Supporting Information

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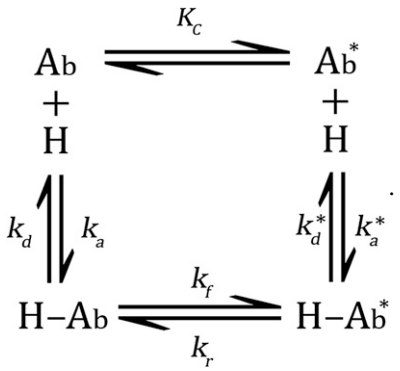
SI Materials and Methods

Kinetic Parameters. The unmutated common ancestor (UCA) and intermediate 2 (I-2) surface plasmon resonance (SPR) binding data were fit with the following model, in which the Fab with correctly configured CDR H3 (Ab*) binds to HA (H) to form a specific complex H–Ab* and the Fab with CDR H3 in any other configuration (Ab) binds to H to form a nonspecific, and much weaker, complex H–Ab. Both H–Ab and H–Ab* contribute to the measurement of bound Fab:



The rate constants for the association and dissociation phases fit with two exponential components are (assuming that the nonspecific complex H–Ab is slow to form; i.e., $k_a \sim 0$, and $k_r \sim 0$) (Eq. **S1**)

$$k_{on,1} = \frac{K_C}{1+K_C} k_a^* [Ab] + k_d^*, \quad \textbf{[S1]}$$

(Eq. **S2**)

$$k_{off,1} = k_d^*, \text{ and} \quad \textbf{[S2]}$$

(Eq. **S3**)

$$k_{on,2} = k_{off,2} = k_d + k_f, \quad \textbf{[S3]}$$

where [Ab] is the concentration of Fab for each injection. In Table 2, we report the effective rate constants $k_a' = \{K_C/(1+K_C)\}k_a^*$ and k_d^* determined by first fitting $k_{on,1}, k_{on,2}, k_{off,1}$, and $k_{off,2}$ for each binding experiment and then fitting k_a' and k_d^* to $k_{on,1}, k_{off,1}$ at different concentrations [Ab]. All parameters are determined using functions implemented in Mathematica. For all of the antibodies, the values thus obtained are within factors of two to three of those values from a 1:1 Langmuir model, and the overall conclusion, that the principle contribution to enhanced affinity comes from an increased association rate, is insensitive to the model chosen.

Binding Simulations. Simulations of Fab binding to HA were prepared by placing the Fab at various distances from the position of CH65 within the CH65–HA complex. Specifically, the Fab was translated along the vector that connects the center of mass of the contact residues in CH65 to the center of mass of the contact residues in HA—a residue in CH65 (or HA) was considered a contact residue if it had atoms within a distance of 4.5 Å from some atom in HA (or CH65). Although the relative orientation of the Fab and HA was preserved in the initial placement, during the binding simulations, the Fab could explore a range of different orientations before successfully binding to HA. After the initial placement, Fab and HA were solvated in water, and the system was neutralized electrostatically with corresponding numbers of Na^+ and Cl^- ions at ~0.1 M concentration. The system was then equilibrated for 50 ns using the methods described in *Materials and Methods* with the protein backbone restrained. A typical system was $\sim 10 \times 10 \times 10$ nm^3 in size and contained ~120 K atoms. At the end of the equilibration, there were two to four layers of water molecules between Fab and HA depending on the initial displacement. The equilibrated system was then simulated in the isothermal–isobaric ensemble as described in the text and *Materials and Methods*.

