Multistate design of influenza antibodies improves affinity and breadth against seasonal viruses

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Influenza is a yearly threat to global public health. Rapid changes in influenza surface proteins resulting from antigenic drift and shift events make it difficult to readily identify antibodies with broadly neutralizing activity against different influenza subtypes with high frequency, specifically antibodies targeting the receptor binding domain (RBD) on influenza HA protein. We developed an optimized computational design method that is able to optimize an antibody for recognition of large panels of antigens. To demonstrate the utility of this multistate design method, we used it to redesign an influenza antibody against a large panel of more than 500 seasonal HA antigens of the H1 subtype. As a proof of concept, we tested this method on a variety of known antiinfluenza antibodies and identified those that could be improved computationally. We generated redesigned variants of antibody C05 to the HA RBD and experimentally characterized variants that exhibited improved breadth and affinity against our panel. C05 mutants exhibited improved affinity for three of the subtypes used in design by stabilizing the CDRH3 loop and creating favorable electrostatic interactions with the antigen. These mutants possess increased breadth and affinity of binding while maintaining high-affinity binding to existing targets, surpassing a major limitation up to this point.

Influenza is a yearly threat to global public health. As many as 56,000 deaths and 710,000 hospitalizations annually can be attributed to influenza infection in the United States (1). In addition, vaccine efficacy has been variable depending on the year (2). A major factor that limits influenza vaccine efficacy is the fact that preexisting antibodies frequently lack the ability to react with current circulating strains. Of particular interest are antibodies that target the receptor-binding domain (RBD) of the HA protein, the site at which the viral protein interacts with the host cell receptor, sialic acid. These antibodies typically neutralize virus very potently (3–6), as they directly inhibit binding of virus to the host cell receptor. However, as the region of HA around the RBD is highly variable, antibodies to this domain tend to have restricted specificity to strains within a single subtype (4–6).

Recently, many antibodies have been described that mimic the chemical interactions of the sialic acid receptor with HA (3–6). The existence of such antibodies has suggested that broad, RBD-specific antibodies elicited by vaccination may be sufficient to protect against future strains, and could become one of the primary components of a proposed “universal flu vaccine” (7). One such antibody, C05, has remarkable breadth of recognition of HAs from certain strains within both group 1 and group 2 viruses, and interacts with the HA molecule by using a single antibody hypervariable loop (8). However, this antibody still has incomplete breadth against HAs within a particular subtype: for example, it is unable to recognize H1 strains circulating in humans after the 2009 H1N1 pandemic, primarily because of a lysine insertion at position 133a (3, 9).

Given the limitations of naturally occurring human antibodies, we sought in this study to use computational design to increase the breadth of existing antiinfluenza antibodies. Computational design has been successful in redesigning a single antibody–antigen interaction (10–12); however, until recently, it has been challenging to include multiple antibody–antigen interactions in a single design simulation to optimize the antibody sequence for recognition of multiple antigens simultaneously. We developed a method that significantly improves the computational efficiency of such multispecificity design (13). To further improve the utility of this method, we reconfigured the method to run in parallel on multiple computing nodes, enabling much larger-scale simulations, and validated this method on redesign of antiinfluenza antibodies. As a proof of principle of the utility of this computational method, we applied the method to the redesign of existing human antibodies against viruses of the influenza A H1 subtype. We expressed and tested a panel of computationally generated variants of antibody C05 and identified mutant antibodies that bound

Significance

Influenza is an annual threat to global public health, in part because of constant antigenic drift that facilitates evasion of the antibody response. Rapid changes in the influenza HA protein make it difficult for an antibody to achieve broad activity against different virus subtypes. We developed a computational method that can optimize an antibody sequence to be robust against seasonal variation. As a proof of concept, we tested this method by redesigning a known antibody against a set of diverse HA antigens and showed that the variant redesigned antibodies have improved activity against the virus panel, as predicted. This work shows that computational design can improve naturally occurring antibodies for recognition of different virus strains.

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 600UL).

