLLIYKVSNFGPDRFSGSGTDFTLKI | DVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYHLWYLQKPGQSKLLIYKVSNFGPDRFSGSGTDFTLKI |
| SRVEAEDLVYFCQSHVPPTFGGGTKLEIKR- | SRVEAEDLVYFCQSHVPPTFGGGTKLEIKR- |

**1BLN Heavy**

| V region of 1T2Q | Germline sequence of 1BLN Heavy |
|-----------------|--------------------------------|
| EVILVESGGGLVKPGGLKLSCAASGFTFSSYTMWSRQTPKREWATISSGGNTYYPDSVKGRTISRDNAKNLNY | EVILVESGGGLVKPGGLKLSCAASGFTFSSYTMWSRQTPKREWATISSGGNTYYPDSVKGRTISRDNAKNLNY |
| LQMSSLRSEDATLYCARYYREAWFASWQGTLTVVS | LQMSSLRSEDATLYCARYYREAWFASWQGTLTVVS |
**1BLN Light**

DVLMTQTTPSLSVSLGDQASISCRSSQVISIVHTGNTLEYELWQLKPQSPKKLLYIKISNRFSGVPPDRFSGSGSTDFTLKI
SRVEAEDLGYYCFQASHAPRTFGGGTKLEIKR-
SRVEAEDLGYYCFQGSHPWTFGGGTKLEIKRA

**1IGF Heavy**

EVQLVESGGDLVKPGGSLKLSCAASGFTFSRCAMSWRQTPKEKRLEWAVGISGGYSYTYPFDTVKGRFIISRNNARNTLS
LQMSSLRSEDTAICYSTSSDPFHFDYWQGQTTLTVS
LQMSSLRSEDTAMYYCARYSDPFFEYFDYWQGQTTLTVS

**1IGF Light**

DVLMTQTTPSLSVSLGDQASISCRSSQVISIVHTGNTLEYELWQLKPQSPKKLLYIKISNRFSGVPPDRFSGSGSTDFTLKI
SRVEAEDLGYYCFQGSHVPPTFGGGTKLEIKR-
SRVEAEDLGYYCFQGSHVPPTFGGGTKLEIKRA

**1RUR Heavy**

EVQLEESGPELVVRPGTSVKISCKAGYTFTNYWLQWKQRPelahGFEWIGDIIPGQYITTNEKFRTGKAITLADTSSTAY
QVQLQQSGAELVRPGTSVKMSCKAGYTFTNYWLQWKQRPelahGFEWIGDIIPGQYITTNEKFRTGKAITLADTSSTAY
MQLSSLTSEDAYYFCARAGGYTYGDYWQGTSVTS
MQLSSLTSEDAYYCARAGGYTYGDYWQGTSVTS

**1RUR Light**

DIVLTQAASNPVLGASISCRSSKSLLNSGIIHYMYWYLQKPGQSPQDLYQMSKLASPDRFSGSGSTDFTLRI
SRVEAEDVGYYCAQLNLELPYTFGGGTKLEIKR-
SRVEAEDVGYYCAQLNLELPYTFGGGTKLEIKRA
| 1RZ7 Heavy | 1RZ7 Light | 1T2Q Heavy | 1T2Q Light | 2AGJ Heavy |
|------------|------------|------------|------------|------------|
| EVQLVQSGAEVKPGATVKISCKASGYTFSDFYMYWVRQAPKGKEWMGLIDPEADTMYAEEKFRGRVTIADTSTDTGY | EVQLVQSGAEVKPGATVKISCKVSGYTFTDYMYHWYQQAPKGKEWMGLVDPEDGETIYAEEKFQGRVTIADTSTDTAY | LESSLRLSEDVTAYYYCAADPWLNAFNWGWGTLVSS | LESSLRLSEDVTAYYYCATDPWELNYFDYWQGTGLVT - | LE SSLRLSEDVTAVY CATDPWELNYFDYWQGTGLVT - |
| * | * | * | * | * |
| * | * | * | * | * |
| * | * | * | * | * |
| * | * | * | * | * |
| * | * | * | * | * |
| MELSSLRLSEDVTAVYCATDPWELNYFDYWQGTGLVT - S | MELSSLRLSEDVTAVYCATDPWELNYFDYWQGTGLVT - S | MELSSLRLSEDVTAVYCATDPWELNYFDYWQGTGLVT - S | MELSSLRLSEDVTAVYCATDPWELNYFDYWQGTGLVT - S | MELSSLRLSEDVTAVYCATDPWELNYFDYWQGTGLVT - S |
| * | * | * | * | * |
| * | * | * | * | * |
| * | * | * | * | * |
| * | * | * | * | * |
| * | * | * | * | * |

