

1 **SARS-CoV-2 S protein ACE2 interaction reveals**
2 **novel allosteric targets**
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21 **Running Title:** *Spike-ACE2 interactions allosterically prime proteolytic processing of Spike*
22 *protein*

23 **Keywords:** Spike, ACE2, allostery, proteolysis, virus-host interactions, fusion peptide, SARS-
24 CoV-2

25 **Abbreviations:** HDXMS, Hydrogen Deuterium Exchange Mass Spectrometry; MD, molecular
26 dynamics, RFU, Relative Fractional deuterium uptake; RMSF, root mean squared fluctuations;
27 PCA, Principal Component Analysis; S, Spike; UPLC, Ultra Performance Liquid
28 Chromatography;

29

30 **Abstract:** The Spike (S) protein is the main handle for SARS-CoV-2 to enter host cells through
31 surface ACE2 receptors. How ACE2 binding activates proteolysis of S protein is unknown. Here,
32 we have mapped the S:ACE2 interface and uncovered long-range allosteric propagation of ACE2
33 binding to sites critical for viral host entry. Unexpectedly, ACE2 binding enhances dynamics at a
34 distal S1/S2 cleavage site and flanking protease docking site ~ 27 Å away while dampening
35 dynamics of the stalk hinge (central helix and heptad repeat) regions ~ 130 Å away. This highlights
36 that the stalk and proteolysis sites of the S protein are dynamic hotspots in the pre-fusion state.
37 Our findings provide a mechanistic basis for S:ACE2 complex formation, critical for proteolytic
38 processing and viral-host membrane fusion and highlight protease docking sites flanking the S1/S2
39 cleavage site, fusion peptide and heptad repeat 1 (HR1) as allosterically exposed cryptic hotspots
40 for potential therapeutic development.

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42 **One Sentence Summary:** SARS-CoV-2 spike protein binding to receptor ACE2 allosterically
43 enhances furin proteolysis at distal S1/S2 cleavage sites

44 The COVID-19 pandemic caused by the SARS-CoV-2 virus has sparked extensive efforts
45 to map molecular details of its life cycle to drive vaccine and therapeutic discovery.(1) SARS-
46 CoV-2 belongs to the family of *Coronaviridae* which includes other human pathogens including
47 common cold causing viruses (hCoV-OC43, HKU and 229E), SARS and MERS-CoV.(2-5)
48 SARS-CoV-2 has a ~30 Kbp long positive RNA genome with 14 open reading frames, encoding
49 4 structural proteins: Spike (S) protein, membrane (M) protein, envelope (E) protein and nucleo-
50 protein; 16 non-structural proteins and 9 accessory proteins.(6-8) An intact SARS-CoV-2 virion
51 consists of a nucleocapsid core composed of nucleoprotein packaged genomic RNA encapsidated
52 as a lipid-protein envelope forming a spherical structure of diameter ~100 nm.(9) The viral
53 envelope is decorated with S, M and E proteins.(9) The S protein is a club-shaped homotrimeric
54 class I viral fusion protein that has distinctive ‘head’ and ‘stalk’ regions (Fig. 1A).

55 A characteristic feature of SARS-CoV-2 is proteolysis of Pre-fusion S protein by host
56 proteases into S1 and S2 subunits. The S1 subunit comprises an N-terminal domain (NTD) and a
57 receptor binding domain (RBD) that interacts with the host receptor Angiotensin converting
58 enzyme-2 (ACE2)(10, 11) to initiate viral entry into the host.(12) Cryo-electron tomography (cryo-
59 ET) has been used to capture the distribution and organization of trimeric S protein on the intact
60 virion,(9) revealing that 25 ± 9 S protein trimers decorate a single virion with a small percentage
61 (3%) of embedded S proteins in a post-fusion state adopting an extended helical conformation.
62 The first virus-host interaction is mediated by the viral S protein with the host ACE2 receptor.(10)
63 Binding to ACE2 primes the S protein for proteolysis at S1/S2 cleavage site into individual S1 and
64 S2 subunits.(13, 14) The S2 subunit is divided into six constituent domains harboring the
65 membrane fusion machinery of the virus. These comprise the fusion peptide (FP), heptad repeat
66 (HR1), heptad repeat 2 (HR2), connector domain (CD), transmembrane domain (TM), and

67 cytoplasmic tail (CT).(15, 16) Extensive structural studies (9, 15, 17, 18) have captured S proteins
68 of coronaviruses in distinct open- (PDB:6VXX)(15) and closed- (PDB:6VYB)(15) conformational
69 states with regards the RBD, as well as the ectodomain orientation in the pre- and post-fusion
70 states, thereby revealing a high intrinsic metastability of the S protein. The S2 subunit promotes
71 membrane fusion and viral entry (Fig. 1B).