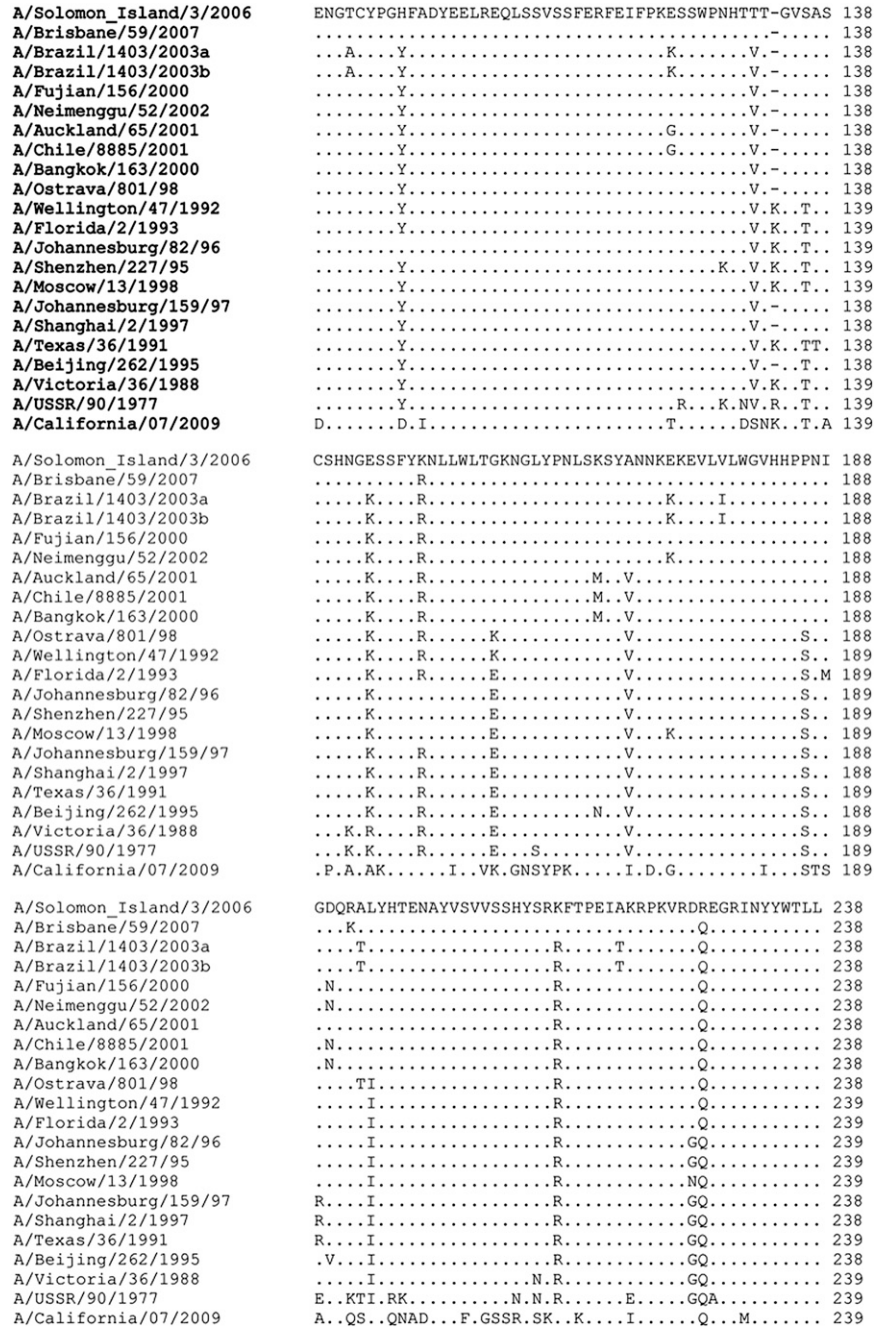


Fig. S1. Comparison of HA receptor-binding domain sequences. A/Solomon Islands/3/2006 is used as reference sequence. Positions of conservation are denoted by a period.