| 1T2Q Light | 1T2Q Light | 2AGJ Heavy |
|------------|------------|------------|
| DFATYYCQQANSF-FTFGGGTKVEIKRT | LEVMLQQLACDTHAIYYCARNRGYSYAMDSWGWQGTSTVS | TLTNZLDPVDTATYCCCARTSGWDDIEFYWQGTLVTVS |
| DFATYYCQQANSFPLTFGGGTKVEIKRT | SRVEAEDLGYYCFQGSHVPLTFGAGTKLELKR | TMNMDPVDTATYCAHRSGWDDIYFDYWQGTLVTVS |
| | | |
| | | |
| | | |
2AGJ Light
EIVLTQSPGTLSSPGERAQLSCRASQTVNDKVAVYQQKPGQPRLYIYGGASRATGPDRFSGSGTDFTLISQGELPE
   |*|***|****|*****|********|********|********|********|********|********|********|
EIVLTQSPGTLSSPGERAQLSCRASQTVSSSYLAWYQQKPGQPAPRLYIYGGASRATGPDRFSGSGTDFTLISRLEPE
DFVYYYCQQYASSPRTFGQGTKEIKRT
   ||*|***|***|****|*******|********|********|********|********|********|********|
DFAVYYCQQYGGSPWTFQGQTKEIKRL

3KYM Heavy
EVQLLESGGGLVQPGGSLRLSCAASGTFSIYPMFWVRQAPGKLEWVSIGPGSGITKYADSVKGRFTISRDNSKNTLYLQ
   |*|***|****|*****|********|********|********|********|********|********|********|
EVQLLESGGGLVQPGGSLRLSCAASGTFSIYAMSVQRQPAGKLEWVSASIGSGSTYYADSVKGRFTISRDNSKNTLYLQ
MNSLRAEDTATYYCAKEHNYFYLWGRGTLVTSE
   |**|*****|*******|********|********|********|********|********|********|********|********|
MNSLRAEDTAVYYCAKEHNYFYLWGRGTLVTSE

3KYM Light
DIQMTQSPGTLSSPGERAQLSCRASQTVSSYLYQQKPGQAPRLYIYDASNRATGPDRFSGSGTDFTLISQGELPE
   |*|***|****|*****|********|********|********|********|********|********|********|
DIQMTQSPGTLSSPGERAQLSCRASQTVSSNLWYQQKPGQAPRLYIYDASNRATGPDRFSGSGTDFTLISQGELPE
FAVYYCQQYDKWPLTFGGGCTKEIK
   |***|*******|********|********|********|********|********|********|********|********|********|
FAVYYCQQYNWPLTFGGGCTKEIK

3QRG Heavy
ILKESGPTLTVKPTQLTTLCTFSGFSLSTSGMVGGWIRQPPGKALEWHLAYNDDKRYNPSLRSRLTIKDTSNQVVL
   |*|***|****|*****|********|********|********|********|********|********|********|
ILKESGPTLTVKPTQLTTLCTFSGFSLSTSVVGWIRQPPGKALEWHLAYNDDKRYNPSLRSRLTIKDTSNQVVL
TMTNMDPVTATYCAHLGYFTYGFAYWQGTLVTSE
   |***|********|********|********|********|********|********|********|********|********|********|
TMTNMDPVTATYCAHLGYFTYGFAYWQGTLVTSE

3QRG Light
DIVMTQSPDLASLGERATINCASQSVDY--NGISYMHWYQQKPGQPPKKLLYIAASNPEGVPDREFSGSGTDFTLTI
   |*|***|****|*****|********|********|********|********|********|********|********|
DIVMTQSPDLASLGERATINCASQSVLYSSNKYNLAWYQQKPGQPPKKLLYIYASTRESGVPDREFSGSGTDFTLTI
SSLQAEDVAVYYCQQQIIEDPWTFQGQTKEIKRT
   |***|********|********|********|********|********|********|********|********|********|********|
SSLQAEDVAVYYCQQYYSPWTFQGQTKEIKRT
4YGV Heavy
QVQLVQSGAEVKPGASKVSLGAQYFTFDTYYHWVRQAPGQGLEWMGETNHRRNGTYYNEKFGKATMTRDTSTSTAYM
ELSSLRSEDATAVYCTIERTYDFMYWGQGTIVTLS