72 Despite extensive cryo-EM studies, how ACE2 binding at the RBD domain primes
73 enhanced proteolytic processing at the S1/S2 site is entirely unknown. Amide hydrogen/deuterium
74 exchange mass spectrometry (HDXMS) is a powerful complementary tool for both virus dynamics
75 (19) and mapping protein-protein interactions.(20) Here, we describe the dynamics of free S
76 protein, the S:ACE2 complex and describe ACE2 binding-induced allosteric conformational
77 changes across the distal regions of S protein, particularly at the stalk and protease docking sites
78 flanking the S1/S2 cleavage sites. These distal ‘hotspots’ are critical for the first step of SARS-
79 CoV-2 infection and represent novel targets for therapeutic intervention.

80 **Results and Discussion**

81 **Localizing subunit specific dynamics and domain motions of S protein trimer**

82 Structural snapshots of the ACE2 binding to the SARS-CoV-2 S protein interface have
83 been obtained with the RBD alone.(10, 16, 21-23) In this study, we have mapped this interface for
84 the S protein construct (1-1208) with mutations at the S1/S2 cleavage site (PRRAS to PGSAS)
85 and proline substitution at 986-987,(16) to block proteolysis during expression and purification
86 (Fig. S1A). The S protein and isolated RBD constructs showed high affinity binding to ACE2 (Fig.
87 S1B). We measured dynamics of a trimer of this near-full length S protein by amide hydrogen-
88 deuterium exchange mass spectrometry (HDXMS). Pepsin proteolysis generated 321 peptides
89 with high signal to noise ratio, accounting for ~87% of the entire S protein (Fig. S2). Glycosylation

90 of at least 22 sites have been predicted on S protein.(24) Average deuterium exchange at these
91 reporter peptides was monitored for comparative deuterium exchange analysis of S protein, ACE2
92 receptor and S:ACE2 complex, along with a specific ACE2 complex with the isolated RBD. While
93 glycosylation is an important posttranslational modification, our HDXMS study has measured
94 deuterium exchange of non-glycosylated segments of S protein alone. Deuterium exchange ($t = 1$
95 and 10 min) across all peptides of the free S protein trimer are shown in (Fig. 2). We built an
96 integrative model of the full-length S protein trimer using experimental structures of prefusion S
97 ectodomain in the open conformation (PDB: 6VSB)(16) and the HR2 domain from SARS S
98 protein as templates. Mapping the relative deuterium exchange across all peptides onto this S
99 protein model showed the greatest deuterium exchange at the stalk region. (Fig. 2A) This is
100 consistent with earlier studies showing at least 60° sweeping motions of the three identified hinge
101 regions of the stalk.(18) This was further verified via all-atom MD simulations of the S protein
102 model embedded in a viral model membrane, which showed significant motions of the S protein
103 ectodomain as a result of the flexible stalk region, (Fig. 2B) as well as large atomic fluctuations
104 around the HR2 domain, compared to the rest of the protein (Fig. S3, Fig. S4).

105 The deuterium exchange heat map showed the highest relative exchange in the S2 subunit
106 (Fig. S3) and helical segments, while peptides spanning the fusion peptide showed relatively lower
107 deuterium exchange. Individually, S1 and S2 subunits showed different intrinsic deuterium
108 exchange kinetics, where the average relative fractional deuterium uptake (RFU) of S1 subunit
109 (~0.25) was lower than the average RFU (~0.35) of S2 subunit (Fig. S3, Table S1). Moreover,
110 peptides connecting the RBD to the remainder of the S protein showed greater deuterium
111 exchange, reflecting its role as a ‘hinge’ facilitating the RBD populating an ensemble of open- and
112 closed- conformational states (red arrow, Fig. 2C). Indeed, in our simulations of the S protein (Fig.

113 2B), the RBD oriented initially in an ‘up’- conformation exhibited spontaneous motion towards
114 the ‘down’- conformation relative to the hinge region (Fig. 2D, Fig. S4A). Interestingly, a part of
115 the receptor binding motif, specifically residues 476-486, exhibited a higher degree of flexibility
116 based on its average atomic fluctuations (Fig. 2A, 4C), suggesting that binding to ACE2 receptor
117 would be required to stabilize its motion.

118 The NTD of the S protein showed low overall RFU (~ 0.2), consistent with its well-
119 structured arrangement of β -sheets connected by loops (Fig. 1B). Importantly, certain regions
120 showed significantly higher deuterium exchange (~ 0.4), of which two loci (136-143, 243-265)
121 span the dynamic interdomain interactions with the RBD. This is supported by the high per-residue
122 root mean square fluctuations (RMSF) and large principal motions observed for residues 249-259
123 during simulations (Fig. 2C, Fig. S4C). One locus (291-303) at the C-terminal end of the NTD
124 connecting to the RBD showed high deuterium exchange, indicative of relative motions of the two
125 domains. The RBD (Fig. 1D) showed relatively higher deuterium exchange (RFU ~ 0.35), with the
126 peptides spanning the hinge-regions (318-336) showing greatest deuterium exchange (~ 0.6).
127 Peptides spanning residues 351-375 and 432-452 showed significantly increased deuterium
128 uptake, and these correspond to the NTD interdomain interaction sites. Interestingly, loci of the
129 RBD implicated in the interface (453-467, 491-510) with ACE2 showed relative higher exchange.

