

# Structures of synthetic nanobody–SARS-CoV-2–RBD complexes reveal distinct sites of interaction and recognition of variants

David Margulies (✉ [dhm@nih.gov](mailto:dhm@nih.gov))

Molecular Biology Section <https://orcid.org/0000-0001-8530-7375>

Javeed Ahmad

NIAID/NIH

Jiansheng Jiang

NIAID/NIH

Lisa Boyd

National Institutes of Health/NIAID

Allison Zeher

NCI/NIH

Rick Huang

NCI/NIH

Di Xia

National Institutes of Health <https://orcid.org/0000-0002-2748-0679>

Kannan Natarajan

National Institute of Allergy and Infectious Diseases

---

## Article

**Keywords:** SARS-CoV-2, receptor-binding domain (RBD), X-ray crystal structures

**Posted Date:** June 16th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-625642/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at Journal of Biological Chemistry on September 16th, 2021. See the published version at <https://doi.org/10.1016/j.jbc.2021.101202>.

1 **Structures of synthetic nanobody–SARS-CoV-2–RBD complexes reveal distinct**  
2 **sites of interaction and recognition of variants**

3 **Authors**

4 Javeed Ahmad<sup>1†</sup>, Jiansheng Jiang<sup>1†</sup>, Lisa F. Boyd<sup>1</sup>, Allison Zeher<sup>2</sup>, Rick Huang<sup>2</sup>, Di Xia<sup>2</sup>, Kannan  
5 Natarajan<sup>1</sup>, and David H. Margulies<sup>1\*</sup>

6 **Affiliations**

7 <sup>1</sup>Molecular Biology Section, Laboratory of Immune System Biology, National Institute of Allergy  
8 and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892, USA

9 <sup>2</sup>Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National  
10 Institutes of Health, Bethesda, MD, 20892, USA

11 \*Correspondence and requests for materials should be addressed to D.H.M  
12 (email:[dmargulies@niaid.nih.gov](mailto:dmargulies@niaid.nih.gov)).

13 †These authors contributed equally.

14

15 **Abstract**

16 The worldwide spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and  
17 emergence of new variants demands understanding the structural basis of the interaction of  
18 antibodies with the SARS-CoV-2 receptor-binding domain (RBD). Here we report five X-ray  
19 crystal structures of sybodies (synthetic nanobodies) including binary and ternary complexes of  
20 Sb16–RBD, Sb45–RBD, Sb14–RBD–Sb68, and Sb45–RBD–Sb68; and Sb16 unliganded. These  
21 reveal that Sb14, Sb16, and Sb45 bind the RBD at the ACE2 interface and that the Sb16 interaction  
22 is accompanied by a large CDR2 shift. In contrast, Sb68 interacts at the periphery of the interface.  
23 We also determined cryo-EM structures of Sb45 bound to spike (S). Superposition of the X-ray  
24 structures of sybodies onto the trimeric S protein cryo-EM map indicates some may bind both "up"  
25 and "down" configurations, but others may not. Sensitivity of sybody binding to several recently  
26 identified RBD mutants is consistent with these structures. (151 words)

27

28 SARS-CoV-2, a  $\beta$ -coronavirus, is remarkable for its high infectivity, rapid world-wide  
29 dissemination, and evolution of highly infectious new variants <sup>1-4</sup>. The virus exploits its trimeric  
30 spike (S) glycoprotein to adsorb to the host cell-surface receptor, angiotensin converting enzyme  
31 (ACE) 2 <sup>5</sup>, resulting in proteolytic processing and conformational changes required for membrane  
32 fusion and cell entry <sup>6</sup>. Understanding the fundamental molecular and cell biology and chemistry  
33 of the viral life cycle and the nature of the host immune response offer rational avenues for  
34 developing diagnostics, therapeutics, and vaccines <sup>7,8</sup>. Emerging viral variants that exhibit  
35 increased infectivity and virulence emphasize the need for continued improvement in  
36 immunization and therapeutic approaches. Specifically, the B.1.1.7 (UK), B.1.351 (South Africa),  
37 P.1 (Brazil), and other strains demand careful attention <sup>9-14</sup>. Exploring the detailed structures of

38 anti-viral antibodies can provide critical understanding of means to attenuate viral adsorption and  
39 entry, and to prevent or retard ongoing infection and communal spread. An evolving database of  
40 X-ray and cryo-EM structures of the SARS-CoV-2 S and RBD and their interactions with ACE2  
41 or various antibodies contributes to the design of effective antibodies or immunogens <sup>15</sup>. Recent  
42 studies indicate the value of single domain antibodies derived from camelids (nanobodies) <sup>16</sup> or  
43 camelid-inspired synthetic libraries (sybodies) <sup>17,18</sup>, and the potential effectiveness of multivalent  
44 constructs <sup>19</sup>. Many properties of nanobodies make them well-suited for structural studies and drug  
45 development <sup>20</sup>. Here, we take advantage of available sequences of five SARS-CoV-2 RBD-  
46 directed sybodies – Sb14, Sb15, Sb16, Sb45, and Sb68 (previously designated Sb#14, Sb#15,  
47 Sb#16, Sb#45, and Sb#68 <sup>18</sup>). These sybodies effectively inhibit the ACE2–RBD interaction and  
48 neutralize viral infectivity <sup>18</sup>. We describe binding studies and X-ray structures of binary  
49 complexes of Sb16–RBD and Sb45–RBD; ternary complexes of Sb14-RBD-Sb68 and Sb45–  
50 RBD–Sb68; and Sb16 unliganded. In addition, we report cryo-EM structures of Sb45 complexed  
51 with trimeric S and evaluate sybody interactions with several mutant RBD representative of newly  
52 evolving variants.

53

## 54 **Results**

55 **Binding and affinity analysis.** Sybodies were expressed in *E. coli* and purified via metal-affinity  
56 chromatography to high purity. These sybodies behaved as monomers by size exclusion  
57 chromatography (SEC) <sup>21</sup> (Extended Data Fig. 1), and we confirmed their activity in binding to  
58 the bacteria-expressed RBD as visualized by SEC (Extended Data Fig. 1). As determined by  
59 surface plasmon resonance (SPR), all five sybodies bind to immobilized RBD with  $K_D$  values of

60 6.8 to 62.7 nM, consistent with previous determinations using RBD-YFP or RBD-Fc molecules  
61 by related techniques<sup>18</sup> (Fig.1).

62 **Structure of Sybody-RBD binary and ternary complexes.** To gain insight into the precise  
63 topology of the interaction of four of these sybodies with the RBD, we determined crystal  
64 structures of their complexes: Sb16–RBD, Sb45–RBD; the ternary Sb45–RBD–Sb68 and Sb14–  
65 RBD–Sb68; and of Sb16 alone. These crystals diffracted X-rays to resolutions from 1.7 to 2.6 Å  
66 (Table 1). After molecular replacement, model building, and crystallographic refinement (see  
67 Methods), we obtained structural models that satisfied standard criteria for fitting and geometry  
68 (Table 1). Illustrations of the quality of the final models as compared with the electron density  
69 maps are shown in Extended Data Fig. 2.

70 The structure of the RBD of these complexes (Fig. 2a, b) revealed little difference between  
71 insect-expressed<sup>22</sup> and our bacteria-expressed and refolded RBD. Each of the sybodies has a barrel  
72 of two β-sheets stabilized by a single disulfide-linked loop of 75 or 76 amino acids characteristic  
73 of an IgV fold<sup>23,24</sup>. The Sb16–RBD complex (Fig. 2a, 3a) illustrates that CDR2 (residues 50-60)  
74 and CDR3 (residues 98-106) bestride the saddle-like region of the ACE2-binding surface of the  
75 RBD (see sequence alignment in Fig. 2f). Sb16 angulates over the RBD by 83°. However, Sb45  
76 (Fig. 2b and 3b) straddles the RBD saddle in the opposite orientation, at an angle of -36°, and  
77 frames the interface with CDR2 (residues 50-59) and CDR3 (residues 97-111). CDR1s of both  
78 sybodies (residues 27-35) lie between the CDR2 and CDR3 loops. Superposition of the two  
79 structures, based on the RBD, emphasizes the diametrically opposite orientation of the two (Fig.  
80 2c), revealing that the CDR2 of Sb16 and CDR3 of Sb45 recognize the same epitopic regions.

81 Exploring conditions using mixtures of two or three sybodies and the RBD, we obtained  
82 crystals and solved the structures of ternary complexes consisting of Sb45–RBD–Sb68 at 2.6 Å

83 resolution (Table 1 and Fig. 2d) and Sb14–RBD–Sb68 at 1.7 Å resolution (Fig. 2e). The refined  
84 models revealed that while Sb14 and Sb45 interact with the ACE2 interface of the RBD, Sb68  
85 binds the RBD at a distinct site (Fig. 2d, e). In the ternary complex, Sb45 binds in an identical  
86 orientation to that observed in the binary Sb45–RBD structure (RMSD of superposition, 0.491 Å  
87 for 1981 atoms), but Sb68 addresses a completely different face of the RBD – similar to that bound  
88 by Fab of CR3022 on RBD of SARS-CoV-2<sup>25</sup> and by V<sub>HH</sub>72 on RBD of SARS-CoV-1<sup>26</sup>. Of  
89 particular interest, whereas Sb45 CDR2 and CDR3 span the RBD saddle as noted above, the  
90 distinct contacts of Sb68 to the RBD are through the longer CDR3, with only minor contributions  
91 from CDR1 and CDR2. Walter et al visualized similar distinct interactions in cryo-EM maps of  
92 two sybodies (Sb15 and Sb68) bound to S protein with local resolution of 6-7 Å<sup>18</sup>. Similarly,  
93 Sb14, which interacts via distinct sybody residues with the RBD at the ACE2 site (see description  
94 below), still permits Sb68 to bind to its epitope as seen in the Sb45–RBD–Sb68 structure (Fig. 2d).

95         Scrutiny of the different interfaces provides insights into the distinct ways each sybody  
96 exploits its unique CDR residues for interaction with epitopic residues of the RBD (Fig. 3).  
97 (Compilation of the contacting residues for each of the four sybodies to the RBD is provided in  
98 Supplementary Table 1). Both Sb16 and Sb45 use longer CDR2 and CDR3 to straddle the RBD,  
99 positioning CDR1 residues over the central crest of the saddle (Fig. 2a-c; Fig.3a,b and  
100 Supplementary Table 1). Also, several non-CDR residues (Y37, E44, and W47 for Sb16), derived  
101 from framework 2<sup>27</sup>, provide additional contacts to the RBD (see Supplementary Table 1). By  
102 contrast with Sb16 and Sb45, Sb14, despite interacting with a large surface area of the RBD, uses  
103 both CDR2 and CDR3 on the same side and exploits many non-CDR residues, particularly sheets  
104 of β-strand as its binding surface (Fig. 3c and Supplementary Table 1). The interface of Sb68 with

105 RBD (Fig. 3d) is quite different, predominantly exploiting nine CDR3, four CDR2, and one CDR1  
106 residues at the interface (see Supplementary Table 1).

107 **Sybodies block ACE2-RBD interaction in discrete ways.** To evaluate the structural basis for the  
108 ability of these four sybodies to block the interaction of RBD with ACE2, we superposed each of  
109 three sybody–RBD structures onto the ACE2–RBD structure and examined the steric clashes (Fig.  
110 4a). Sb16 and Sb45 directly impinge on the ACE2 binding site, offering a structural rationale for  
111 their viral neutralization capacity<sup>18</sup>. Sb68, which also blocks viral infectivity, binds to RBD at a  
112 site which appears to be noncompetitive for ACE2 binding. The carbohydrate at ACE2 residues  
113 N322 and N546 provides an explanation (Fig. 4a).