4YGV Light
DIVMTQTPLSLVTPTGPASQCRQSSQIVSDQGNYELWYKVQKPGPQKLLPLYGKSYRFSGVDPDRFSGSGSTDFLTKIS
RVEAEDVGYYCYCQAPFGTPGKLEIKRT

1.3 Comparison of Flexibility Calculations Across Ensemble Generation Methods

In this work, we have considered multiple ensemble generation methods in conjunction with FIRST-PG analysis to determine the flexibility of CDR-H3 loops. Of all methods used in this paper to generate structural ensembles, only MD simulations have previously been coupled with flexibility analysis\(^8\text{–}^{11}\). Rosetta FastRelax, KIC, and RosettaAntibody have not been used previously for flexibility analysis. MD simulations permit for fluctuations between low-energy states and variations in hydrogen bonding networks, effectively capturing the “flickering” nature of hydrogen bonds. The Rosetta-based methods consider hydrogen-bonding energy, but do not involve dynamic motion in the same way as MD simulations. The Rosetta FastRelax protocol generates ensembles representative of the local energy minimum through side-chain repacking and gradient-based energy minimization, so flexibility analysis of these ensembles should be comparable to flexibility analysis of crystal structures. Rosetta KIC on the other hand generates ensembles of low-energy CDR-H3 loop conformations by de novo modeling of the CDR-H3 loop. RosettaAntibody generates ensembles of low-energy antibody conformations, building on KIC motions through additional V\(_{H}\)-V\(_{L}\) docking.

We compared FIRST-PG calculations on Rosetta FastRelax, RosettaAntibody, and MD ensembles, for three well-studied antibodies, excluding KIC ensembles from analysis because they are effectively superseded by RosettaAntibody ensembles. Qualitatively, the FIRST-PG results agree for all methods for 48G7 and the anti-fluorescein antibody. The Rosetta FastRelax results differ from the other methods for the anti-influenza, with the naïve showing significantly more rigidity. This is most likely due to the difference in quality between the crystal structures. Quantitatively, the \(\Delta AUC\) values for RosettaAntibody and MD ensembles for all three antibody pairs compare well (Supplemental Table 2). Additionally, we compared only RosettaAntibody and MD ensembles for three naïve-reverted/mature antibody pairs, where we found that the \(\Delta AUC\) values roughly agree for two out of three antibody pairs. Taken together, these results indicate that flexibility analyses on RosettaAntibody homology model ensembles are similar to analyses on MD ensembles.
Supplementary Figures and Tables