130 Overall, the S2 subunit showed relatively higher RFU than the S1 subunit, with each
131 domain exhibiting specific conformational changes (Fig. 1E, Fig. S4). Peptides spanning the
132 region immediately downstream of the S1/S2 cleavage site showed the highest deuterium uptake
133 (0.6), reflecting the rapid dynamics it undergoes for facilitating cleavage of S protein into two
134 subunits. Congruently, our MD simulations revealed the unstructured loop housing the S1/S2
135 cleavage site (residues 677-689) to be highly dynamic (Figure S4C), with RMSFs reaching >1.0

136 nm. It is important to note that the S1/S2 cleavage site has been abrogated in the construct of the
137 S protein used in this study to block proteolytic processing into S1 and S2 subunits during
138 expression in host cells. We thus observed lower deuterium uptake (and lower RMSF values) at
139 peptides in the central helix and connector domain, suggesting that these act as the central core of
140 prefusion S, while the peptides spanning hinge-segments and heptad repeats (HR1 and HR2)
141 showed high deuterium uptake and RMSF values, indicative of the inherent metastability of S to
142 adopt prefusion, fusion and post-fusion conformations.

143 **Domain-specific and global effects of ACE2 binding to the RBD**

144 Comparative HDXMS analysis of the S protein and S:ACE2 complex revealed large-scale
145 effects upon binding of ACE2. The main target for direct interactions was the RBD. We therefore
146 set out to characterize the effects of ACE2 binding with RBD ('RBD_s') present on full S protein
147 (Fig. 4A, 4B) and compared this to an isolated construct of the RBD ('RBD_{isolated}') (Fig. 3, Fig.
148 S6). Several peptides of the RBD_s showed decreased exchange upon complexation with ACE2
149 (Fig. 3B). These include peptides 340-359, 400-420, 432-452 and 487-502 in the RBD_s:ACE2
150 complex (Fig. 4). These sites are consistent with the interface of the SARS-CoV-2 S protein RBD
151 bound to the ACE2 receptor resolved by X-ray crystallography.⁽¹⁰⁾ The high-resolution structures
152 showed that the RBD and ACE2 receptor interact via an extensive interface. However, not all
153 peptides at the interface contribute equally to the binding energetics. HDXMS reveals the residues
154 at the core of this interface to be those within peptides spanning residues 340-359, 400-420, 432-
155 452 and 491-510 (Fig. 4A, 4D, Fig. S3). Interestingly, loci showing large-magnitude deuterium
156 exchange correlate to mutational hotspots⁽²⁵⁾.

157 A close-up of RBD_{isolated}:ACE2 complex also showed decreased exchange in peptides
158 spanning these regions (Fig. 3). However, the magnitude of decrease in exchange was significantly

159 greater in RBD_{isolated} than in RBD_S, indicating the higher flexibility in the full-length S trimer than
160 in free RBD. High resolution structures have identified the RBD interface interacting with ACE2
161 spanning the peptide covering residues 448-501 (Y449, Y453, N487, Y489, G496, T500, G502,
162 Y505, L455, F456, F486, Q493, Q498 and N501) using only the RBD from the S protein.(23)
163 Cryo-EM studies have shown that each RBD in the trimeric S protein can adopt an open
164 conformation irrespective of other RBDs, indicating an absence of cooperativity between the three
165 RBDs within a trimer.(9) Therefore, we compared the deuterium exchange profiles of RBD_{isolated}
166 with RBD_S and observed differences in dynamics imposed by quaternary contacts (Fig. 3A, Fig.
167 3B). Overall, the loci with high and low deuterium exchange profiles were similar when compared
168 between RBD_{isolated} and RBD_S, both at the disordered ACE2 receptor binding region as well as the
169 folded region at the N- and C -termini. In solution RBD_S toggles between open- and closed-
170 conformations resulting in an average readout of deuterium exchange measurements.

171 ACE2 binding to RBD_{isolated} and RBD_S resulted in similar effects, where we observed
172 deuterium exchange protection at the peptide regions spanning the known binding interface of
173 RBD. Notably, increased deuterium exchange was observed at the hinge region (Fig. 3C, Fig. S4)
174 indicating allosteric conformational changes, associated with restricting the open- and closed-
175 states interconversion. Therefore, the destabilization/ local unfolding observed at the hinge region
176 as a result of ACE2 binding enables RBD to maintain open conformation. It therefore seems likely
177 that small molecules and biologics targeting the hinge region to lock RBD in the closed state would
178 be of potential high therapeutic value.