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one influenza strain not recognized by C05 and increased affinity against a strain that is recognized with low affinity by C05.

Results

Experimental Workflow. We sought to use RECON (restrained convergence) multistate design to increase the breadth of certain antiinfluenza monoclonal antibodies. The RECON algorithm works by allowing each state to design its optimal sequence, then encouraging the sequences to converge on a multistate solution. The quality of designs then is evaluated by the sum of Rosetta energy of all input complexes. Although the design is done on a fixed backbone, design is iterated with rounds of backbone motion to allow for an ensemble of backbones to be optimized. The RECON multistate design method was written originally to process states serially, which limited both the number of states that could be included and the number of designed residues in each state (13). To address this limitation, we refactored the RECON algorithm to run in parallel by handling each state on a separate processor and implementing Message Passing Interface communication between the different processors (SI Appendix, Fig. S1). The improved parallel RECON protocol therefore is able to handle much larger ensembles of input states. We decided to test application of the parallel RECON protocol on redesign of influenza antibodies against a set of seasonal virus variants (Fig. 1). As a proof of principle for this method, we computationally redesigned existing antibodies against an antigenic panel, then expressed and tested antibody variants for improvement in breadth and affinity across the panel.

Benchmark of Large-Scale Design. To test the utility of the parallel RECON protocol, we first sought to redesign an antiinfluenza antibody for increased breadth of binding to diverse HA antigens from a large panel of seasonal influenza virus strains. We created homology models of HA proteins from the sequences of 524 viruses in the Influenza Research Database using the RosettaCM multitemplate comparative modeling protocol (14, 15). We paired each of the 524 modeled viral proteins with antibody C05 (3) and redesigned the antibody sequence for broad recognition of antigens in the viral panel. We successfully ran 50 independent multistate design simulations against this large seasonal virus HA panel in 13.2 h, distributed over 524 processors (SI Appendix, Table S1). The design simulations scaled well with additional states, and the only limitation on number of states was the number of available processors on our computing cluster. The designed models showed significant variability in sequence, specifically in the antibody CDRH3 region, suggesting that the computational method proposes significantly modified CDRH3 sequences to target the diverse panel of antigens.

Design of H1 Subtype Breadth in Influenza HA Antibodies. Next, we sought to design antibodies with increased breadth among the H1 subtype of influenza. We first identified all H1 subtype HA proteins with crystal structures in the Protein Data Bank (PDB) with a resolution better than 3.5 Å (SI Appendix, Table S1), yielding 13 unique antigens. Next, we identified 7 antibodies that are known to bind at least one H1 HA protein in the panel and that have high-resolution (better than 3.5 Å) cocrystal structures in the PDB. We then created complexes of each antibody with all
Fig. 3. C05 mutants show increased affinity against low-affinity strains. Affinity values for C05 variants of interest against the computationally designed panel are shown as a heat map (Left), and the fold change from WT is also shown (Right). Binding affinity was measured on a ForteBio Octet Red system for all strains except A/Solomon Islands/03/2006 and A/Thailand/CU44/2006, for which binding affinity was estimated by using ELISA. Gray indicates that binding was not detected.

13 viral proteins in the panel and ran RECON multistate design to generate antibody variants with increased breadth across the panel (Fig. 2).

Some antibodies, such as mAb 5J8, showed a modest improvement across all targets in the panel, but not a drastic improvement for any target (Fig. 2A). Other antibodies, such as mAbs CH65, CH67, and 641 I9, showed a strong energetic improvement for some targets, with a deleterious effect on other targets. These designs were considered unsuitable for testing because they were predicted to have a decrease in breadth, albeit with an increase in affinity for certain targets. The antibody in this study with the strongest improvement for several targets without sacrificing affinity for others was C05. C05 is structurally distinct among influenza antibodies in that it binds primarily with a long (26-aa) CDRH3 loop and also has a 5-residue insertion in CDRH1 that can contribute to antigen recognition in some cases (3). We decided to validate C05 variants experimentally to test the effects of multistate design mutations on affinity and breadth.