2.1 Supplementary Figures

**Supplementary Figure 1.** Counts of CDR-H3 loop lengths in our crystallographic data set. Colors indicate the number of mutations. The most common loop length is ten, while there is a wide range of loop lengths in the crystallographic data set.
Supplementary Figure 2. Counts of CDR-H3 loop lengths in our crystallographic data set. Colors indicate the species from which the antibody heavy chain is derived. As previously observed, we see that human antibodies have, on average, longer CDR-H3 loops than mouse antibodies.
Supplementary Figure 3. Distribution of mutations in our crystallographic data set. The size of the point represents the number of antibodies possessing that count of heavy and light chain mutations. As expected, mutations are more frequent in the heavy chain than in the light chain.
**Supplementary Figure 4.** FIRST-PG analysis of KIC ensembles of the crystallographic antibody set, with naïve antibody data shown in blue and mature antibody data shown in orange and standard error of the mean shown in a lighter shade of the respective color. Subplots, below each main plot, show the p-value computed by a KS comparison of the naïve and mature DOF distributions for each hydrogen-bonding energy cutoff, with null hypothesis being that the distributions are the same and a dashed line indicating a p-value of 0.05. (Left) When comparing DOFs scaled to a theoretical maximum as a function of hydrogen-bonding energy cutoff for the entire set, the values are similar for both naïve (AUC = \(-5.91 \pm 0.2\)) and mature (AUC = \(-5.81 \pm 0.28\)) antibodies. (Right) Comparison of DOFs for a single length without scaling reveals naïve antibodies to possess a slightly higher DOF value than mature antibodies at the same hydrogen-bonding energy cutoff. AUCs however are within a standard deviation, compare naïve at \(-154.1 \pm 4.8\) and mature at \(-150.4 \pm 7.7\).
Supplementary Figure 5. Average CDR-H3 loop B-factor z-score for antibodies with loops of length 10 split by number of mutations (left) and all antibodies split by heavy-chain species (right). Mature antibodies have at least one mutation. Comparing the difference in mature versus naïve means for length 10 CDR-H3 loops only to a randomized test (as described in the methods) shows only ~2.9% of random permutations have an equal or greater difference. A two-sample KS test yields a p-value of 0.0135 and D of 0.4949, so these distributions appear to be on the threshold of significance. However, that is obviated when bound structures are excluded from analysis, resulting in ~4.8% of random permutations having an equal or greater difference in means than the observed and a KS-test p-value of 0.0989 (with D of 0.3989). It is difficult to quantify if there is a difference in the length 10 set due to low counts (only 11 naïve antibodies), whereas there is no visible difference between the human and mouse antibodies (21.3% of random permutations have an equal or greater difference and the KS-test p-value is 0.6654 [with a D of 0.0748]).
Supplementary Figure 6. Average CDR-H3 loop B-factor z-score compared with either loop length (left) or crystal structure resolution (right). There is not an obvious dependence of CDR-H3 loop B-factor z-score on either.
Supplementary Figure 7. Accuracy of CDR-H3 loop modeling for the paired mature–reverted-naïve antibodies. The loop RMSD versus model energy is shown in what is known as a “funnel plot” for four mature antibodies for which structures are known. Dashed line indicates 1 Å RMSD. Model quality is good for 1A4J and 1IGF with multiple sub-angstrom CDR-H3 loop models at relatively low-energies, while model quality is ok for 1BLN and 1RUR with multiple low-energy models close to achieving sub-angstrom accuracy.
**Supplementary Figure 8.** As in Supplementary Figure 7, accuracy of CDR-H3 loop modeling for the paired mature–reverted-naïve antibodies. The loop RMSD versus model energy is shown in what is known as a “funnel plot” for four mature antibodies for which structures are known. Dashed line indicates 1 Å RMSD, which is an excellent result for de novo loop modeling. Model quality is good for all but 3KYM, with multiple sub-angstrom CDR-H3 loop models at relatively low-energies, while model quality is ok for 3KYM with multiple low-energy models close to achieving sub-angstrom accuracy.
Supplementary Figure 9. As in Supplementary Figures 7 and 8, accuracy of CDR-H3 loop modeling for the paired mature–reverted-naïve antibodies. The loop RMSD versus model energy is shown in what is known as a “funnel plot” for two mature antibodies for which structures are known. Dashed line indicates 1 Å RSMD, which is an excellent result for de novo loop modeling. Model quality is good for both 3QRG and 4YGV, with multiple sub-angstrom CDR-H3 loop models at relatively low-energies.
**Supplementary Figure 10.** CDR loop motions upon antigen binding for four catalytic antibodies. Loop RMSDs (in angstroms) were calculated from the difference in Cα atom positions after alignment of the corresponding (heavy or light) framework Cα atoms. The dashed line indicates 1 Å. PDB IDs for the structures used in these calculations are reported in Supplementary Table 3. For naïve antibodies, greater than 1 Å motions frequently occur in the CDR-H3 loop for all four antibodies, whereas similar motion rarely occurs for the mature equivalents.
Supplementary Figure 11. Difference in CDR loop motions upon antigen binding between naïve and mature antibodies for four catalytic antibodies. RMSD calculations were done in the same manner as for Supplementary Figure 1. The CDR-H3 loops is highlighted in black. The dashed line indicates 1 Å. A more negative value here indicates less motion upon binding in the mature antibody. The effects of affinity maturation on CDR-H3 loop motion in crystal structures are not always significant, with only 2/4 showing motion reduction greater than an angstrom.
Supplementary Figure 12. (Previous page.) CDR-H3 loop B-factor z-scores for three previously studied antibodies, with PDB IDs shown above each plot. B-factor z-scores were calculated with respect to the Fv region and for Cα atoms only. The anti-influenza antibodies have vary in resolution from 2.5 Å for the naïve and mature to 3.0 Å (4HK3) and 3.6 Å (4HKB) for the intermediates. Additionally, the mature anti-influenza antibody has antigen bound affecting the CDR-H3 loop B-factors. We can see that affinity maturation does not always lead to a reduction in CDR-H3 loop B-factor z-scores.
**Supplementary Figure 13.** FIRST-PG analysis of two previously studied antibodies with MD simulations (labelled MD), RosettaAntibody (labelled RAB), and Rosetta FastRelax (labelled relax) used to generate structural ensembles. Naïve antibodies are colored blue and mature antibodies are colored red, while an “intermediate” (4HKB) influenza antibody is shown in green. Subplots, below each main plot, show the p-value computed by a KS comparison of the naïve and mature DOF distributions for each hydrogen-bonding energy cutoff, with null hypothesis being that the distributions are the same and a dashed line indicating a p-value of 0.05. Again, the effects of affinity maturation are not obvious.
**Supplementary Figure 14.** CDR-H3 B-factor z-scores for antigen-bound and free crystal structures of catalytic antibodies 7G12 and 28B4. The 7G12 antibody has higher z-scores for the mature than the naïve antibody for both the (A) unbound and (B) bound structures, indicating a gain in flexibility upon maturation. The 28B4 antibody shows a loss of flexibility upon maturation for the unbound structure comparison (C), but no change in the bound structure comparison (D).
Supplementary Figure 15. CDR-H3 loop B-factor z-scores for antigen-bound and free crystal structures of the catalytic antibody AZ-28 reveal no significant difference between the naïve and mature antibodies.
2.2 Supplementary Tables