114 To compare the epitopic areas captured by these sybodies, we evaluated the buried surface  
115 area (BSA) interfaces between RBD and ACE2 or the sybodies. The BSA at the ACE2–RBD,  
116 Sb14–RBD, Sb16–RBD, Sb45–RBD, and Sb68–RBD interfaces are 844 Å<sup>2</sup>, 1,040 Å<sup>2</sup>, 1,003 Å<sup>2</sup>,  
117 976 Å<sup>2</sup>, and 640 Å<sup>2</sup>, respectively (Fig. 3a-e). Sb16 and Sb45 capture more surface area than ACE2  
118 or other published nanobody or sybody–RBD complexes (see Supplementary Table 2). The  
119 interface with Sb68 is the smallest (640 Å<sup>2</sup>) (Fig. 3d). The total BSA captured by Sb45 and Sb68  
120 in the ternary complex is 1,650 (1,010 plus 640) Å<sup>2</sup> (Supplementary Table 2) and is consistent with  
121 the view that a linked bispecific sybody, as described by Walter et al<sup>18</sup>, would exert strong avidity  
122 effects. Supplementary Table 2 summarizes these BSA values and those of other nanobody–RBD  
123 interactions.

124 Although Sb68 reveals the smallest BSA with the RBD and binds at a distinct site, it still  
125 blocks ACE2 binding. A reasonable explanation for the ability of Sb68 to block the ACE2–RBD  
126 interaction arises on inspection of the sites where Sb68, bound to the RBD, might clash with ACE2.  
127 Scrutiny of a superposition of Sb68–RBD with ACE2–RBD reveals several areas of steric

128 interference. Sb68 loop 40-44 clashes with amino acid side chains of ACE2 (residues 318-320 and  
129 548-552), loop 61-64 with ACE2 N322 carbohydrate, and loop 87-89 (a 3,10 helix) with ACE2  
130 N546 carbohydrate as well as residues 313 and 316-218 (Fig. 4a). The ACE2 used in the  
131 crystallographic visualization of ACE2–RBD <sup>28</sup> was expressed in *Trichoplusia ni* insect cells,  
132 which produce biantennary N-glycans terminating with N-acetylglucosamine residues <sup>29,30</sup>.  
133 Electron density was observed only for the proximal N-glycans at residues N322 and N546, but  
134 larger, complex, non-sialylated, biantennary carbohydrates have been detected in glycoproteomic  
135 analysis of ACE2 in mammalian cells <sup>31</sup>. These carbohydrates are highly flexible, adding greater  
136 than 1500 Da at each position, and are larger than the single carbohydrate residues visualized in  
137 the crystal structure. Additionally, molecular dynamics simulations of ACE2–RBD implicated the  
138 direct interaction of carbohydrate with the RBD <sup>32</sup>. Thus, the ability of Sb68 to impinge on ACE2  
139 interaction with RBD likely involves the steric clash of the N322- and N546-linked glycans.

140 We also obtained a 1.9 Å structure of free Sb16 (Extended Data Fig. 3). Remarkably, the  
141 CDR2 of Sb16 shows Y54 in starkly different positions in the unliganded structure as compared  
142 to the complex: the C $\alpha$  carbon is displaced by 6.0 Å, while the O $\eta$  oxygen of Y54 is 15.2 Å  
143 distant, indicative of dynamic flexibility.

144 **Analysis of cryo-EM maps of Sb45-trimeric S complexes.** To gain further insight into the  
145 interaction of Sb45 with the full S protein, we prepared complexes of Sb45 with HexaPro S (S-  
146 6P) <sup>33</sup> and acquired cryo-EM images as described in Methods. All image processing, 2D class, 3D  
147 reconstruction, and map refinements were performed with cryoSPARC <sup>34-37</sup>, model fitting with  
148 Chimera <sup>38</sup> and refinement with PHENIX <sup>39</sup>. We identified two conformations of S-6P with RBD  
149 in either a 1-up, 2-down (7N0G/EMD-24105) or 2-up, 1-down (7N0H/EMD-24106) position as  
150 determined by 3D classification (3D Ab-initio reconstruction) (Extended Data Fig.4). We have  
151 built in additional loops of the NTD and glycans based on the models of 6XKL, 7KGJ, and 7B62.

152 We used unsharpened maps for the model refinement. The overall correlation coefficients (CC)  
153 (mask/volume/peaks) of models for 7N0G and 7N0H are 0.84/0.84/0.77 and 0.83/0.83/0.77  
154 respectively. The model quality is shown in Table 2. There are three Sb45s binding to the 1-up, 2-  
155 down form of S-6P (7N0G/EMD-24105); one binds the up position of RBD, two bind the down  
156 position of RBD (Fig. 5a) with CC values of 0.51, 0.49 and 0.58 respectively (Extended Data Fig.  
157 5a-c). Only two Sb45s bind to the 2-up, 1-down form of S-6P (7N0H/EMD-24106), with one on  
158 the up position of the RBD, and the other on the down position of the RBD (Fig. 5b) with CC  
159 values of 0.51 and 0.71 respectively (Extended Data Fig. 5d,e). It seems that Sb45 can bind all the  
160 down positions of the RBD. In particular, Sb45-Z binds well to RBD-C with higher CC values  
161 (Extended Data Fig. 5c,e), with additional contacts to the neighboring (up position) RBD-A (Fig.  
162 5a). These variations in saturation of the available conformations by Sb45 reflect the mobility of  
163 the RBD. Notably, the interfaces between Sb45 and RBD of S-6P are the same as those in the  
164 crystal structure (7KGG) (Fig. 2). Moreover, the RBD domains are compressed down towards the  
165 center of S, approximately 2-4 Å as compared with uncomplexed S-6P (6XKL).

166  
167 **Superposition of sybodies on trimeric spike protein models.** To gain additional insight into the  
168 structural consequences of the interactions of each of these sybodies with a trimeric S protein, we  
169 superposed each of the individual sybody–RBD complexes on S-6P of our cryo-EM structures  
170 (7N0G and 7N0H) (see Extended Data Fig. 6). Sb16 and Sb45 may dock on all three RBDs in the  
171 trimeric S in any of the four configurations, without any apparent clash (Extended Data Fig. 6a,b).  
172 Sb14, however, reveals clashes when the Sb14–RBD complex is superposed on trimeric S in any  
173 down position (Extended Data Fig. 6e). Sb68 could not be superposed without clashes to any RBD  
174 of the 3-down or to the 1-up, 2-down position. The only permissible superpositions were to two in  
175 the 2-up, 1-down; and to all three in the 3-up position (Extended Data Fig. 6f). For paired sybodies,  
176 Sb16 and Sb68 (Extended Data Fig. 6b), or Sb45 and Sb68 (Extended Data Fig. 6d), superposition

177 was possible without clashes, with two or more RBDs in the up conformation. Walter et al <sup>18</sup>  
178 suggested that a covalent bispecific Sb15–Sb68 reagent could bind S in both the 2-up and 3-up  
179 configurations, based on cryo-EM maps of complexes of S with Sb15 and Sb68. It appears that  
180 Sb16 binds to S in an orientation similar to but in detail distinct from that of Sb15. This analysis  
181 demonstrates an advantage of the small size of sybodies or nanobodies in accessing epitopic  
182 regions of S.

183 **Binding to RBD mutants.** The major circulating variants, specifically B.1.1.7 (UK), B.1.351  
184 (South Africa), and P.1 (Brazil), contain mutations in the RBD that lead to increased binding  
185 affinity to ACE2 and have the potential to reduce vaccine efficacy <sup>40-43</sup>. Specifically, in addition  
186 to other mutations throughout the S protein and viral genome, all three harbor N501Y. B.1.351  
187 and P.1 also have the E484K substitution, as well as substitution of K417 (to N for B.1.351 and to  
188 T for P.1). To assess the effect that substitution at each of these positions exerts on reactivity with  
189 Sb14, Sb15, Sb16, Sb45, and Sb68, we engineered individual mutations in the RBD and tested  
190 them by SPR (see Fig. 6a). In general, the five sybodies which interact with the parental  
191 (designated wild type (WT)) RBD with  $K_D$  values of  $6.8 \times 10^{-9}$  (for Sb15) to  $6.3 \times 10^{-8}$  M (for  
192 Sb68) (see Fig. 1), showed different patterns of binding to the K417N, E484K, and N501Y  
193 mutants. Sb68 bound each with similar affinity, consistent with its epitope lying outside of the  
194 ACE2 binding site on RBD, while each of the others revealed a distinct pattern. Sb14 binding was  
195 most affected by K417N. Sb15 bound both K417N and E484K less efficiently than N501Y. Sb16,  
196 largely unaffected in binding to K417N showed decreased recognition of N501Y and failed to  
197 interact detectably with E484K. Similar to Sb16, Sb45 also failed to bind E484K and showed  
198 decreased recognition of K417N and N501Y as compared to WT. To understand the structural  
199 basis of these differences in recognition of the different RBD mutants, we generated models based

200 on the sybody–RBD structures (Fig. 6b-e). For Sb16, Sb45, and Sb14, interaction with the N501Y  
201 mutant resulted in displacement of its 496-506 loop by 2.0 Å, 1.0 Å, and 1.5 Å respectively.  
202 Nevertheless, R60 of Sb16 and H103 of Sb45 maintained contact with N501Y. This suggests that  
203 N501Y mutation would not escape recognition by these sybodies. Other cryo-EM studies indicate  
204 modest effects of the N501Y substitution on binding to different antibodies <sup>44</sup>. In contrast to the  
205 effects of N501Y, E484K revealed major incompatibilities due to charge repulsion, in the  
206 interaction with Sb16 via K32 and of Sb45 via R33 (Fig. 6d,e).

207

## 208 **Discussion**

209 Our studies of the X-ray structures of Sb16 alone, Sb16–RBD, Sb45–RBD, the ternary Sb14–  
210 RBD–Sb68 and Sb45–RBD–Sb68 complexes, and the cryo-EM structures of Sb45–S provide  
211 critical detail describing the basis of the inhibition of S binding to the cell surface ACE2 receptor  
212 and the resulting block of viral infectivity. Sybodies and nanobodies, by virtue of their single  
213 domain structure and ability to be expressed in *E. coli* systems, as noted by others <sup>17,19</sup>, offer  
214 advantages over Fab. Barnes et al <sup>45</sup> categorized a host of anti-S and anti-RBD Fabs into four  
215 classes (1-4) based on the location of the footprint, and whether the Fab has access to either the up  
216 only or up and down configuration of the RBD in the context of the full trimer (Extended Data  
217 Fig. 7a). By superposition (Extended Data Fig. 7a), Sb14 clearly belongs to Class 1 because it  
218 completely covers the light chain of the B38 Fab (7BZ5). Sb16 partially clashes with B38 but it  
219 primarily overlaps with the heavy chain of COVA2-39 (7JMP) and it can bind both to up and down  
220 positions of the RBD in S (Extended Data Fig.6), indicating that it belongs to Class 2 (Extended  
221 Data Fig. 7a). Sb45 clashes effectively with the heavy chain of COVA2-39, and our cryo-EM  
222 structures (7N0G, 7N0H) indicate that Sb45 can bind to both up and down forms of S-6P (Fig.5).

223 Thus, Sb45 qualifies as Class 2 (Extended Data Fig. 7a). By contrast, Sb68 competes most with  
224 the CR3022 heavy chain (6W41), V<sub>HH</sub>72 (6WAQ)<sup>26</sup> and V<sub>HH</sub>-U (7KN5)<sup>46</sup> placing it in Class 4.  
225 However, unlike the other class 4 antibodies, Sb68 competes presumably due to its spatial  
226 orientation. Overall, our structural studies not only define the Sb14, Sb16, Sb45, and Sb68 epitopes  
227 at high resolution, but also reveal that these sybodies capture a rather large epitopic area  
228 (Supplementary Table 2), suggesting that a judicious choice of several sybodies or nanobodies  
229 have the potential to effectively saturate the available RBD surface.