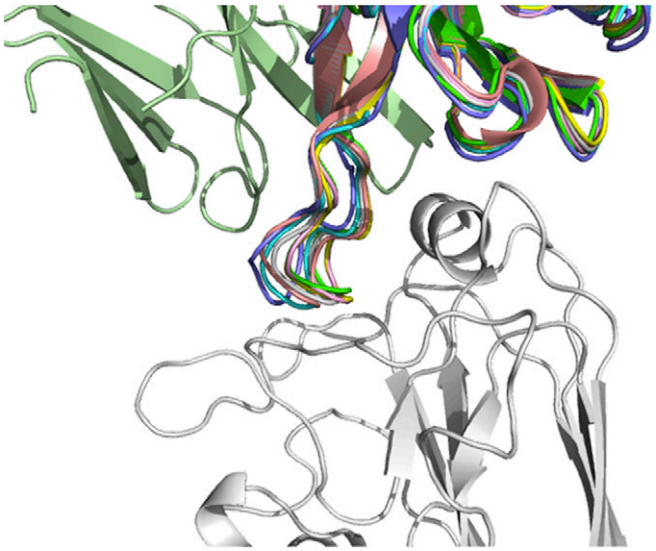


Fig. S2. Comparison of the unbound CH67 CDR H3 loop conformations with their HA head-bound conformation. The six copies of unbound CH67 free Fab V_Hs are structurally aligned to the bound CH67 Fab (V_L is shown in pale green, and V_H is in green) to HA head (silver). The CDR H3 loop on each of the six copies in the asymmetric unit has essentially the same conformation as the bound Fab. Image created with PyMol.