179 **ACE2 binding at the RBD is allosterically propagated to the S1/S2 cleavage site and Heptad**
180 **Repeat**

181 Unexpectedly, ACE2 binding at the RBD induced large-scale changes in deuterium
182 exchange in distal regions of the S protein. Some of the peptides in the stalk of S protein showed
183 decreased exchange in the S:ACE2 complex (Fig. 4C, 4D). This indicates that ACE2 receptor
184 interactions stabilized the hinge dynamics. Decreased exchange was also seen in the distal sites in
185 the S2 subunit, localized at the fusion peptide locus and central helix (CH). Interestingly, increased
186 exchange was seen in multiple peptides flanking the S1/S2 cleavage site, HR1 domain and
187 critically at the S1/S2 cleavage sites (Fig. 4D). Even though the construct used in this study has
188 the proteolysis site mutated, it still resulted in increased dynamics at this S1/S2 locus. Furthermore,
189 this region exhibited high RMSF values during simulations. (Fig. S4B). These results clearly
190 indicate that ACE2 binding induces allosteric enhancement of dynamics at this locus, providing
191 mechanistic insights into the conformational switch from the pre-fusion to fusogenic intermediate.
192 Differences in deuterium exchange between free S protein and S:ACE2 complex shows
193 stabilization at ACE2 interacting site and local destabilization at peptides juxtaposed to S1/S2
194 cleavage site (residues 931-938). This suggests that ACE2 binding potentiates peptide of residues
195 931-938 and other high exchanging regions flanking the S1/S2 cleavage site for enhanced furin
196 protease binding and cleavage. Importantly, these results suggest that the S1/S2 cleavage site is a
197 critical hotspot for S protein dynamic transitions for viral entry into the host, and therefore
198 represents a new target for inhibitory therapeutics against the virus.

199 **Dynamics of ACE2 with RBD and S protein provides insights into viral-entry into host**

200 Considering the indispensable role of ACE2 binding in SARS-CoV-2 infection, it is crucial to
201 assess the effects of S protein and RBD binding on ACE2 dynamics. We therefore mapped the
202 corresponding binding sites of RBD, both isolated and within the Spike, on ACE2. The S:ACE2
203 complex represents the prefusion pre-cleavage state wherein full-length S protein is bound to the

204 ACE2 receptor (Fig. 1B ii), while the RBD_{isolated}:ACE2 complex represents the post-furin cleavage
205 product formed by the S1 subunit and ACE2 (Fig. 1B iii). Previous studies have shown that 14 key
206 amino acids of RBD interact with ACE2, wherein mutations at 6 amino acids resulted in higher
207 binding affinity of SARS-CoV-2.(26) SARS-CoV-2 adopted a different binding mode to ACE2 as
208 a superior strategy for infection in comparison to SARS-CoV-1. A crystal structure of
209 RBD_{isolated}:ACE2 complex has identified 24 key ACE2 residues, spanning across peptides 16-45,
210 79-83, 325-330, 350-357 and Arg393.(27) While most of these residues are conserved in binding
211 to both SARS-CoV-1 and SARS-CoV-2, Arg393 and residues 325-330 are unique to SARS-CoV-
212 1 interaction.(25) Interestingly, we observed increased deuterium exchange at these residues in the
213 S:ACE2 complex compared to ACE2 alone (Fig. S8). Identifying the intrinsic dynamics and
214 allosteric changes due to binding could potentially better inform drug development.