Experimental Validation of C05 Mutants. We next sought to validate the predicted increases in breadth and affinity of binding for C05 variant antibodies. We observed many suggested mutations in the CDRH1 and CDRH3 loops of C05 (Fig. 2B). The majority of these mutations were focused in the distal end of the CDRH3 loop, which is in close contact with the antigen. We modeled the effects of each suggested mutation as a single or double amino acid substitution and measured the effect on the energy of the antibody–antigen complex (SI Appendix, Fig. S3). Of the mutations introduced, only a small number appeared to contribute the majority of the energetic improvement. We focused our subsequent experimental efforts on mutations that were predicted to have the largest impact on energy of binding. Among 71 single or double amino acid mutants introduced by multistate design, 27 passed a quantitative and qualitative evaluation for experimental characterization (SI Appendix, Table S2). The quantitative filter allows mutations with an improvement in fitness of greater than 0.5 SD. The qualitative filter consisted of visual inspection and accounting for known pathologies in the Rosetta score function. As part of the qualitative assessment, we relied primarily on energetic terms such as van der Waals interactions, hydrogen bonding, and backbone stabilization via favorable φ-ω angles, and paid less attention to terms such as internal rotamer energy, which tends to disfavor rare rotamers even if they are well resolved in the crystal structure, and reference energy, which is derived empirically to optimize sequence recovery on a global scale (16). The selected mutations are predicted to have several effects, including establishing new hydrogen bonds across the interface (14 of 27) and within the antibody (8 of 27), improving van der Waals interactions (6 of 27), stabilizing loop conformations (2 of 27), and relieving clashes with antigenic residues (3 of 27).

We next synthesized a group of cDNAs for antibody variable genes encoding 33 C05 variant antibodies, comprising 27 single or double amino acid mutants that passed the previously discussed filters and 6 combinations of mutations that were predicted to result in the greatest improvement in stability and binding affinity. We expressed and purified the variant antibodies as IgG molecules and measured their activity and binding kinetics by using the ForteBio Octet system. SDS/PAGE was used to confirm correct molecular weight and high purity of purified IgG molecules (SI Appendix, Fig. S4). Binding traces of all interactions reported in this study are shown in SI Appendix, Fig. S5. For reference, we include a list of all influenza strains tested experimentally in this study and for which assays they were used (SI Appendix, Table S3).

We observed two mutants that exhibited increased affinity and breadth across the panel (Fig. 3 and Table 1). The majority of the effect on affinity was focused on strains that were recognized by C05 with low affinity, namely the avian strain A/mallard/Alberta/35/1976 and the human strain A/Puerto Rico/8/1934. Full binding data of C05 mutants over all strains tested in this study are shown in Fig. 3, Table 1, and SI Appendix, Fig. S5. Mutations V110P and A117E in the CDRH3 loop increased affinity for A/mallard/Alberta/35/1976 by approximately four- and threefold, respectively, with the combination of both mutations improving affinity by approximately a factor of four to five. Interestingly, the single mutations each increased the on-rate of the antibody–antigen interaction, with a slight decrease in off-rate. The double mutant showed a greater increase in on-rate with a concomitant increase in off-rate, which limited the total effect on affinity. The V110P mutation also increased breadth by facilitating binding to a new strain that was not recognized by WT C05, A/Puerto Rico/8/1934. C05 V110P recognized A/Puerto Rico/8/1934 with a modest but observable affinity in the micromolar range, whereas the WT

Table 1. Binding kinetics of C05 variants to strains A/Puerto Rico/8/1934 and A/mallard/Alberta/35/1976