230         The significance of the ternary structures of Sb45–RBD–Sb68 (7KLW) and Sb14–RBD–  
231 Sb68 (7MFU) is shown in a recent paper<sup>46</sup>. Koenig et al<sup>46</sup> determined a ternary nanobody structure  
232 of V<sub>HH</sub>-E–RBD–V<sub>HH</sub>-U (7KN5) which illustrates the binding to two distinct epitopic sites. The  
233 ternary structure may also be considered as illustrative of the potential behavior of a bispecific  
234 construct linking two nanobodies. The bivalent or multivalent binding by antibody or nanobody  
235 would be expected to increase neutralization potential<sup>19,46-48</sup>. Superposition of Sb14–RBD–Sb68  
236 or Sb45–RBD–Sb68 on V<sub>HH</sub>-E–RBD–V<sub>HH</sub>-U indicates that Sb14, Sb45 and V<sub>HH</sub>-E represent class  
237 1 and class 2 in recognizing the epitopic region but do so in somewhat different orientations  
238 (Extended Fig. 7b). Sb45 exploits its two lengthy CDR2 and CDR3 loops which ride along both  
239 sides of the RBD surface, and Sb14 uses both CDR2 and CDR3 on the same side close to Sb68,  
240 while V<sub>HH</sub>-E uses a long CDR3 loop engaging one side of the RBD surface. Furthermore, Sb14  
241 and Sb68 in Sb14–RBD–Sb68 (7MFU) show contacts (Y57-E44, G55-E44, and T54-H108)  
242 between two specific sybodies on the RBD surface (Extended Data Fig. 7c), which emphasizes the  
243 importance of bivalent and multivalent antibodies/nanobodies against the virus.

244         Recently, several SARS-CoV-2 spike variants have been isolated and characterized with  
245 respect to their infectivity and severity of disease. The UK-SARS-CoV-2 variant has multiple

246 substitutions including N501Y in the RBD <sup>1</sup>. The mutation of E484K leads to repulsion of charged  
247 residues of antibody/nanobody/sybodies (Fig.6). To accommodate such a mutation, the  
248 complementary charged residues of the antibody/nanobody/sybody should also reverse their  
249 charge. Alternatively, employing another antibody/nanobody/sybody with opposite charge could  
250 capture such a escape mutation. Indeed, knowledge of the location of common or recurrent escape  
251 mutations and their potential resistance to antibody/nanobody/sybodies would provide a rational  
252 basis for either sequential or simultaneous use of reagents with complementary specificity. Thus,  
253 precise mapping of anti-RBD antibody, nanobody, and sybody epitopes, especially for those that  
254 are developed for clinical trials, has implications not only for mechanistic understanding of the  
255 interactions of the RBD with ACE2, but also for evaluating the potential susceptibility of newly  
256 arising viral variants to currently administered vaccines and antibodies.

257

## 258 **Online Methods**

259 **Subcloning, expression and purification of RBD, spike, and sybody proteins.** The sequences  
260 encoding the RBD of the SARS-CoV-2 spike protein (amino acids 333 to 529) were subcloned  
261 into pET21b(+), (Novagen) via *NdeI* and *EcoRI* restriction sites, using pcDNA3-SARS-CoV-2-  
262 RBD-8his (Addgene #145145, <sup>49</sup>) as template. The primers used were forward primer, 5'-  
263 TGCAGTCATATGAATCTTTGTCCGTTCCGGTGAG and reverse primer, 5'-  
264 TGCAGTGAATTCTCACCTTTTTGGGCCCAAACT. The RBD was expressed as  
265 inclusion bodies in *E. coli* strain BL21(DE3) (Novagen). Expression, isolation of inclusion bodies,  
266 denaturation and reduction was done in 6 M guanidine hydrochloride and 0.1 mM DTT as  
267 described elsewhere <sup>50</sup>. Briefly, refolding was carried out in a refolding buffer supplemented with  
268 oxidized and reduced glutathione and arginine for 3 days at 4 °C followed by dialysis against

269 HEPES buffer (25 mM HEPES, pH 7.3, 150 mM NaCl). Concentrated and filtered protein was  
270 analyzed by size-exclusion chromatography on a Superdex 200 10/300 GL column (GE  
271 Healthcare) equilibrated with HEPES buffer. The peak corresponding to 24 kDa (monomeric)  
272 protein was collected, concentrated, and further purified by ion-exchange chromatography on  
273 Mono-Q® (Cytiva). Mutant RBD were generated by Site directed mutagenesis, performed with  
274 the QuikChange Lightning Multisite mutagenesis kit (Agilent, Santa Clara, CA, USA). All mutants  
275 were sequenced through GeneWiz and protein expression, refolding, and purification were done  
276 as described above.

277 Plasmids pSb-init encoding sybodies Sb14, Sb15, Sb16, Sb45. and Sb68 (Addgene #15322,  
278 153523, #153524, #153526, and #153527, respectively) were originally reported by Walter et al  
279 <sup>18</sup> and generously made available. All plasmids were verified by DNA sequencing. Purification of  
280 the recombinant proteins from the periplasm of *E. coli* MC1061 was based on a protocol described  
281 elsewhere <sup>21</sup>. Briefly, *E. coli* MC1061, transformed with a sybody-encoding plasmid, was grown  
282 in Terrific Broth (TB) medium (Gibco) supplemented with 25 µg/ml chloramphenicol, at 37 °C  
283 with shaking at 160 rpm for 2 hrs. The temperature was then decreased to 22 °C until A<sub>600</sub> reached  
284 0.5. Protein expression was induced by addition of L-(+)-arabinose (Sigma) to a final concentration  
285 of 0.02% (w/v) and expression continued overnight at 22 °C and 160 rpm. The next day cells were  
286 collected by centrifugation at 2000 x g for 15 minutes. The cell pellet was then washed twice in  
287 PBS and resuspended in periplasmic extraction buffer (50 mM Tris/HCl pH 8.0, 0.5 mM EDTA,  
288 0.5 µg/ml lysozyme, 20% w/v sucrose (Sigma)) at 4 °C for 30 min followed by addition of TBS  
289 (pH 8.0) and 1 mM MgCl<sub>2</sub>. Cells were then centrifuged at 10,000 rpm (Fiberlite™ F21-8 x 507  
290 Fixed Angle Rotor) for 30 min. Following transfer of the supernatant to a fresh tube, imidazole  
291 was added to a final concentration of 10 mM. Ni-NTA resin (Qiagen) equilibrated with TBS was

292 added to the supernatant and incubated for 1 hr at RT with mild agitation. The resin was collected,  
293 washed three times with buffer supplemented with 30 mM imidazole and sybody proteins were  
294 eluted with 300 mM imidazole in TBS.

295 Plasmid encoding spike HexaPro (designated “S” throughout) was procured from Addgene  
296 (#154754) <sup>33</sup> and transfected into Expi293F™ cells (ThermoFisher Scientific) using  
297 manufacturer’s protocol. Briefly, Expi293F™ cells were seeded to a final density of  $2.5-3 \times 10^6$   
298 viable cells/ml and grown overnight at 37 °C in Expi293™ Expression Medium (Gibco). The  
299 following day, cell viability was determined, and cell density was adjusted to  $3 \times 10^6$  viable  
300 cells/ml with fresh, prewarmed Expi293™ Expression Medium. Transfection was then done as per  
301 manufacturer’s instructions using 1 µg/ml plasmid DNA. Cultures were grown for 6 days  
302 following transfection and supernatant was collected, filtered through a 0.22 µm filter and passed  
303 over Ni-NTA resin for affinity purification. Further purification was accomplished by size-  
304 exclusion chromatography using a Superose 6 10/300 GL column (Cytiva) in a buffer consisting  
305 of 2 mM Tris pH 8.0, 200 mM NaCl. Purification of sybodies, RBD, and 6-SP is shown in  
306 Extended Data Fig.8.

307 **Preparative and analytical size-exclusion chromatography.** Sybodies purified by Ni-NTA  
308 affinity chromatography were concentrated using Amicon 10K MWCO concentrators and purified  
309 on a Sepax SRT-10C SEC100 column at a flow rate of 1 ml/min. Monomeric sybodies elute at a  
310 retention volume of 11–12.5 ml from the Sepax SRT-10C SEC100 column. Monomeric peak  
311 fractions were collected and analyzed by SDS-PAGE. Analytical SEC of RBD-sybody complexes  
312 was performed on a Shodex KW-802.5 column at a flow rate of 0.75 ml/min in TBS buffer (pH.  
313 8.0). (The interaction of individual sybodies with the column matrix is a well-documented  
314 phenomenon <sup>21</sup>).

315 **Surface Plasmon Resonance.** SPR experiments were performed on a Biacore T200 (Cytiva) at  
316 25 °C in 10 mM HEPES pH 7.2, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20. RBD was  
317 immobilized on a series S CM5 sensor chip (Cytiva) by amine (NHS/EDC) coupling to flow cells.  
318 For background subtraction a reference cell was mock coupled. Binding and kinetic studies were  
319 performed multiple times for each sybody. Graded and increasing concentrations of SB16, SB45  
320 and SB68 were injected over the RBD-immobilized surface at a flow rate of 30ml/min with an  
321 association time of 120 s and dissociation time of 2000 s. Binding data were analyzed by surface  
322 site affinity distribution analysis by EVILFIT<sup>51,52</sup>. In general, these values were consistent with  
323 fits to the Langmuir binding equation for a 1:1 interaction model using Biacore T200 Evaluation  
324 Software v3.0, but revealed better statistics.

325 **Thermal stability.** Thermal melt analysis of the recombinant proteins was performed in triplicate  
326 in 96-well plates in a QuantStudio 7 Flex real time PCR machine (Applied Biosystems). Each  
327 well contained 2-4 mg protein in buffer (25mM TRIS pH 8, 150 mM NaCl) and 5x Sypro Orange  
328 (Invitrogen, stock 5000x) in a total volume of 20 ml. Following an initial two-minute hold at 25  
329 °C, the plate was heated to 99 °C at a rate of 0.05 °C/sec. Data were analyzed with Protein Thermal  
330 Shift Software v1.3 (Invitrogen) to obtain  $T_m$  values for RBD, S, Sb14, Sb15, Sb16, Sb45, and  
331 Sb68 (Extended Data Fig. 9).

332 **Crystallization, data collection, structure determination and crystallographic refinement.**

333 Purified sybodies (Sb14, Sb15, Sb16, Sb45 and Sb68) and RBD were mixed in approximate 1:1  
334 molar ratio to a final concentration of 8 mg/ml. The protein mixtures were incubated on ice for 1  
335 hour prior to screening. Initial screening for crystals was carried out using the hanging drop vapor  
336 diffusion method using the Mosquito robotic system (sptlabtech.com). Crystals of SB16–RBD and  
337 SB45-RBD complexes and Sb16 alone were observed within one week using Protein Complex

338 (Qiagen) and Wizard Classic 4 (Rigaku). Conditions for Sb16–RBD were either 0.1M HEPES pH  
339 7.0, 15% PEG 20000, or 0.1M HEPES pH 7.0, 18% PEG 12000; and for Sb45–RBD was 18%  
340 PEG 12000 and 12% PEG 8000, 0.1 M HEPES pH 7.5, 0.2 M NaCl. Crystallization condition for  
341 Sb14–RBD–Sb68 was 12% PEG 8000, 0.1 M MOPS, pH 7.5, 0.1 M Mg Acetate. Sb16 alone  
342 crystallized in 20% PEG 4000, 0.1 M MES, pH 6.0, 0.2 M LiSO<sub>4</sub>. We also screened mixtures of  
343 two or three sybodies with RBD. Crystals of Sb45–RBD–Sb68 were obtained after one month  
344 following mixing the three proteins in an equimolar ratio in 10% PEG 8000, 0.1M sodium  
345 cacodylate pH 6.0.