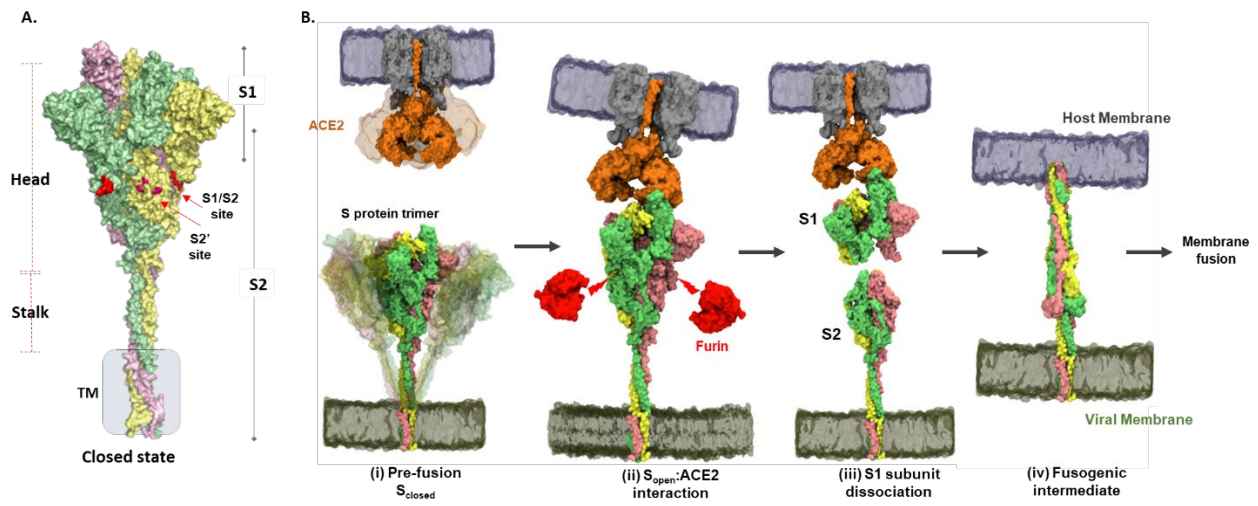
215 Simulations of the ACE2 dimer complexed with the B⁰AT1 amino acid transporter (PDB:
216 6M1D)(12) in a model epithelial membrane revealed a large motion of the peptidase domain (PD),
217 which recognizes the S protein RBD, with respect to the transmembrane and juxtamembrane
218 domains (Fig. S7). This large motion is reminiscent of the flexible tilting displayed by the S protein
219 ectodomain itself, suggesting that both S protein and ACE2 have adaptable hinges that allow for
220 orientational freedom of the domains involved in recognition. To understand how S protein
221 binding affects ACE2 dynamics, we performed HDXMS experiments of monomeric ACE2 alone,
222 S:ACE2 and RBD:ACE2 complexes (Fig. S7, Fig. S8) and mapped the deuterium exchange values
223 on a deletion construct of ACE2 (PDB: 1R42)(27) (Fig. S7, Fig. S8). We observed a reduction in
224 deuterium exchange across both RBD_{isolate}:ACE2 and larger S:ACE2 complexes compared to free
225 ACE2 (Figure S8B and S8C). Differences in deuterium exchange between RBD_{isolated}:ACE2
226 complex and free ACE2 showed that RBD binding stabilizes ACE2 globally, specifically large

227 differences at the binding site (peptides 21-29, 30-39, and 75-92), and also at distal regions
228 (peptides 121-146, 278-292, 575-586) from RBD binding site of ACE2 (Fig. S8D). Cryo-EM
229 studies have shown that a dimeric full length ACE2 receptor can stably bind to one trimer of the S
230 protein.(12)

231 **Conclusions**

232 Here a combination of HDXMS and MD simulations provide a close-up of S protein
233 dynamics in the pre-fusion, ACE2 bound and other associated conformations. Our results reveal
234 the energetics of the S:ACE2 complex interface. ACE2 binding to the isolated RBD and S protein
235 alike lead to binding and stabilization. Interestingly, ACE2 binding to the RBD induces global
236 conformational changes across the entire S protein. Importantly, the stalk region undergoes
237 dampening of conformational motions while causing increased deuterium exchange in the protease
238 sites. Regions highlighting the allosteric propagation of ACE2 binding represent cryptic targets
239 for small molecule inhibitor/antibody development as therapeutics.