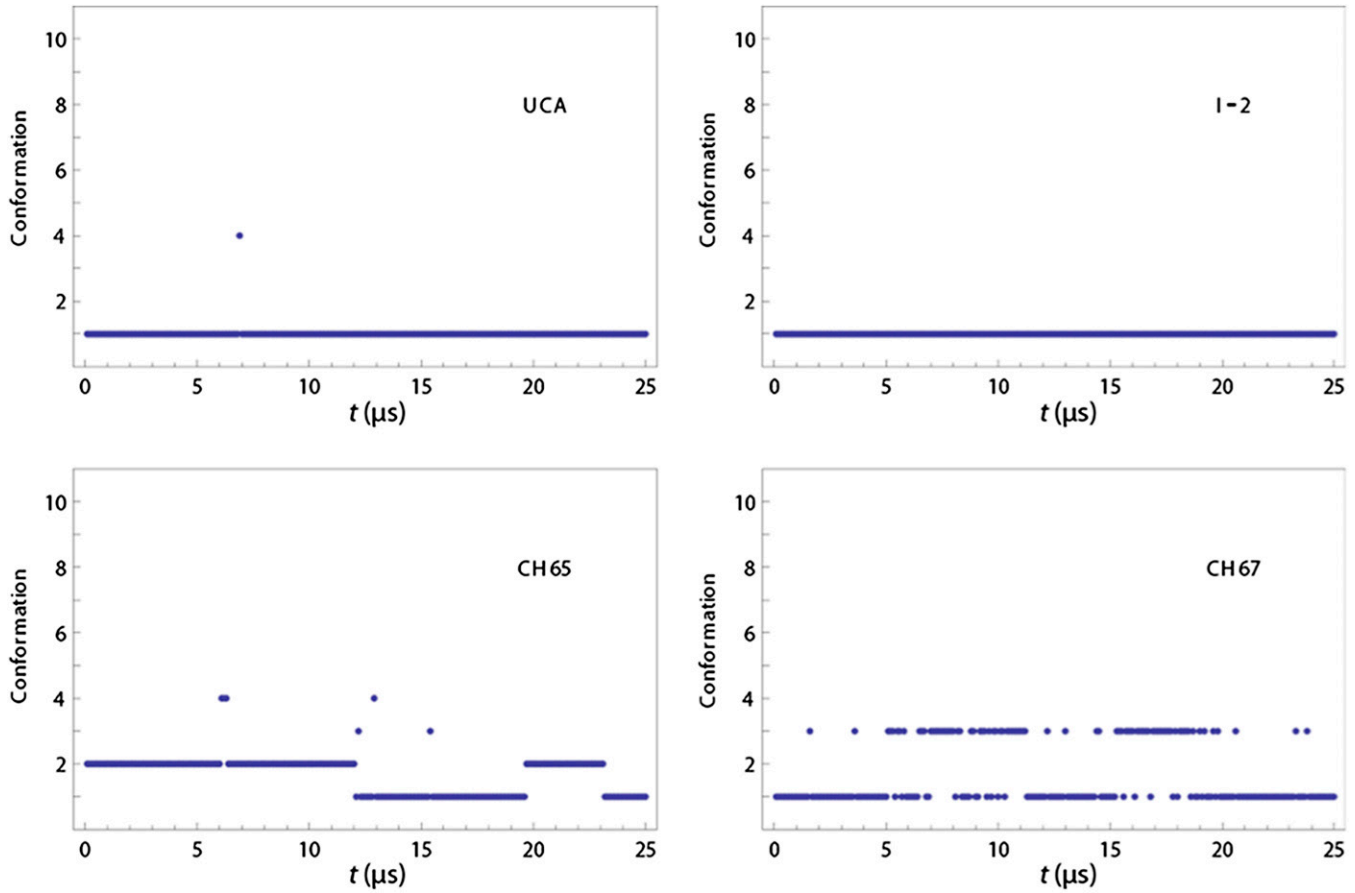


Fig. S3. Conformational dynamics of CDR H3 in the simulations of antibody–HA complexes. Conformations of the loop were assigned to clusters as described in the text. The cluster assignment of each conformation in the complex is plotted against the simulation time. In these simulations, the CDR H3 loops in the UCA and I-2 predominantly occupy conformations in one cluster (cluster 1), whereas the CDR H3 loops in CH65 and CH67 each occupy conformations in one additional cluster (cluster 2 for CH65 and cluster 3 for CH67). The conformations in clusters 1, 2, and 3 are very similar (Fig. S4).

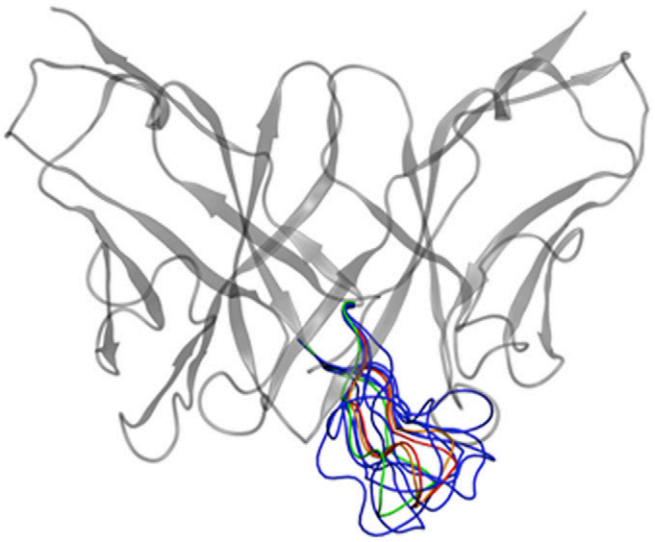


Fig. S4. The mean conformations of the 10 conformational clusters of the CDR H3 loop. The bound conformation common to all four antibodies is shown in red, the additional bound conformation observed in the CH65–HA complex is in green, the additional bound conformation observed in the CH67–HA complex is in orange, and the other seven conformations are in blue. The rest of the Fab is shown in only one conformation in gray. The atomic coordinates of the 10 unique conformations are available upon request.