346 Crystals of protein complexes were optimized with slight adjustments of the concentration  
347 of PEG components. Crystals were cryoprotected in mother liquor containing 5% ethylene glycol  
348 and 5% glycerol and flash frozen in liquid nitrogen for data collection. Diffraction data were  
349 collected at the Southeast Regional Collaborative Access Team (SER-CAT) beamline 22ID-D at  
350 the Advanced Photon Source, Argonne National Laboratory and data were processed with XDS  
351 <sup>53</sup>. Multiple data sets were collected for the protein complexes from 2.3-3.2 Å resolution. The  
352 initial model of Sb16 and Sb45 for the molecular replacement search were built by the MMM  
353 server ([manaslu.fiserlab.org/MMM](http://manaslu.fiserlab.org/MMM) <sup>54</sup>), using the heavy chain V domain and the RBD of the Fab  
354 B38–RBD complex (PDBid: 7BZ5) <sup>22</sup>. The initial model of Sb68 for molecular replacement was  
355 built based on the V<sub>H</sub> domain of 7BZ5. Molecular replacement solutions were found using Phaser  
356 <sup>39,55</sup>. Subsequent refinements were carried out with PHENIX <sup>56</sup>. CDR loops were manually rebuilt  
357 by fitting to the electron density maps with Coot <sup>57</sup>. In particular, Sb68 CDR loops were deleted  
358 before refinement and built in manually based on electron density maps. Illustrations and  
359 calculations of superpositioned models were prepared in PyMOL (The PyMOL Molecular

360 Graphics System, Version 2.4.0 Schrödinger, LLC). Calculation of hinge relationships of domains  
361 was accomplished with HINGE  
362 (<https://collab.niaid.nih.gov/sites/research/DIR/LIG/SIS/Lists/Programs/homeview.aspx>)  
363 provided courtesy of Peter Sun, NIAID. Buried surface area (BSA) calculations were performed  
364 with PISA (<https://www.ebi.ac.uk/pdbe/pisa/>). The final structures for the RBD-SB16 and RBD-  
365 SB45 complexes showed  $R_{\text{work}}/R_{\text{free}}$  (%) 25.4/27.7 and 18.6/21.6 respectively, and for SB16 alone  
366 with  $R_{\text{work}}/R_{\text{free}}$  22.4/25.9. Data collection and structure refinement statistics are provided in Table  
367 1.

368 **Cryo-EM sample preparation and data collection.** Freshly purified S-6P was incubated with  
369 Sb45 in a 1:3 molar ratio and repurified by size exclusion chromatography. Negative stain  
370 screening was accomplished with a Tecnei T12 120-keV microscope (Thermo Fisher). The protein  
371 complexes were concentrated to 0.7-1 mg/ml and 3  $\mu\text{l}$  of the sample was applied onto holey-carbon  
372 cryo-EM grids (Cu R1.2/1.3, 300 mesh, Quantifoil), which had been glow discharged for 60  
373 seconds, blotted for 3 seconds, and plunge frozen into liquid ethane with a Vitrobot (Thermo Fisher  
374 Scientific) at 4 °C and 95% humidity. Cryo-EM data in selected grid regions were collected on a  
375 Titan Krios 300-keV microscope (Thermo Fisher). Images were acquired automatically with  
376 SerialEM<sup>58</sup> on a BioQuantum-K2 summit detector (Gatan) with a 20eV energy filter slit in super-  
377 resolution mode at 130x nominal magnification (1.052 Å binned pixel size) and a defocus range  
378 from  $-0.7$  to  $-2.0$   $\mu\text{m}$ . An exposure time of 8s at 0.2s per frame was recorded with a total exposure  
379 of about 56 electrons/Å<sup>2</sup>. Two raw data sets were collected on two frozen grids: one with 1,780  
380 micrographs and one with 7,945 micrographs.

381 **Image processing and structure solution.** All image processing, 2D class, 3D reconstruction,  
382 and map refinements were performed with cryoSPARC v3.1 and v3.2<sup>36 34,35,37</sup>. A total of 9,725

383 micrographs was imported into cryoSPARC. Following “patch motion correction” and “patch CTF  
384 estimation,” the number of micrographs was reduced to 9,703. Micrographs were inspected by  
385 “curate exposures,” in which outliers of defocus range, defective micrographs, and those with a  
386 low-resolution estimation of the CTF fit ( $>5 \text{ \AA}$ ) were discarded, resulting in 9,237 micrographs.  
387 “Blob picker” was used with the particle diameter between 128 and 256 angstroms for picking  
388 particles. After “inspect particles” with NCC (Normalized Correlation Coefficient) 0.28 and  
389 “power threshold” between 500 and 1000 (which removed ice and aggregates), the number of  
390 particles was 1,876,941. To determine the “box size,” we performed several trials indicating that  
391 the box size should be larger than 336 pixels, and finally used a box size of 400 pixels and extracted  
392 1,433,963 particles. After “2D classification” (100 classes), 18 2D classes were selected, retaining  
393 662,994 particles. The particles were submitted to a series of “Ab initio 3D reconstruction”  
394 classification and divided into 2 or 4 sub-groups. After removing the particles of un-recognized or  
395 “defective” shape, a total of 417,460 particles with shape resembling spike remained. These  
396 particles were subjected to “homogeneity refinement,” followed by “CTF global and local  
397 refinement” and “non-uniform refinement.” No symmetry was imposed aside from C1 during the  
398 map refinements. The map after refinement could reach  $2.84 \text{ \AA}$  resolution by the gold-standard  
399 FSC estimation with a 0.143 cut-off criterion. We then identified further the two conformations of  
400 S-6P as previously described<sup>33</sup>. One sub-class of 214,171 particles revealed the conformation of  
401 “1-up, 2-down” of RBD (Extended Data Fig. 4c), and one sub-class of 61,062 particles showed  
402 the conformation of “2-up, 1-down” (Extended Data Fig. 4c). The maps of “1-up,2-down” and “2-  
403 up,1-down” were refined at  $3.02 \text{ \AA}$  and  $3.34 \text{ \AA}$  resolution respectively. Local resolution plots for  
404 each map are shown in Extended Data Fig. 4d,e. The maps are deposited in EMDB as EMD-24105  
405 and EMD-24106.

406 An initial model for S-6P was generated using PDB 6XKL and was fit as a rigid body into  
407 the map using Chimera<sup>38</sup> followed by PyMOL. The Sb45–RBD (7KGJ) crystal structure was  
408 superimposed onto the S-6P model in PyMOL. We used real space refinement in PHENIX<sup>39</sup>  
409 including rigid-body refinement. The model was split into subdomains, NTD (24-289) and RBD  
410 (334-528) for rigid-body refinement. Simulated annealing (SA) was performed initially, including  
411 a local grid search and ADP refinement, using secondary structure restraints. We noticed that the  
412 original 6XKL model lacked some loops in RBD and NTD domains, which were replaced by the  
413 RBD domain from 7KGJ, and the NTD domain from 7B62<sup>59</sup> with all loops. For the model of the  
414 “1-up” form of S-6P, the CC was 0.84/0.78 (volume/peaks) with three Sb45 domains bound to  
415 three RBDs. However, the CC for three Sb45-X, Sb45-Y, and Sb45-Z are 0.51, 0.49, and 0.58  
416 respectively, which indicates that the Sb45 does not fully bind to S-6P. For the “2-up” form of S-  
417 6P, we first generated the model by superimposing the A-chain of the “1-up” form of S-6P onto  
418 B-chain and replaced B-chain for the real space refinement, the resulting model with an overall  
419 CC of 0.83/0.76 (volume/peaks), but with only two Sb45 domains, one Sb45-X binds to A-chain  
420 (up RBD) and one Sb45-Z binds to C-chain (down RBD) with CC 0.44 and 0.68 respectively.  
421 These two models are deposited in PDB as 7N0G and 7N0H. Data processing, refinement  
422 statistics, and model validation are listed in Table 2.

423 **Acknowledgments.** We appreciate the help of Joy (Huaying) Zhao and Peter Schuck, NIBIB, NIH  
424 in analyzing SPR data, and thank Peter Sun, NIAID, NIH for access to his program, HINGE. We  
425 thank Barney Graham, NIAID, NIH for plasmids used in initial aspects of the work, and Apostolos  
426 Gittis, NIAID, NIH, for help in protein characterization. We appreciate the advice of Michael  
427 Mage and D. K. Taylor during this work. This work was supported by the Intramural Research  
428 Program of the NIAID, NIH, including funds from the CARES Act. X-ray data were collected at

429 Southeast Regional Collaborative Access Team (SER-CAT) 22-ID (or 22-BM) beamline at the  
430 Advanced Photon Source, Argonne National Laboratory. SER-CAT is supported by its member  
431 institutions ([www.ser-cat.org/members.html](http://www.ser-cat.org/members.html)) and equipment grants (S10\_RR25528 and  
432 S10\_RR028976) from the National Institutes of Health. Use of the Advanced Photon Source was  
433 supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences,  
434 under Contract No. W-31-109-Eng-38. The Electron Microscopy Resource is supported by the  
435 National Cancer Institute and NIH Intramural Research Program Cryo-EM Consortium (NICE).

436 **Author contributions.** J.A., J.J., K.N., and D.H.M. conceived the project; J.A., K.N., and L.F.B.  
437 engineered constructs, purified protein, and performed binding analyses; J.J. screened for crystals,  
438 processed X-ray data, and refined the structures; J.A., J.J., and A.Z. did negative staining and  
439 sample preparation; R.H. and A.Z. collected images, and pre-processed cryo-EM data; J.J., A.Z.,  
440 and R.H. solved and refined cryo-EM structures and with X.D. analyzed cryo-EM data; all authors  
441 contributed to the final manuscript.

442 **Competing interests.** The authors declare no competing interests.

443 **Data and materials availability.** All data are included in the paper or in the supplementary  
444 material. X-ray structure factors and coordinates are deposited at the protein data bank  
445 ([www.pdb.org](http://www.pdb.org)) under accession numbers 7MFU, 7KGG, 7KGJ, 7KLW, and, 7MFV for Sb14-  
446 RBD-Sb68, Sb16-RBD, Sb45-RBD, Sb45-RBD-Sb68, and Sb16 respectively. Cryo-EM maps  
447 of SB45+S-6P (1-up, 2-down) and Sb45+S-6P (2-up, 1-down) have been deposited in the EMDB  
448 under accession numbers EMD-24105 and EMD-24106 and their respective models under 7N0G  
449 and 7N0H.

450

451 **Figure Legends**

452 **Fig. 1| Sybodies bind RBD with  $K_D$  values in the nanomolar range.** RBD was coupled to a  
453 biosensor chip as described in Methods. Graded concentrations (31 to 500 nM) of each of the  
454 indicated sybodies were offered to the coupled surface (from  $t=0$  to  $t=160$  s), followed by buffer  
455 washout, and measurement of net binding (in resonance units, RU). Experimental curves were fit  
456 by global analysis using BIAeval 2.0 (Cytiva). Curves shown are representative of at least two  
457 determinations.

458 **Fig. 2| Overall structures of Sb14, Sb16, Sb45 and Sb68 complexed with SARS-CoV-2 RBD.**  
459 Ribbons (sybodies) and ribbons plus surface (RBD) representations of the complex of (a) Sb16  
460 (slate) with RBD (grey) (7KGK); (b) Sb45 (cyan) with RBD (7KGJ), (d) Sb45 and Sb68 (purple)  
461 with RBD (7KLW) and (e) Sb14 (blue) and Sb68 (magenta) with RBD (7MFU). Sb16-RBD and  
462 Sb45-RBD superimposed based on the RBD are shown in (c) to highlight CDR loops, which are  
463 color coded as CDR1 (pink), CDR2 (orange) and CDR3 (red). The CDR2 of Sb16 and CDR3 of  
464 Sb45 interact similarly with the RBD surface. Panel (f) shows a sequence alignment of the four  
465 sybodies.

466 **Fig. 3| Interfaces and interactions of sybodies with RBD.** (a) Sb16-RBD, (b) Sb45-RBD, (c)  
467 Sb14-RBD, and (d) Sb68-RBD. (Individual contacting residues are listed in Supplementary Table  
468 1). CDR1, CDR2, CDR3 regions are painted pink, orange and red respectively. Additional non-  
469 CDR region contacting residues are colored lime. On the RBD surface, the epitopic residues that  
470 contact the sybodies are colored according to the sybody CDR.