| Variant | Kₐ, μM | Kᵦ fold change | kₐ,m, 1/Ms × 10⁴ | kₐ,m, 1/s × 10⁻¹ | Kₐ, nM | Kᵦ fold change | kₐ,m, 1/Ms × 10⁻³ | kₐ,m, 1/s × 10⁻¹ |
|---------|-------|----------------|-----------------|-----------------|--------|----------------|-----------------|-----------------|
| WT      | ND    | ---            | ND              | ND              | 511 ± 18 | 4.1 ± 0.01 | 2.1 ± 0.03 |
| V110P   | 42 ± 4 | >4.8          | 1.2 ± 0.2       | 5.1 ± 0.2       | 120 ± 4   | 4.3           | 14.5 ± 0.3      | 1.7 ± 0.06       |
| A117E   | ND    | ---            | ND              | ND              | 199 ± 21  | 2.6           | 5.1 ± 0.3       | 1.0 ± 0.09       |
| V110P-A117E | ND | ---            | ND              | ND              | 106 ± 7   | 4.8           | 202 ± 11        | 21.5 ± 0.5       |

Binding kinetics were measured on a ForteBio Octet Red system with four dilutions of antibody. Data were fit to a 2:1 binding model and the high-affinity component is reported. WT Kₐ against A/Puerto Rico/8/1934 is estimated to be >200 μM. ND, not detected.
antibody did not show any binding activity, even when tested at high concentrations. We estimate that this mutation contributed to an increase of affinity by a factor of at least 4.8 for this antigen.

To confirm that this modest binding was not an artifact of the testing format, we repeated the assay with C05 WT or V110P immobilized to the biosensor and HA used as the analyte. We observed a similar pattern in this format, with no detectable affinity for C05 WT and an affinity of 2.3 μM for V110P (SI Appendix, Fig. S6). These mutants were also tested for binding to the remaining members of the panel; however, apart from the two previously discussed strains and two high-affinity strains (A/Solomon Islands/03/2006 and A/Hong Kong/1/1968 (H3 HK68), no binding was observed for the WT or variant antibodies (Fig. 3).

Therefore, C05 variant antibodies possessed increased affinity for two weakly bound strains; however, we were interested in whether these variant antibodies lost affinity for strains that were recognized previously. Several groups have reported a tradeoff between affinity and breadth, in which mutated antibodies that have gained affinity for several targets lose affinity for other targets (9, 17, 18). This pattern has been observed for antibody C05 in experiments designed to improve affinity for H1 and H3 viruses (9). We observed that the mutants in this study maintained high affinity for strains in the panel that were previously recognized by C05 (SI Appendix, Fig. S7). We compared the binding activity of the mutant from this study, V110P-A117E, to an experimentally derived mutant from a study by Wu et al. (9), referred to as VVSSGW. Relative K0, was determined by ELISA for V110P-A117E and by the Octet system for VVSSGW.

Advantage in preserving high-affinity binding across a panel, at least in this analysis.

To characterize the biological activity of these antibody variants, we performed hemagglutination inhibition (HAI) assay with a panel of influenza viruses (Table 2). Although the mutants V110P and V110P-A117E show improved binding affinity against viruses A/Puerto Rico/8/1934 and A/mallard/35/1976, the variant antibodies as well as the WT failed to inhibit hemagglutination of these two viruses. We attribute this to the binding affinities, which, although improved, are still outside of the range necessary for viral neutralization, as well as high dissociation rates. In addition, we tested the V110P and V110P-A117E variants against a panel of viruses neutralized by C05 with high affinity. In general, the variants maintain potent neutralization against these viruses, consistent with the ELISA binding data showing that these variants do not sacrifice affinity for previously recognized strains.

**Structural Characterization of a Redesigned C05 Variant.** We next sought to confirm the accuracy of our models of the C05 double mutant V110P-A117E. Therefore, we determined the crystal structure of the HA1 subunit from A/Hong Kong/1/1968 (H3N2) in complex with the V110P-A117E C05 variant at a resolution of 3.25 Å (SI Appendix, Table S4) (19). Although the modeling simulations were performed with HA model structures, we observed the same pattern in this format, with no detectable affinity for C05 WT and an affinity of 2.3 μM for V110P (SI Appendix, Fig. S6). These mutants were also tested for binding to the remaining members of the panel; however, apart from the two previously discussed strains and two high-affinity strains (A/Solomon Islands/03/2006 and A/Hong Kong/1/1968), no binding was observed for the WT or variant antibodies (Fig. 3).