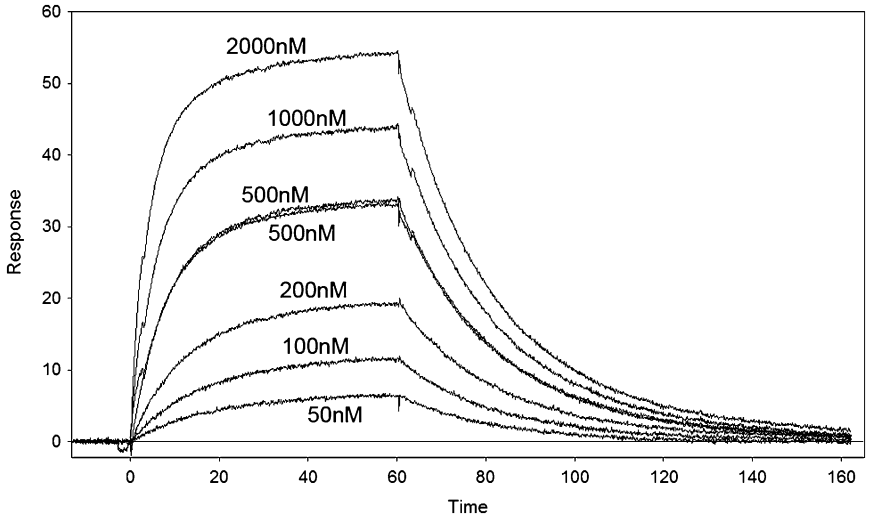
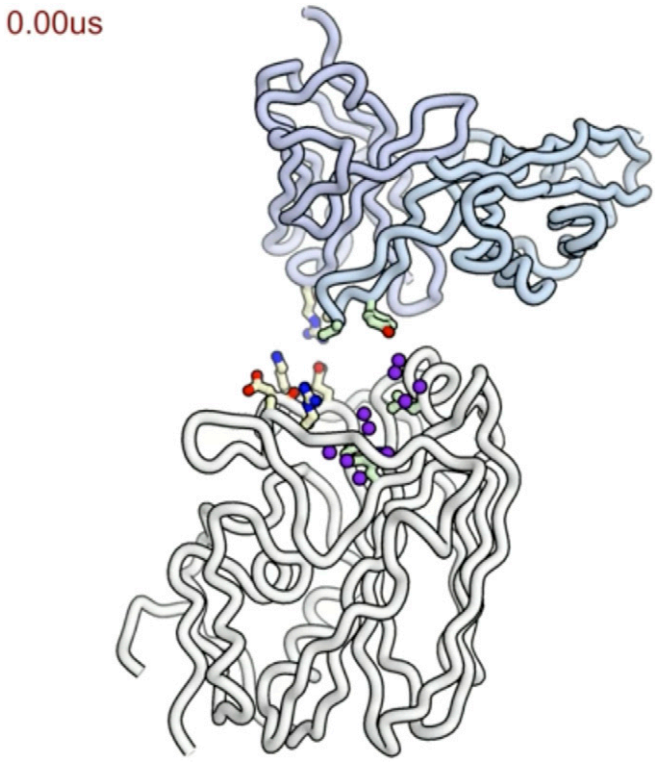


Fig. S5. A representative SPR trace. HA head was coupled to the chip, and CH65 Fab was flowed over as described in *Materials and Methods*. The concentrations used for all SPR experiments ranged from low nanomolar to high micromolar depending on the Fab used. Concentrations used for this particular experiment are labeled. The data were fit as described above and in *Materials and Methods*.

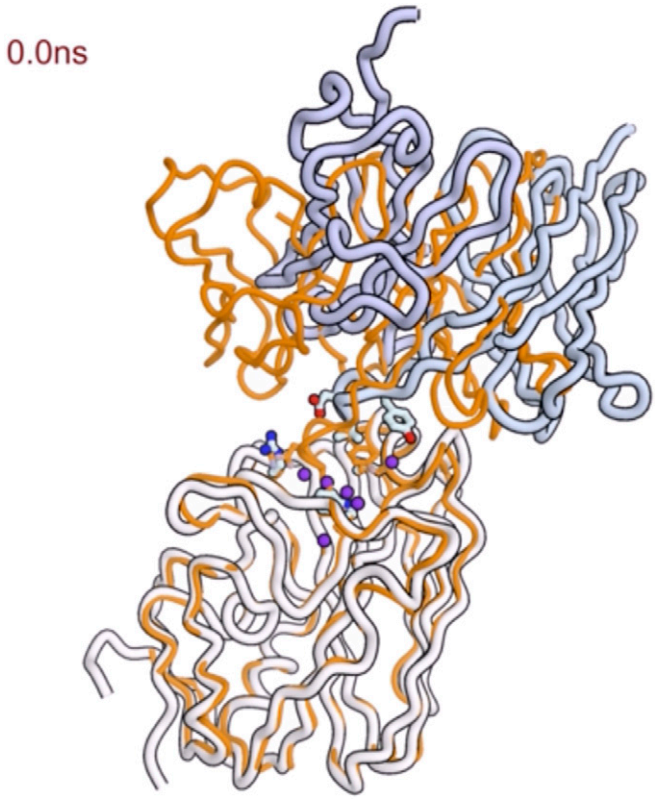
Table S1. X-ray data collection and refinement statistics