471 **Fig. 4| Sybodies clash with ACE2 in RBD complex structures.** (a) Sb16 (slate), Sb45 (cyan),  
472 Sb14 (blue), and Sb68 (purple) – RBD complexes were superposed on the ACE2–RBD structure

473 (salmon) (6M0J) based on the RBD. Views of Sb16 (b), Sb45 (c), and Sb14 (d) are shown alone  
474 as well. Sb14 and Sb16 are buried inside ACE2; Sb45 is partially buried in ACE2; and Sb68 has  
475 major clashes with two N-glycan sites (N322 and N546) of ACE2 (inset). (e) Epitopic area (on  
476 RBD) captured by ACE2 (salmon) is indicated along with its BSA.

477 **Fig. 5| X-ray model of sybody superposed on cryo-EM Structures of SB45–S-6P.** (a) Model  
478 of Sb45+S-6P (1-up, 2-down) is fitted to the map with Sb45-X bound to RBD-A (up), Sb45-Y to  
479 RBD-B (down), and Sb45-Z to RBD-C (down), and CC (Sb45-X/Sb45-Y/Sb45-Z) are  
480 0.52/0.49/0.57 respectively; (b) Model of Sb45+S-6P (2-up, 1-down) is fitted to the map with  
481 Sb45-X bound to RBD-A (up), and Sb45-Z bound to RBD-C (down), and CC (Sb45-X/Sb45-Z)  
482 are 0.47/0.70 respectively.

483 **Fig. 6| RBD mutations affect sybody binding.** (a) SPR binding of each of the indicated sybodies  
484 (across top) to each of the individual RBD mutants. Inset shows binding of sybodies to wild type  
485 RBD (from Fig. 1). Experimental tracings are shown in red, curve fits in black and  $k_d$  ( $s^{-1}$ ) and  $K_D$   
486 (M) values as determined from global fitting with BIAeval 2.0 are provided in each panel. (b)  
487 Location of contacts of Sb16, Sb45, and Sb14 are shown. E484, K417 and N501 of RBD (wild  
488 type) interact with K32, Y54 and R60 of Sb16 respectively; E484 and N501 of RBD (wild type)  
489 interact with R33 and H103 of Sb45 respectively; and E484, K417 and N501 of RBD (wild type)  
490 interact with Q39, E35, and Y60 of Sb14 respectively. (c) Comparison of complex structures with  
491 minimized models involving the N501Y mutation. *In silico* mutagenesis of N501Y was performed  
492 using 7KGGK (Sb16+RBD), 7KGGJ (Sb45+RBD), and 7MFU (Sb14+RBD+Sb68). Following amino  
493 acid substitution in Coot, local energy minimization (within 15 to 20 Å of the mutant residue) was  
494 performed through three rounds in PHENIX. For the Sb16-RBD complex, when N501 is mutated  
495 to Y501, the loop (496-506, from yellow to wheat) extends about 2.4 Å, but R60 (revealing a

496 double conformation) still forms hydrogen bonds with the Y501 loop; for the Sb45-RBD complex,  
497 when N501 is mutated to Y501, the loop (496-506, from yellow to wheat) extends about 1.0 Å,  
498 but H103 of Sb45 would still interact with Y501; for the Sb14-RBD complex, when N501 is  
499 mutated to Y501, the loop (496-506, from yellow to wheat) is extended about 2.0 Å, but T58 and  
500 K65 still the hydrogen bonds with Y501; (d) The surface charge of Sb16, K32 forms a hydrogen  
501 bond with E484 of RBD with the opposite charge; the surface charge of Sb45, R33 forms a  
502 hydrogen bond with E484 of RBD with the opposite charge; the surface charge of Sb14, Q39 (a  
503 neutral residue) interacts with E484 of RBD; (e) Surface charge of wild type of RBD and surface  
504 charge of RBD with the three mutations (E484, K417N, and N501Y). When E484 is mutated to  
505 K484, the surface charge is changed from negative to positive, therefore the hydrogen bonds are  
506 broken – pushing Sb16 and Sb45 out of contact, while since Q39 of Sb14 is not a charged residue,  
507 it still may interact with K484 of the mutated RBD.  
508

509 **Table 1| X-ray data collection and refinement statistics**

|   | <b>Sb16-RBD</b>        | <b>Sb45-RBD</b>        | <b>Sb14-RBD-Sb68</b>   | <b>Sb45-RBD-Sb68</b>   | <b>Sb16</b>            |
|---|------------------------|------------------------|------------------------|------------------------|------------------------|
| <b>PDBID</b>  | <b>7KGK</b>            | <b>7KGJ</b>            | <b>7MFU</b>            | <b>7KLW</b>            | <b>7MFV</b>            |
| <b>Data collection</b>                                  |                        |                        |                        |                        |                        |
| Space group   | P6 <sub>s</sub> 22     | P3 <sub>2</sub> 21     | P2 <sub>1</sub>        | C222 <sub>1</sub>      | P6 <sub>s</sub> 22     |
| Cell dimensions   |                        |                        |                        |                        |                        |
| <i>a, b, c</i> (Å)*                                     | 65.64, 65.64, 344.69   | 62.55, 62.55, 168.82   | 66.82, 83.05, 92.83    | 74.50, 102.40, 138.97  | 68.92, 68.92, 107.17   |
| <i>α, β, γ</i> (°)□                                     | 90.0, 90.0, 120.0      | 90.0, 90.0, 120.0      | 90.0, 106.71, 90.0     | 90.0, 90.0, 90.0       | 90.0, 90.0, 120.0      |
| Resolution (Å)  | 57.34-2.60 (2.69-2.60) | 45.59-2.30 (2.38-2.30) | 42.17-1.70 (1.76-1.70) | 44.12-2.60 (2.69-2.60) | 34.46-1.90 (1.97-1.90) |
| <i>R</i> <sub>sym</sub> or <i>R</i> <sub>merge</sub>    | 0.080 (0.455)          | 0.101 (0.849)          | 0.086 (0.765)          | 0.095 (0.739)          | 0.074 (3.39)           |
| <i>I</i> /σ( <i>I</i> )                                 | 18.0 (3.3)             | 14.9 (3.4)             | 8.9 (1.7)              | 13.1 (2.1)             | 15.2 (0.7)             |
| Completeness (%)  | 98.8 (99.1)            | 99.3 (98.3)            | 98.4 (93.8)            | 98.8 (98.7)            | 94.5 (85.0)            |
| Redundancy  | 10.3 (10.9)            | 7.9 (8.2)              | 3.1 (3.1)              | 7.2 (7.4)              | 12.4 (12.6)            |
| <i>R</i> <sub>pim</sub>                                 | 0.024 (0.134)          | 0.038 (0.293)          | 0.057 (0.510)          | 0.038 (0.287)          | 0.022 (1.05)           |
| CC <sub>1/2</sub>                                       | 0.999 (0.987)          | 0.997 (0.919)          | 0.995 (0.640)          | 0.998 (0.895)          | 0.999 (0.526)          |
| Estimated twin fraction                                 | 0.0 (none)             | 0.06 (-h, -k, l)       | 0.0 (none)             | 0.0 (none)             | 0.0 (none)             |
| <b>Refinement</b>                                       |                        |                        |                        |                        |                        |
| Resolution (Å)  | 56.09-2.60 (2.69-2.60) | 45.59-2.30 (2.38-2.30) | 42.27-1.70 (1.76-1.70) | 36.72-2.60 (2.69-2.60) | 34.46-1.90 (1.97-1.90) |
| No. reflections   | 13219 (1185)           | 17592 (1687)           | 105129 (9993)          | 16508 (1627)           | 11788 (1025)           |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> (%) | 25.8/27.7 (36.3/44.2)  | 18.6/21.6 (24.1/29.8)  | 18.1/21.5 (27.0/31.6)  | 20.6/25.5 (29.3/34.5)  | 22.8/25.8 (34.5/33.5)  |
| No. atoms   | 2486                   | 2641                   | 7798                   | 3552                   | 976                    |
| Protein   | 2486                   | 2500                   | 6798                   | 3456                   | 907                    |
| Water + ligands   | 0                      | 141                    | 962+38                 | 96                     | 65+4                   |
| B-factor Wilson/Ave                                     | 39.3/59.8              | 26.9/32.9              | 20.3/26.9              | 33.9/31.5              | 31.8/45.0              |
| Protein   | 59.8                   | 32.8                   | 25.7                   | 31.5                   | 45.0                   |
| Water + ligands   | 0                      | 34.7                   | 34.5+40.0              | 29.5                   | 45.1+55.8              |
| R.m.s. deviations                                       |                        |                        |                        |                        |                        |
| bond length (Å)   | 0.002                  | 0.005                  | 0.004                  | 0.003                  | 0.013                  |
| bond angle (°)  | 0.54                   | 0.74                   | 0.74                   | 0.64                   | 1.17                   |
| Ramachandran  |                        |                        |                        |                        |                        |
| favored (%)   | 92.9                   | 97.4                   | 98.3                   | 96.3                   | 95.6                   |
| allowed (%)   | 7.1                    | 2.6                    | 1.5                    | 3.7                    | 3.5                    |
| outliers (%)  | 0.0                    | 0.0                    | 0.2                    | 0.0                    | 0.9                    |

510

511 \*Values in parenthesis are for highest resolution shell.

512

513 **Table 2** | Cryo-EM data collection, refinement and validation statistics

|     | Sb45+S-6P (1-up,2-down)               | Sb45+S-6P (2-up,1-down) |
|-----|---------------------------------------|-------------------------|
| 514 |                                       |                         |
| 515 | EMDB ID                               | EMD-24105               |
| 516 | PDB-ID                                | 7N0G                    |
| 517 | Data collection and processing        |                         |
| 518 | Magnification                         | 130,000                 |
| 519 | Voltage (kV)                          | 300                     |
| 520 | Electron exposure (e <sup>-</sup> /Å) | 56                      |
| 521 | Defocus range (μm)                    | -0.7 to -2.0            |
| 522 | Pixel size (Å/pixel)                  | 0.526 (1.052 binned)    |
| 523 | Raw micrographs (no.)                 | 9,725                   |
| 524 | Extract particles (no.)               | 1,447,993               |
| 525 | Selected 2D particles (no.)           | 662,994                 |
| 526 | Refined particles (no.)               | 417,460                 |
| 527 | Particles for final map (no.)         | 214,171                 |
| 528 | Symmetry imposed                      | C1                      |
| 529 | Map resolution (Å)                    | 3.02                    |
| 530 | FSC threshold                         | 0.143                   |
| 531 | Refinement                            |                         |
| 532 | Initial model used                    | 6XKL, 7KGJ              |
| 533 | Model composition                     |                         |
| 534 | Atoms                                 | 29,062                  |
| 535 | Residues                              | 3,592                   |
| 536 | Ligands (NAG)                         | 73                      |
| 537 | Overall B-factor (Å <sup>2</sup> )    |                         |
| 538 | Protein (min/max/mean)                | 36.8/589.6/157.0        |
| 539 | Ligands (min/max/mean)                | 55.3/340.1/129.9        |
| 540 | R.m.s. deviations                     |                         |
| 541 | bond length (Å)                       | 0.003                   |
| 542 | bond angle (°)                        | 0.548                   |
| 543 | CC (mask/volume/peaks)                | 0.84/0.84/0.77          |
| 544 | Validation                            |                         |
| 545 | MolProbity score                      | 1.62                    |
| 546 | Clashscore                            | 7.71                    |
| 547 | Poor rotamers                         | 0.00                    |