Therefore, C05 variant antibodies possessed increased affinity for two weakly bound strains; however, we were interested in whether these variant antibodies lost affinity for strains that were recognized previously. Several groups have reported a tradeoff between affinity and breadth, in which mutated antibodies that have gained affinity for several targets lose affinity for other targets (9, 17, 18). This pattern has been observed for antibody C05 in experiments designed to improve affinity for H1 and H3 viruses (9). We observed that the mutants in this study maintained high affinity for strains in the panel that were previously recognized by C05 (SI Appendix, Fig. S7). We compared the binding activity of the mutant from this study, V110P-A117E, to an experimentally derived mutant from a study by Wu et al. (9), referred to as VVSSGW (Fig. 4). Although the VVSSGW variant possesses increased H1 affinity by a greater magnitude, this improvement came at the cost of H3 affinity, which was reduced. The V110P-A117E mutation increased affinity by a more moderate factor, but did not reduce affinity for either of these two strains. Interestingly, the H3 subtype was not included in any design simulations. However, by including diverse H1 strains in the computational panel, we were able to focus on the most highly conserved residues, which has the side effect of maintaining H3 affinity as well.

In addition, we compared the binding activity to a panel of strains of different subtypes (SI Appendix, Fig. S7). In general, the V110P-A117E mutation is able to maintain high-affinity binding to these heterosubtypic strains. Notably, neither of the two strains that were accounted for in the computational panel displayed a significant effect on binding. In the case of the H2N2 strain A/Japan/305+/1957, we did detect an approximately two-fold decrease in binding; however, it is worthwhile to note that the VVSSGW variant has no detectable binding to this strain (9).

![Fig. 4.](Image)

Table 2. HAI activity of WT C05 and redesigned variants

| Subtype | Virus          | C05 WT | V110P | V110P-A117E |
|---------|----------------|--------|-------|-------------|
| H1      | A/Puerto Rico/8/1934 | >      | >     | >           |
|         | A/Solomon Islands/03/2006 | 0.3    | 0.3   | 0.6         |
|         | A/mallard/Alberta/35/1976 | >      | >     | >           |
|         | A/New Caledonia/20/1999 | 0.2    | 0.2   | 0.2         |
| H3      | A/Hong Kong/1/1968 | 0.04   | 0.04  | 0.04        |

Shown is the concentration in micrometers per milliliter at the endpoint titer. The “>” symbol indicates that HAI was not observed when testing concentrations as high as 100 μg/mL.
Fig. 5. Crystal structure of the C05 V110P-A117E double mutant in complex with A/Hong Kong/1/68 head domain confirms the accuracy of the computational models. (A) Structure of V110P-A117E is shown in complex with A/Hong Kong/1/68, with the 2Fo-Fc electron density contoured at 1.0 σ. (B) Model of C05 V110P-A117E in complex with A/mallard/Alberta/35/1976, with predicted hydrogen bonding shown as dashed lines. rmsd measurements in Angströms over all atoms and Ca atoms are shown as dashed lines.

(Fig. 5). This hypothesized mechanism is also consistent with the observation that A117E is not universally favorable for all antigens: it confers an increase in affinity for A/mallard/Alberta/35/1976 with a slight decrease in affinity for A/Puerto Rico/8/1934 (Fig. 3), as evidenced by the fact that the double mutant is unable to recognize A/Puerto Rico/8/1934 whereas V110P can.

As a complementary approach, we characterized the interaction of C05 V110P-A117E with the head domain of A/Solomon Islands/03/2006 HA by using hydrogen–deuterium exchange MS. We mapped the perturbation of hydrogen–deuterium exchange upon antibody–antigen binding on the epitope and paratope (SI Appendix, Fig. S8). We observed peptides originating from the CDRH3 loop to be most solvent occluded in the antibody–antigen complex, most notably at short time points (<1 min). This is in accordance with the predicted binding mode from the Rosetta models. In addition, the epitope peptides shielded upon binding are located along the rim of the RBD, which is in agreement with the models and crystal structure (SI Appendix, Fig. S8).