Supplementary Table 1. List of antibodies analyzed in this study. The following 922 antibodies were studied (attached separately).
Supplementary Table 2. Rrigidity changes according to several methods. Changes in the rigidity of the 48G7 antibody CDR-H3 loop according to several methods. Unbound is denoted by (U) and bound is denoted by (B). A positive number indicates an increase in rigidity upon affinity maturation. Changes for B-factors are calculated as the difference in the average CDR-H3 loop B-factor between the naïve and mature crystal structure: \( \Delta B = B_{\text{naive}} - B_{\text{mature}} \pm \sqrt{s^2_{\text{naive}} + s^2_{\text{mature}}} \). Changes in FIRST-PG are calculated as the percent change between the AUC of the CD\( \text{R-H3 melting curve for naïve and mature antibodies: } \Delta \text{AUC} = 100 \times \frac{\text{AUC}_\text{mature} - \text{AUC}_\text{naive}}{\text{AUC}_\text{naive}}. \) Finally, changes in MD RMSD or RMSF are calculated as the difference in average CDR-H3 loop RMSF or RMSD between the MD simulations of the naïve and mature antibodies: \( \Delta R = R_{\text{naive}} - R_{\text{mature}} \pm \sqrt{s^2_{\text{naive}} + s^2_{\text{mature}}} \). (*) Only bound crystal structures were available for the 4-4-20 antibody, but Relax, KIC, RA and MD simulations were run without antigen. (#) Only an unbound naïve and bound mature crystal structures were available for the anti-influenza antibody, but Relax, KIC, RA and MD simulations were run without antigen.

| Antibody         | ΔB-Factor | ΔRelax AUC (%) | ΔKIC AUC (%) | ΔRA AUC (%) | ΔMD RMSD   | ΔMD RMSF   | ΔMD AUC (%) |
|------------------|-----------|----------------|--------------|-------------|------------|------------|-------------|
| 48G7 (U)         | 2.14 ± 0.62 | 3.9           | 13.0         | 0.2         | -1.04 ± 0.65 | -0.71 ± 0.64 | -1.3        |
| 48G7 (B)         | 1.21 ± 0.89 | -6.2          | -8.9         | -           | -          | -          | -           |
| 4-4-20 (U)       | -         | -             | -            | -6.2        | 0.85 ± 0.53 | -0.35 ± 0.36 | -4.1        |
| 4-4-20 (B)*      | 0.46 ± 0.77 | -8.4          | 2.8          | -           | -          | -          | -           |
| Influenza (U)    | -         | -             | -            | 6.1         | 2.25 ± 1.33 | 0.44 ± 1.14 | 9.1         |
| Influenza (B)#   | 1.85 ± 1.64 | -15.2         | 1.7          | -           | -          | -          | -           |
Supplemental Table 3. Incomplete list of previously studied naïve–mature antibody pairs, with crystal structures. The naïve antibody here does not always have zero mutations, but rather was designated as naïve by the authors of the original study. Crystal structures may have unresolved residues. Asterisk (*) indicates a catalytic antibody. Additional crystal structures, not listed in the table, include: 11Q9L and 1Q9V; 21Q9R and 1Q9T; 31Q9T; 41FLR; 54HK3 (unbound intermediate); 64FQ2; and 74S1R and 4S1S. Antibody 4JPK* is bound to a designed antigen, rather than the natural one.