541 **References**

542

- 543 1. Conti, P. et al. The British variant of the new coronavirus-19 (Sars-Cov-2) should not create a  
544 vaccine problem. *J Biol Regul Homeost Agents* **35**(2021).
- 545 2. Kirby, T. New variant of SARS-CoV-2 in UK causes surge of COVID-19. *Lancet Respir Med*  
546 (2021).
- 547 3. Tang, J.W., Tambyah, P.A. & Hui, D.S. Emergence of a new SARS-CoV-2 variant in the UK. *J*  
548 *Infect* (2020).
- 549 4. Wibmer, C.K. et al. SARS-CoV-2 501Y.V2 escapes neutralization by South African COVID-19  
550 donor plasma. *bioRxiv*, 2021.01.18.427166 (2021).
- 551 5. Wang, Q. et al. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell*  
552 (2020).
- 553 6. Shang, J. et al. Cell entry mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A* **117**, 11727-  
554 11734 (2020).
- 555 7. Baden, L.R. et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med*  
556 (2020).
- 557 8. Cao, Y. et al. Potent Neutralizing Antibodies against SARS-CoV-2 Identified by High-Throughput  
558 Single-Cell Sequencing of Convalescent Patients' B Cells. *Cell* **182**, 73-84 e16 (2020).
- 559 9. Cerutti, G. et al. Structural Basis for Accommodation of Emerging B.1.351 and B.1.1.7 Variants by  
560 Two Potent SARS-CoV-2 Neutralizing Antibodies. *bioRxiv* (2021).
- 561 10. Edara, V.V. et al. Reduced binding and neutralization of infection- and vaccine-induced antibodies  
562 to the B.1.351 (South African) SARS-CoV-2 variant. *bioRxiv* (2021).
- 563 11. Planas, D. et al. Sensitivity of infectious SARS-CoV-2 B.1.1.7 and B.1.351 variants to neutralizing  
564 antibodies. *Nat Med* (2021).
- 565 12. Ramanathan, M., Ferguson, I.D., Miao, W. & Khavari, P.A. SARS-CoV-2 B.1.1.7 and B.1.351  
566 Spike variants bind human ACE2 with increased affinity. *bioRxiv* (2021).
- 567 13. Singh, J. et al. Structure-Function Analyses of New SARS-CoV-2 Variants B.1.1.7, B.1.351 and  
568 B.1.1.28.1: Clinical, Diagnostic, Therapeutic and Public Health Implications. *Viruses* **13**(2021).
- 569 14. Wang, P. et al. Antibody resistance of SARS-CoV-2 variants B.1.351 and B.1.1.7. *Nature* (2021).
- 570 15. Xu, C. et al. Conformational dynamics of SARS-CoV-2 trimeric spike glycoprotein in complex with  
571 receptor ACE2 revealed by cryo-EM. *Sci Adv* (2020).
- 572 16. Hanke, L. et al. An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *Nat*  
573 *Commun* **11**, 4420 (2020).
- 574 17. Schoof, M. et al. An ultrapotent synthetic nanobody neutralizes SARS-CoV-2 by stabilizing inactive  
575 Spike. *Science* (2020).
- 576 18. Walter, J.D. et al. Highly potent bispecific sybodies neutralize SARS-CoV-2. *bioRxiv* (2020).
- 577 19. Xiang, Y. et al. Versatile and multivalent nanobodies efficiently neutralize SARS-CoV-2. *Science*  
578 **370**, 1479-1484 (2020).
- 579 20. Ingram, J.R., Schmidt, F.I. & Ploegh, H.L. Exploiting Nanobodies' Singular Traits. *Annu Rev*  
580 *Immunol* **36**, 695-715 (2018).
- 581 21. Zimmermann, I. et al. Generation of synthetic nanobodies against delicate proteins. *Nat Protoc* **15**,  
582 1707-1741 (2020).

583 22. Wu, Y. et al. A noncompeting pair of human neutralizing antibodies block COVID-19 virus binding  
584 to its receptor ACE2. *Science* **368**, 1274-1278 (2020).

585 23. Natarajan, K., Mage, M.G. & Margulies, D.H. Immunoglobulin Superfamily. in *eLS* (John Wiley &  
586 Sons, Ltd, Chichester, 2015).

587 24. Halaby, D.M., Poupon, A. & Mornon, J. The immunoglobulin fold family: sequence analysis and  
588 3D structure comparisons. *Protein Eng* **12**, 563-71 (1999).

589 25. Huo, J. et al. Neutralization of SARS-CoV-2 by Destruction of the Prefusion Spike. *Cell Host*  
590 *Microbe* **28**, 445-454 e6 (2020).

591 26. Wrapp, D. et al. Structural Basis for Potent Neutralization of Betacoronaviruses by Single-Domain  
592 Camelid Antibodies. *Cell* **181**, 1004-1015 e15 (2020).

593 27. Wu, T.T. & Kabat, E.A. An analysis of the sequences of the variable regions of Bence Jones proteins  
594 and myeloma light chains and their implications for antibody complementarity. *J Exp Med* **132**, 211-  
595 50 (1970).

596 28. Lan, J. et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2  
597 receptor. *Nature* **581**, 215-220 (2020).

598 29. Rudd, P.M. et al. Hybrid and complex glycans are linked to the conserved N-glycosylation site of  
599 the third eight-cysteine domain of LTBP-1 in insect cells. *Biochemistry* **39**, 1596-603 (2000).

600 30. Hsu, T.A. et al. Differential N-glycan patterns of secreted and intracellular IgG produced in  
601 *Trichoplusia ni* cells. *J Biol Chem* **272**, 9062-70 (1997).

602 31. Shajahan, A. et al. Comprehensive characterization of N- and O- glycosylation of SARS-CoV-2  
603 human receptor angiotensin converting enzyme 2. *Glycobiology* (2020).

604 32. Zhao, P. et al. Virus-Receptor Interactions of Glycosylated SARS-CoV-2 Spike and Human ACE2  
605 Receptor. *Cell Host Microbe* **28**, 586-601 e6 (2020).

606 33. Hsieh, C.L. et al. Structure-based design of prefusion-stabilized SARS-CoV-2 spikes. *Science* **369**,  
607 1501-1505 (2020).

608 34. Punjani, A. & Fleet, D.J. 3D Variability Analysis: Resolving continuous flexibility and discrete  
609 heterogeneity from single particle cryo-EM. *BioRxiv*, bioRxiv 2020.04.08.032466 (2020).

610 35. Punjani, A. & Fleet, D.J. 3D Flexible Refinement: Structure and Motion of Flexible Proteins from  
611 Cryo-EM. *BioRxiv*, bioRxiv 2021.04.22.440893 (2021).

612 36. Punjani, A., Rubinstein, J.L., Fleet, D.J. & Brubaker, M.A. cryoSPARC: algorithms for rapid  
613 unsupervised cryo-EM structure determination. *Nat Methods* **14**, 290-296 (2017).

614 37. Punjani, A., Zhang, H. & Fleet, D.J. Non-uniform refinement: Adaptive regularization improves  
615 single particle cryo-EM reconstruction. *BioRxiv*, bioRxiv 2019.12.15.877092 (2019).

616 38. Pettersen, E.F. et al. UCSF Chimera--a visualization system for exploratory research and analysis.  
617 *J Comput Chem* **25**, 1605-12 (2004).

618 39. Adams, P.D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure  
619 solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-21 (2010).

620 40. Boehm, E. et al. Novel SARS-CoV-2 variants: the pandemics within the pandemic. *Clin Microbiol*  
621 *Infect* (2021).

622 41. Wang, Z. et al. mRNA vaccine-elicited antibodies to SARS-CoV-2 and circulating variants. *Nature*  
623 **592**, 616-622 (2021).

624 42. Wibmer, C.K. et al. SARS-CoV-2 501Y.V2 escapes neutralization by South African COVID-19  
625 donor plasma. *Nat Med* **27**, 622-625 (2021).

626 43. Starr, T.N. et al. Prospective mapping of viral mutations that escape antibodies used to treat COVID-  
627 19. *Science* **371**, 850-854 (2021).

628 44. Zhu, X. et al. Cryo-electron microscopy structures of the N501Y SARS-CoV-2 spike protein in  
629 complex with ACE2 and 2 potent neutralizing antibodies. *PLoS Biol* **19**, e3001237 (2021).

630 45. Barnes, C.O. et al. SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies.  
631 *Nature* (2020).

632 46. Koenig, P.A. et al. Structure-guided multivalent nanobodies block SARS-CoV-2 infection and  
633 suppress mutational escape. in *Science* 2021/01/14 edn (2021).

634 47. Xu, J. et al. Multimeric nanobodies from camelid engineered mice and llamas potently neutralize  
635 SARS-CoV-2 variants. *bioRxiv* (2021).

636 48. Hansen, J. et al. Studies in humanized mice and convalescent humans yield a SARS-CoV-2 antibody  
637 cocktail. *Science* **369**, 1010-1014 (2020).

638 49. Chan, K.K. et al. Engineering human ACE2 to optimize binding to the spike protein of SARS  
639 coronavirus 2. *Science* **369**, 1261-1265 (2020).

640 50. Li, H., Natarajan, K., Malchiodi, E., Margulies, D. & Mariuzza, R. Three-dimensional structure of  
641 H-2D(d) complexed with an immunodominant peptide from human immunodeficiency virus  
642 envelope glycoprotein 120. *Journal of Molecular Biology* **283**, 179-191 (1998).

643 51. Zhao, H., Boyd, L.F. & Schuck, P. Measuring Protein Interactions by Optical Biosensors. *Curr*  
644 *Protoc Protein Sci* **88**, 20 2 1-20 2 25 (2017).

645 52. Zhao, H., Gorshkova, II, Fu, G.L. & Schuck, P. A comparison of binding surfaces for SPR  
646 biosensing using an antibody-antigen system and affinity distribution analysis. *Methods* **59**, 328-35  
647 (2013).

648 53. Kabsch, W. Xds. *Acta Crystallogr D Biol Crystallogr* **66**, 125-32 (2010).

649 54. Rai, B.K. & Fiser, A. Multiple mapping method: a novel approach to the sequence-to-structure  
650 alignment problem in comparative protein structure modeling. *Proteins* **63**, 644-61 (2006).

651 55. McCoy, A.J. et al. Phaser crystallographic software. *J Appl Crystallogr* **40**, 658-674 (2007).

652 56. Liebschner, D. et al. Macromolecular structure determination using X-rays, neutrons and electrons:  
653 recent developments in Phenix. *Acta Crystallogr D Struct Biol* **75**, 861-877 (2019).

654 57. Emsley, P., Lohkamp, B., Scott, W.G. & Cowtan, K. Features and development of Coot. *Acta*  
655 *Crystallogr D Biol Crystallogr* **66**, 486-501 (2010).

656 58. Mastronarde, D.N. Automated electron microscope tomography using robust prediction of specimen  
657 movements. *J Struct Biol* **152**, 36-51 (2005).

658 59. Custodio, T.F. et al. Selection, biophysical and structural analysis of synthetic nanobodies that  
659 effectively neutralize SARS-CoV-2. *Nat Commun* **11**, 5588 (2020).