To test the effect of mutations on the thermodynamic stability of the antibody, we measured the melting temperature of variants by using differential scanning fluorimetry (DSF). The variants mostly exhibited two melting transitions, one at ~62 °C and another at ~69 °C, corresponding to the Fc and Fab domains, respectively (SI Appendix, Fig. S9). This finding agrees with previous data on IgG melting transitions (21). To confirm these domain assignments, we repeated the experiment with a cleaved Fab protein and observed the transition at ~70 °C (SI Appendix, Table S5). As predicted, mutation V110P did increase the antibody stability by approximately 0.5 °C, although addition of A117E reduced stability by 0.6 °C (Table 3). Notably, several other mutations also increased the melting temperature by a significant margin, including mutations that had a neutral or negative impact on binding affinity. Previous studies suggested that an increase of 1 °C is sufficient to increase affinity 10-fold (22). However, these results suggest that an increase in stability does not necessarily confer an increase in binding affinity.

Table 3. Thermodynamic stability of C05 mutants as measured by DSF

| Variant              | Transition 2, °C | Change from WT, °C | Significance |
|----------------------|-----------------|-------------------|-------------|
| C05 WT               | 69.8            | —                 |             |
| S27G F28P V110P      | 70.8            | 1.0               | **          |
| F28E                 | 70.4            | 0.6               | *           |
| Y35H V110P           | 70.8            | 0.9               | **          |
| V110P                | 70.3            | 0.5               | *           |
| V110P A117E          | 69.2            | —0.6              | *           |
| A117E                | 69.2            | —0.6              | *           |
| D118R                | 70.6            | 0.8               | **          |
| D120R                | 70.3            | 0.5               | *           |
| 6-aa mutant          | 71.9            | 2.1               | **          |

Statistical significance was assessed using a two-tailed t test: **P < 0.005 and *P < 0.05.

Tradeoff in Breadth and Affinity. A common theme in design of antibody breadth is a tradeoff between breadth and affinity. This relationship has been shown theoretically (17, 23) and in practice (9), and is a major motivation for computational methods that can account for hundreds of antigens during design, such as the RECON algorithm. To test if an antibody must sacrifice affinity for an individual target to acquire breadth, we repeated design of C05 against each of the antigens in the panel by using single-state design against each of the targets individually instead of RECON multistate design. The results showed a tradeoff between breadth and affinity, as the single-state–designed antibody was consistently better against each target than the multistate solution in terms of their evaluation by the Rosetta energy function (SI Appendix, Fig. S10). For each of the antigens, the redesigned C05 for single-state–optimized binding had lower total score than the optimal multistate C05, and 10 of 13 had lower predicted binding affinity for single-state design than multistate design. This finding supports the idea that, at least in our in silico simulations, multistate design achieves a compromise between the optimal single-state solutions.

Discussion

Summary of Results. In this study, we report a protocol for multistate design of large, parallelized panels of influenza virus strains. We adapted a previously reported protocol for multistate design to run in parallel on a computing cluster and showed that this protocol can scale to very large (>500) panels of antigens. As a proof of principle, we applied this methodology to designing antinfluenza antibodies for increased breadth against panels of seasonal H1 viruses. We report redesigned antibodies that have approximately a fivefold increased affinity against one strain and now detectable binding to another strain in the panel, while maintaining high-affinity binding for previously targeted members of the panel.

Large-Scale Panels in Multistate Design. Multistate design has been successful in a number of different applications; however, it is generally applied to modulating specificity in protein–protein binding partners (24–26) or modeling conformational ensembles of a single protein (27). We instead focused here on design of an antibody against a large ensemble of targets. In typical computational antibody design approaches, a single antigen or a small panel of representative antigens is modeled and assumed to represent the scope of antigenic variability (10, 28). However, by using the protocol reported here, it should be possible to include a much larger panel of targets, easily making an antibody robust to antigenic variation. In this work, the affinity increases that we report (approximately fivefold) are modest compared with the increases reported in other studies that use experimental approaches (9) or computational approaches with single antigens (22). We expect that, as the size of the target panel increases, it will become increasingly difficult to find mutations that can improve affinity for some targets in the panel without sacrificing affinity for any other targets. Therefore, a modest increase in affinity may be more realistic when designing against large and diverse antigenic panels, especially when considering already high-affinity complexes. However, the advantage of this approach is that the affinity-enhancing mutations can be selected to be compatible with all targets, which is often not the
case with experimental approaches that do not account for multiple states (9).