| Antibody | H3 Length | Naïve Unbound | Naïve Bound | Mature Unbound | Mature Bound | References |
|----------|-----------|---------------|-------------|----------------|--------------|------------|
| 7G12*    | 5         | 1NGZ          | 1N7M        | 1NGY           | 1NGW         | Yin et al.12 |
| 28B4*    | 8         | 1FL5          | 1FL6        | 1KEL           | 1KEM         | Hsieh-Wilson et al.13 and Yin et al.14 |
| AZ-28*   | 11        | 1D5I          | 1D6V        | 1D5B           | 1AXS         | Ulrich et al.15 and Mundorff et al.16 |
| S25-2/S45-18 | 11   | 1Q9K1        | 1Q9Q2       | 1Q9O           | 1Q9W3        | Nguyen et al.17 |
| 48G7*    | 5         | 2RCS          | 1AJ7        | 1HKL           | 1GAF         | Wedemayer et al.18,19 and Patt et al.20 |
| D44.1/F10.6.6 | 7   | 1MLB          | 1MLC        | 2Q76           | 1P2C         | Braden et al.21, Acierno et al.22, and Cauerhoff et al.23 |
| H26/H63/H8 | 5      | 1DQQ          | 1DQJ/1NDM   | -              | 1NDG         | Li et al.24,25 |
| 4-4-20   | 7         | -             | 1T66        | -              | 4FAB4        | Terzyan et al.26 and Herron et al.27 |
| Anti-influenza | 17    | 4HK0          | -           | 4HKB3          | 4HKX         | Schmidt et al.28 |
| PGT121   | 24        | 4FQQ          | -           | 4FQ16          | 4FQC         | Mouquet et al.29 |
| NIH45-46 | 16        | 4JDV          | 4JDT        | 3U7W           | 3U7Y         | Scharf et al.30 and Diskin et al.31 |
| VRC01    | 12        | 4JPI          | 4JKP8       | -              | 4S1Q7        | Jardine et al.32 and Wu et al.33 |
| VRC03    | 14        | 5JOF          | -           | 5JXA           | 3SE8         | Wu et al.34 and Davenport et al.35 |
| VRC26    | 36        | 4ODH          | -           | 4OD1           | -            | Doria-Rose et al.36 |
Supplementary Table 4. Brief summaries of previous work considering the effects of affinity maturation on antibody flexibility.