324 **Figures**

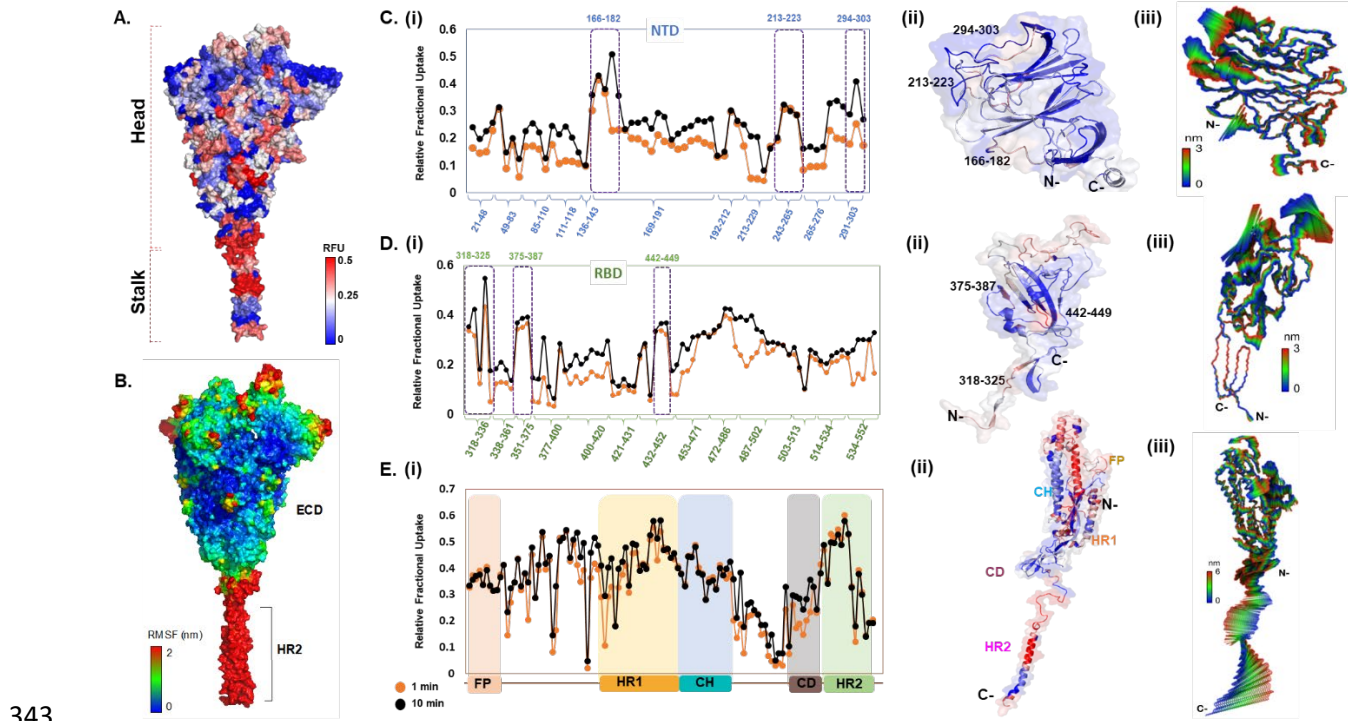


325

326 **Figure 1: Structure and domain organization of trimeric S protein showing steps in the virus-**
327 **host entry initiated by S recognition and binding to ACE2 receptor**

328 **A.** Prefusion S protein trimer in closed conformational state, with monomers shown in yellow,
329 green and pink. S protein construct (1-1245) used in this study showing head, stalk and
330 transmembrane (TM) segments as generated by integrative modeling. The S1/S2 and S2' cleavage
331 sites are in red. Proteolytic processing (Furin) of S protein generates S1 and S2 subunits. **B.**
332 Schematic of viral entry into host cell mediated by S:ACE2 interactions as previously outlined(28):
333 (i) Intrinsic dynamics of pre-fusion S protein trimer decorating SARS-CoV-2 and host ACE2
334 dimeric structure showing sweeping motions of S protein and ACE2 to facilitate S:ACE2
335 recognition. (ii) In the open conformation (S_{open}), RBD adopts an 'up' orientation to recognize and
336 bind the host membrane-bound ACE2 receptor (PDB: 1R42). ACE2 binding induces
337 conformational changes promoting Furin (red) proteolysis at the S1/S2 cleavage site (red arrows,
338 leading to dissociation of S1 and S2 subunits, mechanism of which is unknown. (iii) The residual
339 ACE2-bound S1 subunit stably bound to ACE2 and S2 subunits dissociate (iv) Conformational

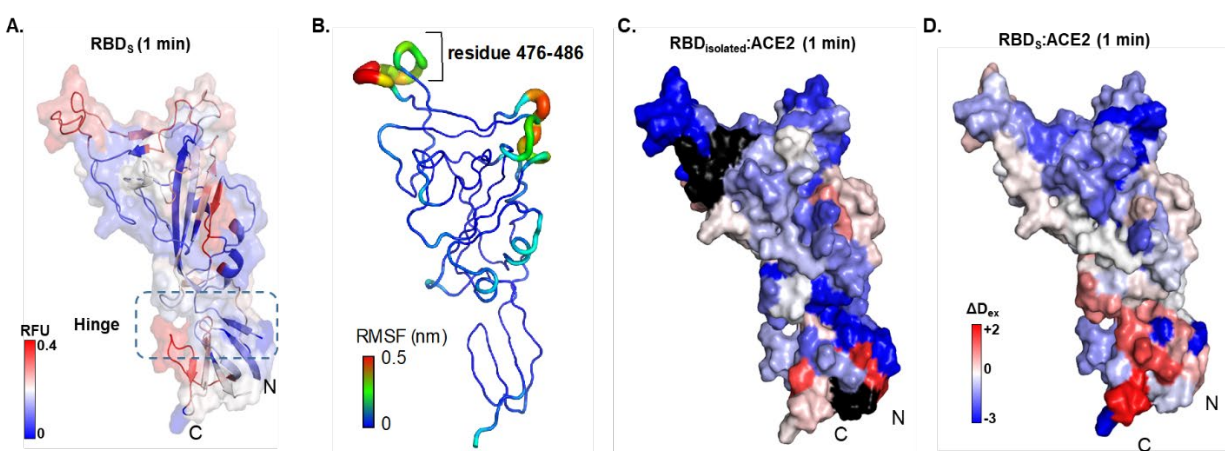
340 changes in the separated S2 subunit promote formation of an extended helical fusogenic
 341 intermediate (PDB ID: 6M3W),(17) for fusion into the host cell membrane, membrane fusion and
 342 viral entry into the host cell.(11)