We note that, although we ran design simulations on seven anti-influenza antibodies, we experimentally validated mutants of only one antibody, C05. The computational results of the remaining six antibodies suggest that not all antibodies are suitable for multistate design, as some are likely to be already close to optimal for breadth and/or affinity.

Our work focuses on the problem of antibody redesign, wherein a known antibody targeting a defined epitope can be reengineered to increase binding breadth. However, we do not address the more challenging problem of designing an antibody de novo against a novel epitope. Such a problem has been approached computationally by several groups (11, 12), and we expect that our multistate design approach could be applied in the future to a de novo engineered antibody to further increase the power of the method.

Mechanisms of Action. We hypothesize that the V110P mutant reported in this work enhances affinity by increasing the thermodynamic stability of the antibody. It is common that affinity-enhancing mutations are located in positions that do not directly contact antigen and function by increasing antibody stability and rigidifying CDR loops (29, 30). This phenomenon is seen in silico-engineered antibodies, mutations introduced by directed evolution, and naturally occurring somatic mutations from mature antibodies. We also generated several mutants that increased thermodynamic stability but failed to improve binding affinity, showing that increased stability is not sufficient by itself for increase of binding to a target. A mutant with increased affinity but decreased thermodynamic stability, A117E, is predicted to increase affinity by establishing electrostatic contacts on the antigen, which has traditionally been a difficult task in Rosetta protein interface design. This mutation is also more selective than V110P, improving affinity for only strains that have the correct electrostatic partner in position to make contact. The difference in mechanism between these two mutations illustrates the balance between breadth and affinity in antibody evolution.

Mutations that improve only antibody stability without directly contacting the antigen are more likely to be beneficial across a panel of targets, whereas mutations that require specific electrostatic partners are likely to be more selective.

Implications for Influenza Studies. The antibody highlighted in this work, C05, is a clinically relevant antibody of interest for therapeutic and vaccine development. As it targets a very small epitope on the RBD of influenza, it potently neutralizes certain viruses from H1 and H3 subtypes (3). By using Rosetta design and a trimeric linker, Strauch et al. (31) were able to engineer a protein binder targeting the influenza RBD that was based on the C05 epitope. Our work suggests that C05 can be optimized further for affinity and breadth. One limitation to C05 binding is that it is susceptible to the 133a insertion that emerged after the 2009 H1 pandemic (3). Interestingly, one of the strains that was bound more tightly by C05 variants, A/mallard/Alberta/35/1976, is an avian virus that contains the lysine insertion at 133a characteristic of C05 escape (9). This finding suggests that C05 recognition of K133a strains may be possible with further optimization, improving this already potent antibody further.

Materials and Methods

Template structures were downloaded from the PDB and processed to remove waters and nonprotein residues. To generate mock complexes of antibody and antigen, the antigens in the panel were aligned to the antigen in the cryocystal structure of each antibody. We considered any residue on the antibody with a heavy atom within 7 Å of a heavy atom on the HA for design. We ran RECON multistate design with four rounds of a ramping sequence constraint (13). A total of 100 design simulations were performed for each antibody. For experimental characterization, point mutants of antibody C05 were generated by using site-directed mutagenesis with the QuikChange II kit (Agilent Technologies), using the recommended protocol, in an Ig expression vector. Antibody variants were expressed by transient transfection of Exp293F HEK cells and purified from supernatant by using protein A. Binding kinetics were determined using biolayer interferometry with an Octet Red instrument. Viral neutralization was measured by HAI assay. Full methods are detailed in SI Appendix, Materials and Methods.

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