| Authors         | Year | Journal  | Short Summary                                                                                                                                                                                                 |
|-----------------|------|----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Foote and Milstein | 1994 | PNAS     | Stopped-flow fluorescence measurements on three antibodies reveal binding kinetics with multiple phases indicating that “ligand binding involved isomerization, as well as associative steps.” The experimentally characterized antibodies were mature, but the authors speculate that “antibodies in the primary repertoire may be more prone to isomerism” and “affinity maturation in such cases may include mutations leading to a more favorable isomeric equilibrium.” |
| Patten et al.   | 1996 | Science  | To our knowledge, the first published suggestion of rigidification of the CDR H3 upon maturation, based on studies of the esterolytic antibody 48G7, “affinity maturation appears to play a conformational role, either in reorganizing the active site geometry or limiting side-chain and backbone flexibility of the germline antibody.” But no direct evidence is presented. |
| Wedemayer et al.| 1997 | Science  | This paper reports crystal structures for hapten bound/unbound and naïve/mature 48G7 antibodies. Comparison of naïve/mature structural rearrangements upon binding reveals reduced CDR H3 motion of the mature antibody. The authors conclude, “The end result of these somatic mutations is a combining site with improved complementarity to hapten … which, in contrast to the germline antibody, binds hapten in a pre-organized fashion.” |
| Chong et al.    | 1999 | PNAS     | 500 ps MD simulation on 48G7 antibody with hapten present found higher RMSFs in the “belly” atoms from the naïve than the mature antibody. |
| Mundorff et al.| 2000 | Biochemistry | Similar to paper #2, this paper compares the naïve/mature bound/unbound structures of catalytic antibody AZ-28. The authors find large rearrangements of the naïve CDR H3 upon hapten binding. |
| Manivel et al. | 2000 | Immunity | In this paper, surface plasmon resonance is used to study anti-peptide antibodies. Comparison of the naïve/mature enthalpic and entropic contributions to binding reveals that reduction of entropic contributions is the primary cause for increased affinity upon maturation. The authors conclude, “high affinity and specificity are simultaneously achieved by the simple device of regulating paratope flexibility.” |
| Yin et al.      | 2001 | Biochemistry | Similar to papers #2 & #3, this paper compares naïve/mature bound/unbound structures of redox antibody 28B4 (half of which were solved in Hseih-Wilson et al. [PNAS, 1996]). The authors find that there is more motion in germline CDRs H3 and L1, than in the mature, concluding, “mutations introduced into the germline antibody … act to decrease the CDR loop flexibility and preorganize hapten
|   | Authors               | Year  | Journal | Details                                                                 |
|---|----------------------|-------|---------|-------------------------------------------------------------------------|
| 6 | Jimenez et al.       | 2003  | PNAS    | Using a chromophore (fluorescin) and three-pulse photon echo peak shift (3PEPS) spectroscopy, this paper compares the dynamics of three antibody-fluorescein complexes (including 4-4-20), demonstrating different mechanisms of antigen recognition, including lock-and-key, induced-fit, and conformational selection. |
| 7 | Yin et al.           | 2003  | JMB     | Similar to papers #2, #3, & #5, crystal structures of naïve antibody 7G12 in complex with cognate hapten, non-cognate jeffamine, and ligand-free reveal that large changes in the CDR H3 loop permit binding of both molecules. Comparison to the mature antibody shows that the maturation process rigidifies the loop in the hapten-bound state and sterically occludes jeffamine binding. |
| 8 | Li et al.            | 2003  | NSM     | To our knowledge, the first published study with protein antigen, rather than small molecules or peptides (as in #1–7). Authors compared crystal structures of antibody–hen-egg-white-lysozyme complexes for a high-affinity and low-affinity antibody. Additional structural comparison was made to crystal structures of unbound antibodies. SPR was used to determine binding parameters. Overall, the results show that binding is enhanced due to increased burial of apolar surface area and improved shape complementarity. Evidence of CDR H3 loop rearrangements is minimal. |
| 9 | Jimenez et al.       | 2004  | PNAS    | Similar to #6, this paper uses 3PEPS to characterize the effects of mutations in the light chain on 4-4-20 antibody–antigen complex flexibility. The authors find significant rigidification of the complex, as exhibited by decreased picosecond and nanosecond time-scale motions by 2.5- and 20-fold in the mature vs. naïve antibody, with the significant caveat that only the light chain was reverted. |
| 10| Zimmerman et al.     | 2006  | PNAS    | A follow-up paper on #9. Using 3PEPS, dynamic Stokes-shift (DSS), SPR, and MD experiments, this paper characterizes the evolution of the antifluorescein antibody 4-4-20. All experiments show that the naïve antibody exhibits more conformational heterogeneity than the mature antibody. MD shows that the naïve motion is primarily in the L1, L2, and H3 CDRs. Therefore, the conclusion is that evolution optimizes a binding site for a specific molecule by preconfiguration. |
| 11| Thorpe et al.        | 2007  | PNAS    | A follow-up paper on #10, with MD simulations of several (varying in maturation) bound/unbound 4-4-20 antibody–antigen complexes. Analysis shows larger fluctuations in the germline unbound simulation than the bound. Calculations of the enthalpies and entropies show a larger change entropy (of binding) upon maturation than in enthalpy (of binding). |