660

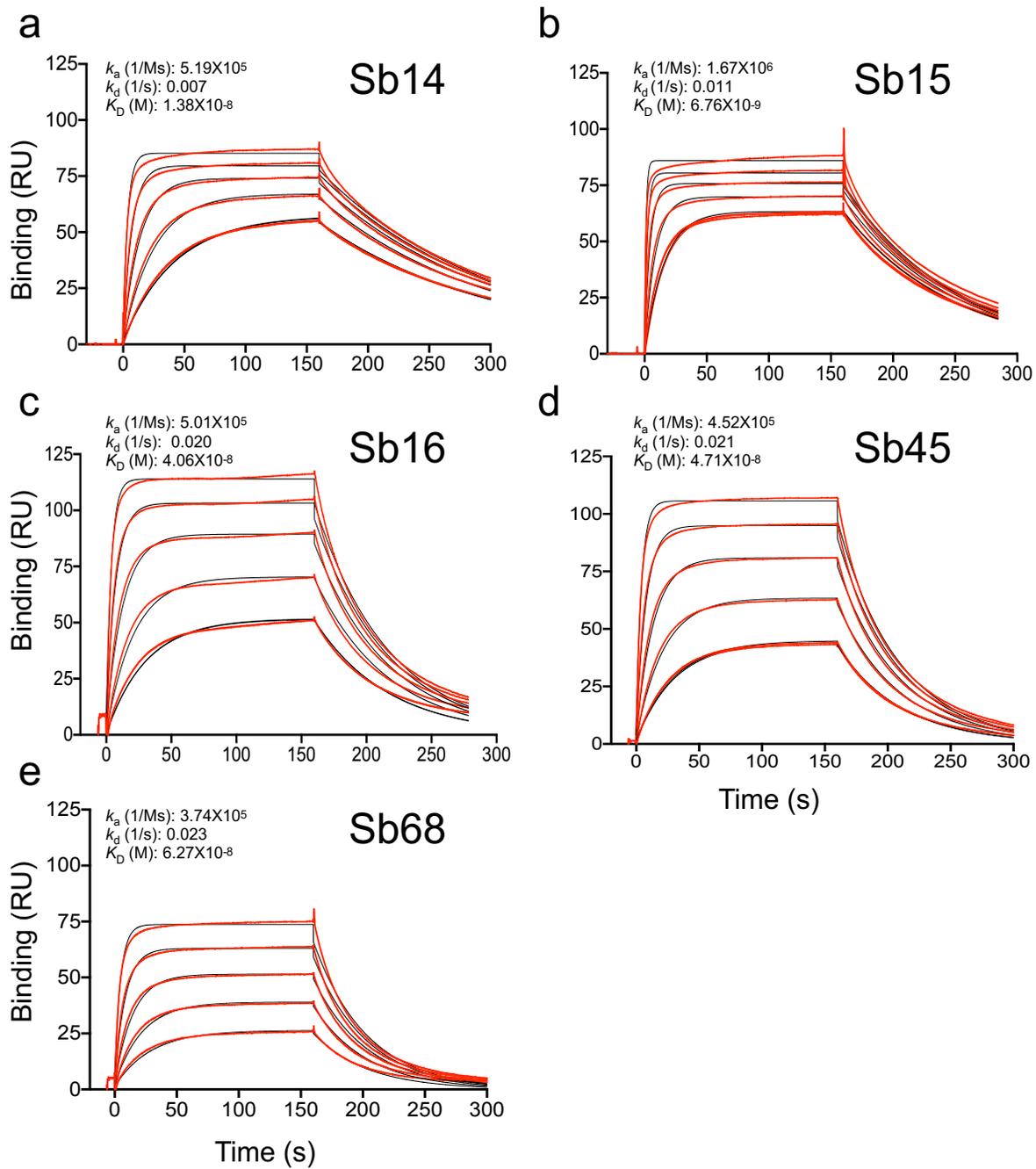


Fig. 1.

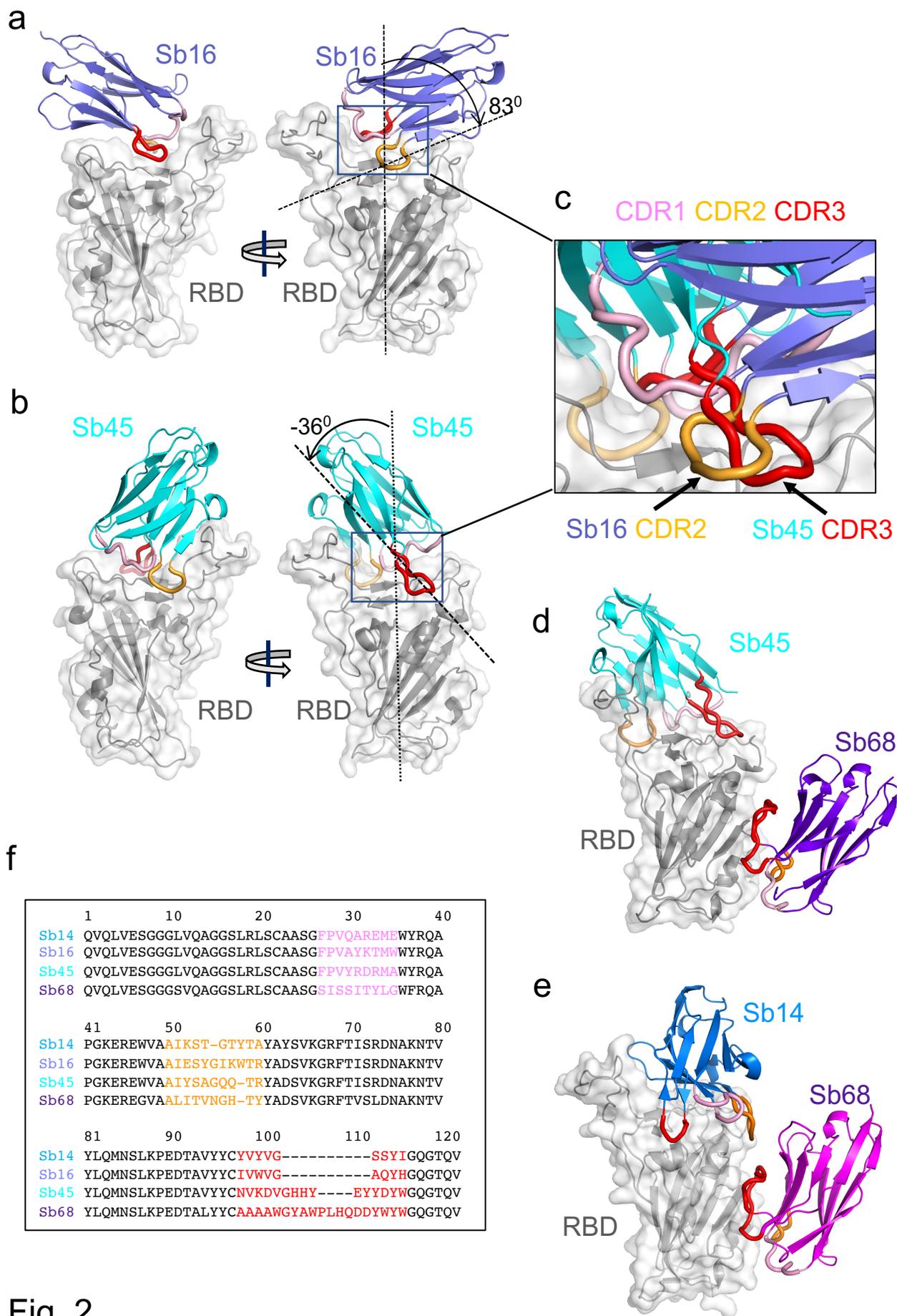


Fig. 2.

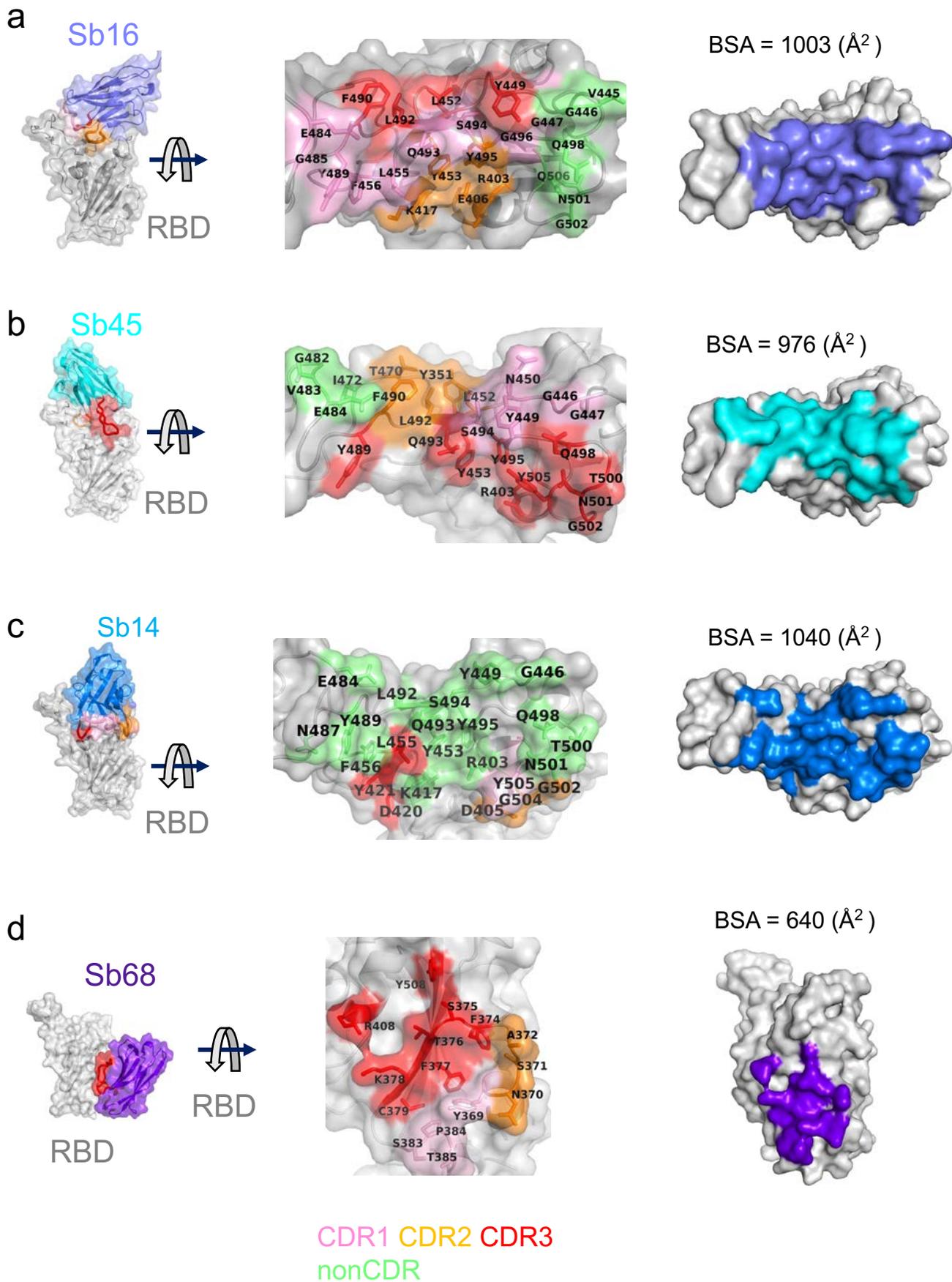


Fig. 3.