	CH67–HA complex	CH67	UCA	I-2
Data collection	APS ID-24-E	APS ID-24-E	APS ID-24-E	ALS 8.2.2
Resolution (Å)	50–2.0	50–3.6	50–2.5	50–2.0
Wavelength (Å)	0.9798	0.97919	0.97919	0.99997
Space group	C2	P212121	P62	P64
Unit cell dimensions (*a*, *b*, *c*; Å)	181.1, 69.8, 80.3	113.2, 123.3, 228.8	102, 102, 163.1	102.5, 102.5, 81.5
Unit cell angles (α, β, γ; °)	90, 90, 107.3	90, 90, 90	90, 90, 120	90, 90, 120
I/σ	18.7	16.62	29.6	42.01
Rsym (%)	12.0	10.7	7.8	6.0
Completeness (%)	91.9	99.8	99.7	100
Number of reflections	406,536	186,854	238,294	487,896
Redundancy	6.7	4.8	7.2	15.0
Refinement				
Working	31,623	35,858	31,487	9,351
Free	1,736	1,956	1,681	494
*R*work (%)	18.6	25.8	20.6	22.06
*R*free (%)	21.9	30.5	25.8	28.96
Ramachandran plot, Percent (favored/disallowed)	96.7/0.5	91/1.5	96.3/0.7	94.0/1.0
rmsd bond lengths (Å)	0.008	0.005	0.013	0.009
rmsd bond angles (°)	1.081	1.378	1.603	1.293
Average B-factor	46.18	122.58	42.62	48.11



Movie S1. CH65 Fab spontaneously binds to HA in a molecular dynamics simulation. HA is shown in white, the heavy chain of CH65 Fab is in light blue, and the light chain is in light purple. Water molecules between the Fab–HA binding interface are represented by dark purple spheres. Residues involved in key interactions between the Fab and HA are shown in ball-and-stick representation. V106 and Y109 of the heavy chain form hydrophobic contacts with W153 and L194 of HA in the complex; these residues are colored light green at the beginning of the movie but are colored dark green when the hydrophobic contacts form. D26 and R29 of the light chain and D107 of the heavy chain form polar interactions with A137 (backbone nitrogen), K222, D225, R226, and E227 of HA. When hydrogen bonds form among these residues, the donors and acceptors are highlighted in orange color with pink lines between them to represent the hydrogen bonds. The crystal structure of CH65–HA complex is superimposed onto the last frame of the movie and shown in orange.

Movie S1



Movie S2. UCA Fab spontaneously binds to HA in the same conformation as CH65. The same molecular representations and colors are used as in Movie S1. The crystal structure of CH65–HA complex is superimposed onto the first frame of the movie (aligning HA) to indicate that UCA Fab starts from a different conformation than the conformation of the bound CH65. The same crystal structure is again superimposed onto the last frame of the movie to show that UCA Fab has spontaneously bound to HA in the same conformation as CH65. Hydrophobic interactions are formed between V106 and Y109 of the heavy chain and W153 and L194 of HA, just as in CH65–HA complex. D107 of the heavy chain forms a hydrogen bond with R226 of HA.

Movie S2

Movie S3. The CDR H3 loop of UCA Fab assumes a variety of conformations that are incompatible with binding to the receptor-binding pocket of HA. The heavy chain is shown in light blue, the light chain is in light purple, and the CDR H3 loop is in orange. In the bound conformation, Y108 of CDR H3 is sandwiched between S31 and W90 of the light chain. The CDR H3 loop moves away from the bound conformation when Y108 exits from that sandwiched position and water molecules (dark purple) seep in.

Movie S3

Other Supporting Information Files

Dataset S1 (DOC)