|    | Authors         | Year | Journal       | Summary                                                                 | Additional Notes                                      |
|----|----------------|------|---------------|------------------------------------------------------------------------|--------------------------------------------------------|
| 12 | Thielges et al.| 2008 | Biochemistry  | This paper analyzes the thermodynamics and dynamics of six              | **antifluorescent antibodies** (including 4-4-20). ITC is used to measure enthalpies and entropies of binding. 3PEPS and transient grading (TG) experiments are used to measure motions on the femto-, pico-, and nano-second timescales. Experiments show antibodies net favorable dG binding is due in some cases primarily to a favorable enthalpy change, but one case due to a favorable entropy change. The authors conclude that the immune system can generate antibodies with a variety of dynamics, from rigid antibodies capable of lock-and-key binding to flexible ones capable of induce-fit or conformational-selection binding.** |
| 13 | Babor et al.   | 2008 | Proteins      | In this paper, the authors use Rosetta Design on antibody crystal       | structures to recover CDR H3 sequences. When the design is constrained to multiple structures, then naïve sequence is more likely to be recovered than the mature sequence. This is taken to indicate that the naïve sequence is optimal for conformational flexibility. |
| 14 | Wong et al.    | 2010 | Proteins      | In this paper, four catalytic antibodies and their naïve equivalents are | modeled using MD simulations. Flexibility was assessed by comparing alpha-carbon B-factors for the six CDR loops. In three of the four studied antibodies, a loss of flexibility in residues contacting antigen was observed following maturation. |
| 15 | Adhikary et al.| 2012 | JBC           | This paper is similar to #12, in that a panel of antibodies was evolved | against a chromophore (MPTS) and they were studied by ITC, 3PEPS, and TG. The results are similar to #12, in that the antibodies had varying dynamics and recognized MPTS through multiple mechanisms. The authors conclude that antibodies are initial dynamic with the potential to recognize multiple targets, but eventually are tailored to a single target by evolution. |
| 16 | Schmidt et al. | 2013 | PNAS          | This paper involves crystallization, MD simulation, and SPR studies of   | broadly neutralizing influenza virus antibodies. The authors show with ~30 microsecond MD simulations that the naïve antibody CDR H3 loop is rarely in the bound conformation, whereas the mature antibody CDR H3 loops occupy the bound conformation between 20–70% of the time. SPR shows that ~66% of the improvement in KD can be attributed to a 10-fold decrease in dissociation rate upon affinity maturation. The authors collectively interpret the results to indicate that “increased conformational restriction of the CDR H3 has been the principle consequence of affinity maturation.” |
| 17 | Willis et al.  | 2013 | PLoS Comp. Bio.| Similar to #13, this paper uses multi/single-state design to assess the  | structural preference (sequence recovery) of naïve/mature sequences. The authors design on multiple structures originating from the same VH germline, diverging from the previous study. Their results indicate that multi-state design is more likely to recapitulate the naïve sequence whereas single-state design is more likely to recapitulate the mature sequence. The conclusion is that germline sequences possess high conformational flexibility. |
|   | Authors          | Year | Journal     | Details                                                                                                                                                                                                 |
|---|-----------------|------|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|18 | Adhikary et al. | 2015 | Biochemistry| Similar to #12 & #15, this paper uses ITC, 3PEPS, crystallography, and ELISA to analyze the thermodynamics and dynamics of **anti-MPTS antibodies**. The authors selectively isolate three antibodies with varying specificity for MPTS and other proteins. The authors find that the antibody with the few somatic mutations is the most polyspecific, but also the least dynamic. On the other hand, the antibody with the highest affinity for MPTS is the most dynamic. The authors conclude that affinity maturation can have divergent effects on dynamics towards antigen recognition.  

|19 | Li et al.       | 2015 | PLoS Comp. Bio. | The authors assess the flexibility of three antibodies (**anti-fluorescein, anti-CD3, 48G7**) using MD to generate ensembles and a distance constraint model to evaluate flexibility. The authors note a significant amount of rigidity increases in the CDR H3 loop and flexibility increases in the CDR L2 loop. They believe these effects are compensatory.  

|20 | Davenport et al.| 2016 | Structure    | This paper studies the effects of SHM on the dynamics of three **anti-HIV antibodies** using HXMS. The authors find that most stabilization occurred in the CDR L2, H2, and FW3. This contradicts previous studies. The authors rationalize the contradiction as arising due to the relative complexity of HIV antigen versus the previous studied antigen.  |
### Supplementary Table 5. Manually identified germlines.

| PDB  | Selected Germline  |
|------|--------------------|
| 5ggs | IGKV3D-7*01        |
| 5ibu | IGHV5-51*01        |
| 5wuv | IGHV3-48*01        |
| 5w05 | IGHV5-10-1*01      |
| 5w06 | IGHV5-10-1*01      |
| 5uy3 | IGHV1-8*01         |
| 5v7j | IGLV3-21*02        |
| 5b71 | IGHV3-66*01        |
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