344 **Figure 2: Deuterium exchange heat map and MD simulations reveal domain-specific**
 345 **conformational dynamics of pre-fusion S protein trimer.**

346 **A.** Deuterium exchange heat map (shades of blue (low exchange) and red (high exchange)) of S
 347 protein (residues 1-1208) at t = 1 min deuterium exchange mapped onto structure of S protein. **B.**
 348 Per-residue root mean square fluctuations (RMSF) of the S protein (without TM domain) mapped
 349 on to the surface of the S trimer. Deuterium exchange based dynamics across N-terminal domain
 350 **(C)**, RBD **(D)**, and the S2 subunit **(E)**. (i) Relative fractional deuterium uptake (RFU) plots of
 351 NTD, RBD and the S2 subunit at 1 min (orange) and 10 min (black) deuterium exchange times is
 352 shown, with pepsin digest fragments displayed from N to C-terminus (X-axis), (see Fig. S2, Table

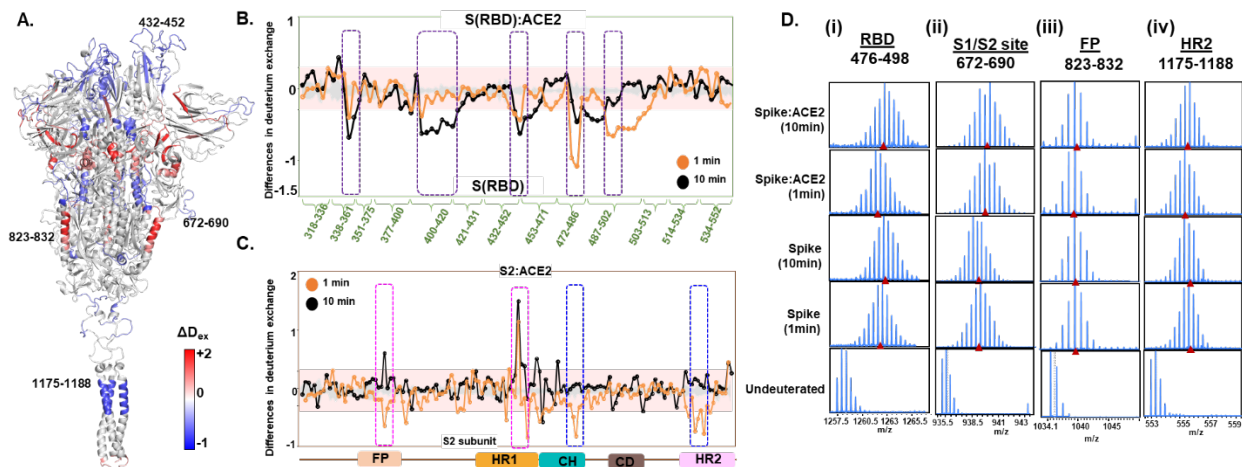
353 S1). (ii) Close-up of the structures of NTD (21-303), RBD (318-552) and the S2 subunit (810-
354 1208). Peptides spanning NTD-RBD interaction sites (166-182, 213-223, 294-303, 318-325, 375-
355 387 and 442-449) showing relatively high deuterium exchange at $t=1$ min are highlighted. (iii)
356 The first principal motion and RMSF values of backbone atoms on the NTD, RBD and the S2
357 subunits. Residues with high RMSF are labelled. Different domains (FP, HR1, CH, CD, HR2)
358 showing domain-specific RFU changes are labeled.
359



360

361 **Figure 3. Map of RBD_{isolated}:ACE2 interactions**

362 (A) Relative fractional deuterium uptake values at $t = 1$ min for RBD (314-547) of S protein
363 (RBD_s) mapped on to the structure of RBD extracted from S protein model (see Table S2). High
364 and low exchanging regions are represented as shown in key, and regions with no coverage are
365 shown in black. (B) The RMSF values of backbone atoms on the RBD showing residues with high
366 RMSF (476-486), as per key. Differences in deuterium exchanged between RBD_{isolated}:ACE2
367 complex and free RBD_{isolated} (C) and RBD_{spike}:ACE with free RBD_{spike} (D) at 1min of deuterium
368 labelling are mapped on to the structure of RBD. Protection from deuterium uptake and increases
369 in exchange are indicated in blue and red respectively. Regions with no coverage are in black.