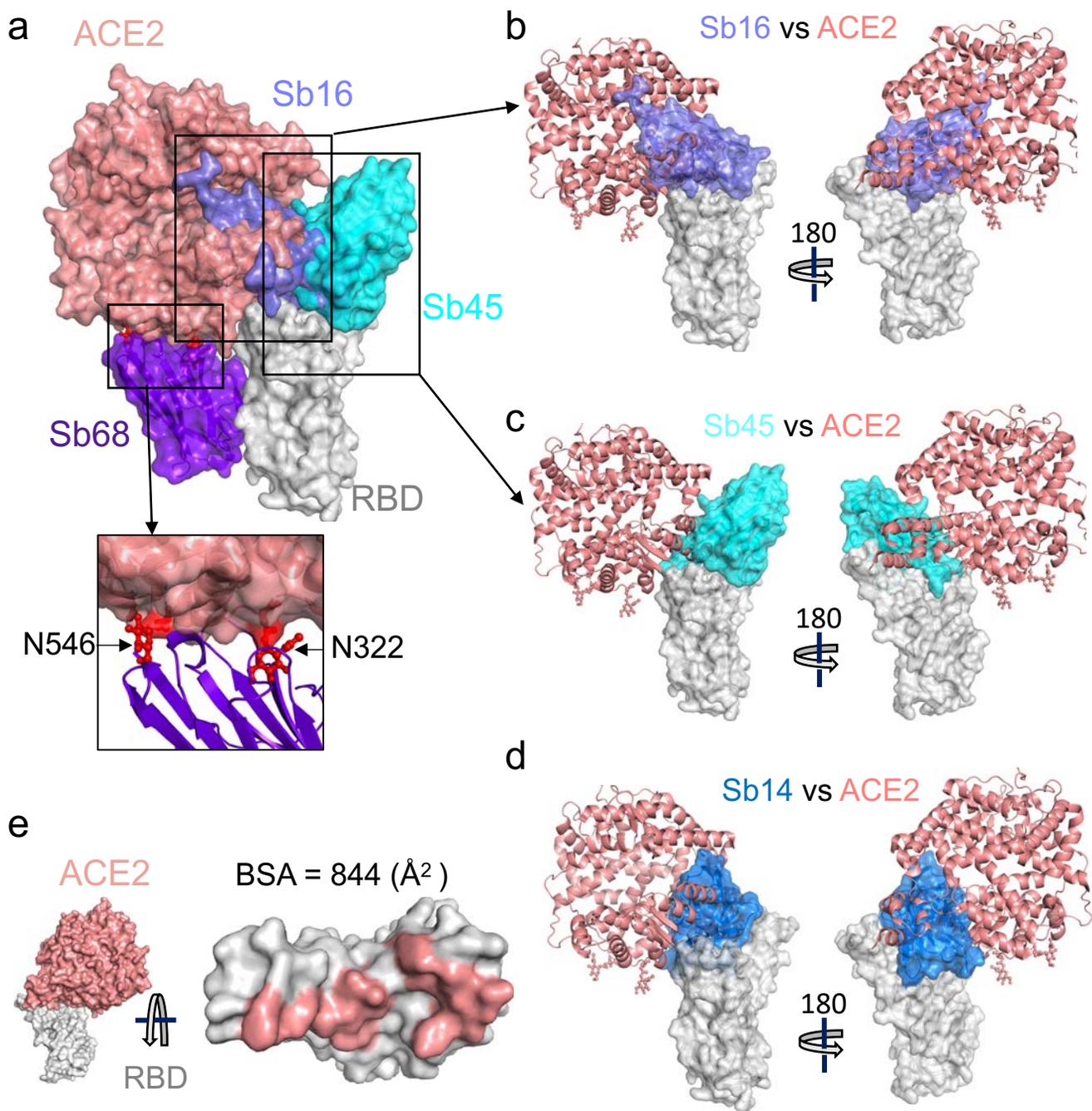


Fig. 4.

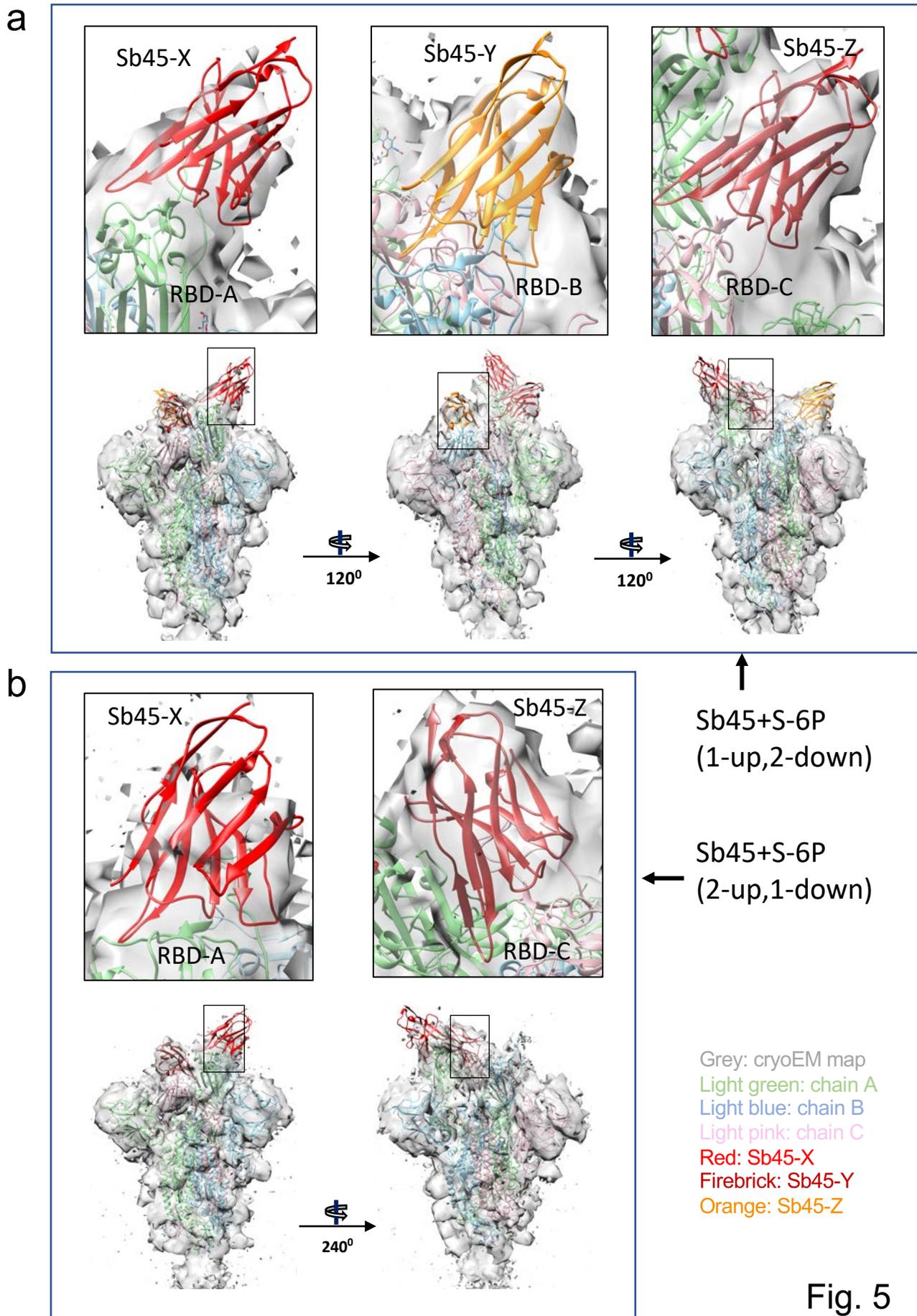


Fig. 5

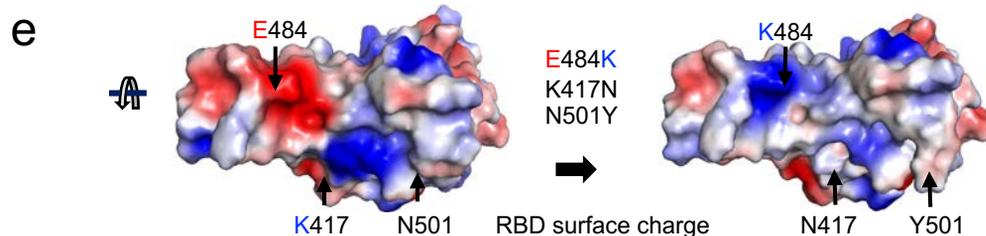
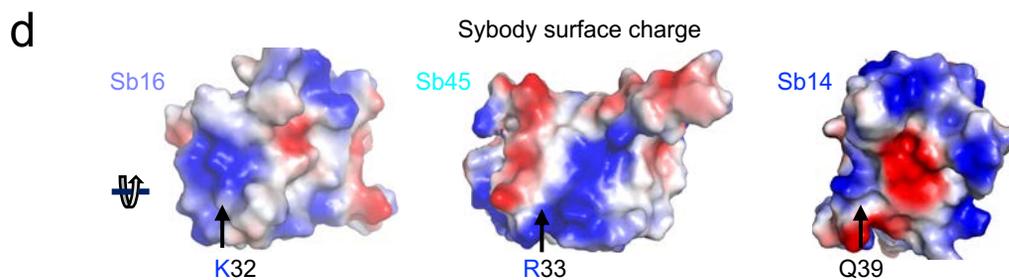
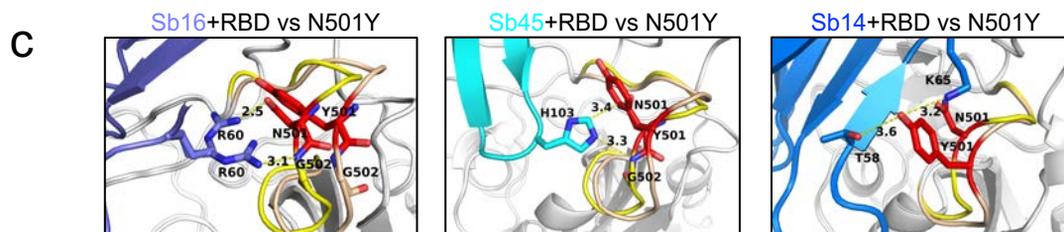
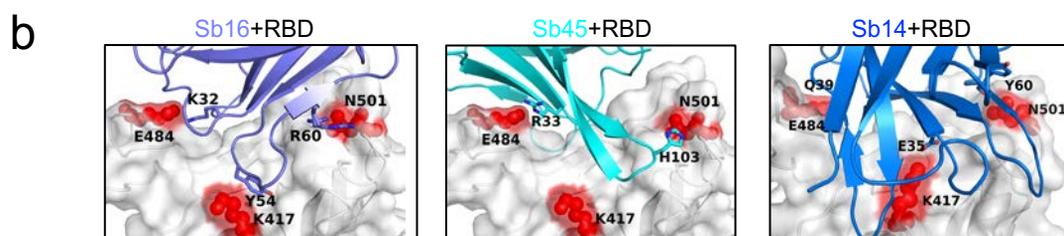
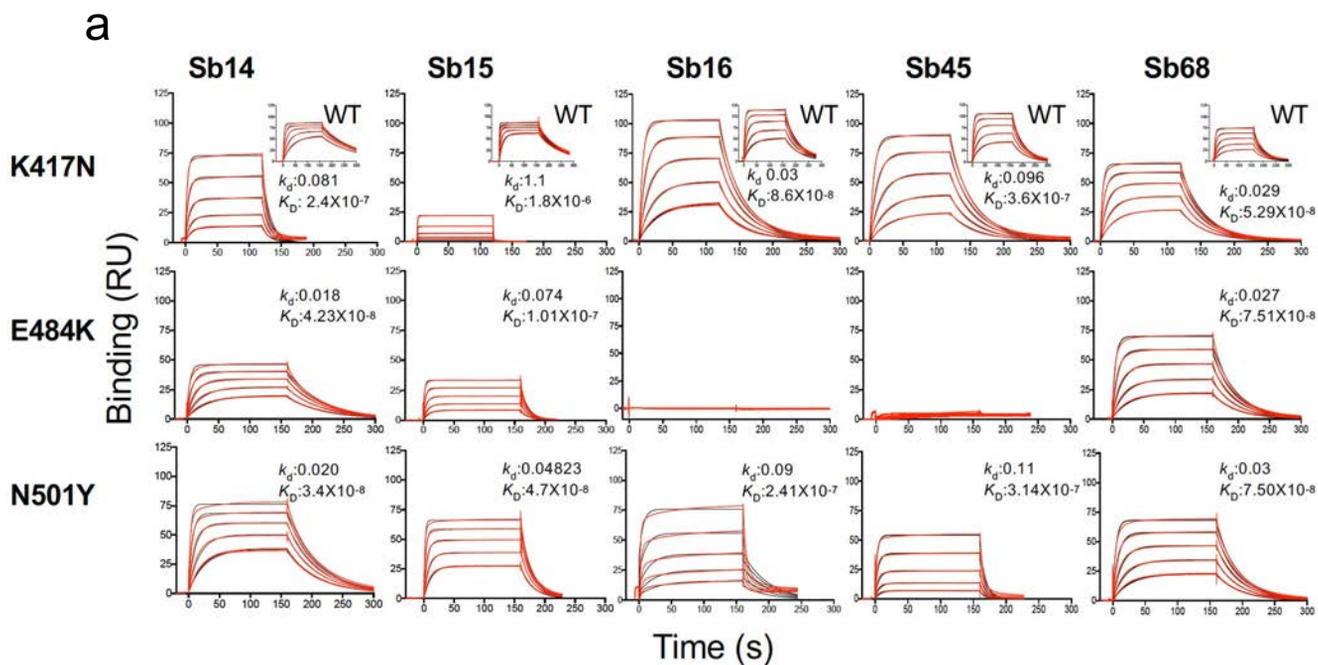


Fig. 6

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AhmadetalSuppTablesExtData.pdf](#)