370

371 **Figure 4: ACE2 interaction induce large scale allosteric changes across S protein**

372 (A) Differences in deuterium exchange (ΔD_{ex}) ($t = 1$ min) in S protein upon binding ACE2 showing
 373 decreased (blue) and increased (red) deuterium exchange, mapped onto structure of S protein.
 374 These differences in deuterium exchange for peptides from (B) RBD and S2 subunit (C) for pepsin
 375 digest fragments (X-axis) are shown. Difference cutoff ± 0.3 D is the deuterium exchange
 376 significance threshold indicated by pink shaded box with standard error values in gray. Positive
 377 differences (>0.3 D) denote increased deuterium exchange and negative differences (<-0.3 D)
 378 denote decreased deuterium exchange in S protein bound to ACE2. (B) Peptides spanning residues
 379 interacting with ACE2 are in purple. (C) Peptides spanning fusion peptide (FP) and HR1 are
 380 highlighted in pink boxes, while peptides spanning central helix (CH) and heptad repeat 2 (HR2)
 381 are in blue. D. Stacked mass spectra with isotopic envelopes after deuterium exchange ($t = 1, 10$
 382 min) for select peptides from (i) RBD (residues 476-498), (ii) S1/S2 cleavage site (residues 672-
 383 690), (iii) fusion peptide (residues 823-832) and (iv) HR2 (residues 1175-1188) are shown for the
 384 S protein and S:ACE2 complex. Mass spectra of the equivalent undeuterated peptide are shown
 385 for reference. The centroid masses are indicated by red arrow-heads.

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298

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312 Visualization – P.V.R., N.K.T., F.S.; Writing - original draft – P.V.R., N.K.T., G.S.A., F.S., P.J.B.;
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316

317 **SUPPLEMENTARY MATERIALS**

318 Materials and Methods

319 Figs. S1 to S8

320 Tables S1 to S3

321 References (29-55)

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Supplementary Materials for

SARS-CoV-2 S protein ACE2 interaction reveals novel allosteric targets

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This PDF file includes:

Materials and Methods
References (29-55)
Figs. S1 to S8

Other Supplementary Materials for this manuscript include the following:

Captions for Data S1 to S3

31 **Materials and Methods**

32 Materials

33 Mass Spectrometry grade acetonitrile, formic acid and water were from Fisher Scientific
34 (Waltham, MA); Deuterium oxide was from Cambridge Isotope Laboratories (Tewksbury, MA).
35 All reagents and chemicals were research grade or higher and obtained from Merck-Sigma-Aldrich
36 (St. Louis, MO).

37

38 Methods

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40 **Transient expression and purification of recombinant SARS-CoV2 Spike, RBD and ACE2** 41 **receptor**

42 A near full-length Spike (S) protein, excluding transmembrane domain and cytoplasmic
43 tail, of SARS-CoV-2 (1-1208; Wuhan-Hu-1; GenBank: QHD43416.1) was codon optimized for
44 mammalian cell expression and cloned into pTT5 expression vector (National Research Council
45 Canada, NRCC) with a twin strep tag at the C-terminus (Twist Biosciences). This double mutant
46 Spike construct was generated by mutating RRAR (682-685) into GSAS and residues KV (986-
47 987) into PP. A gene encoding SARS-CoV-2-RBD (319-591 of SARS-CoV-2 Spike) (BioBasic)
48 was cloned into the expression vector pHLmMBP-10 (Addgene) following the N-terminal His and
49 mMBP tag. A gene encoding human ACE2 (residues 21-597; GenBank: AB046569.1) fused to a
50 C-terminal Fc tag (Biobasic) was cloned into vector pHL-sec (Addgene) between the signal
51 peptide and c-terminal His tag. SARS-CoV-2- Spike constructs were expressed in HEK293-6E
52 (NRCC) using polyethylenimine (PEI) as the transfection reagent while the isolated RBD
53 ('RBD_{isolated}') and ACE2 constructs were expressed in Expi293F using the Expi293 System
54 (Thermo Fisher). Culture supernatant was harvested on day 7 for HEK293-6E expression and day
55 5 for Expi293F expression. Spike proteins were affinity purified using Strep-Tactin®XT column
56 (IBA). RBD protein was affinity purified using cOmplete™ His-Tag Purification column (Merck).
57 ACE2 receptor was affinity purified using HiTrap® MabSelect™ SuRe™ column (GE
58 Healthcare). Purified proteins were concentrated and buffer exchanged into PBS using VivaSpin
59 (Sartorius) and the purity was assessed by denaturing polyacrylamide gel electrophoresis (Fig.
60 S1A).

61 **Deuterium labelling and quench conditions**

62 Recombinant purified S protein (8 μ M), ACE2 receptor (52 μ M) and RBD (67 μ M)
63 solubilized in phosphate buffer (PBS, pH 7.4) were incubated at 37°C in PBS buffer reconstituted
64 in D₂O (99.90%) resulting in a final D₂O concentration of 90%. S:ACE2 and RBD:ACE2
65 complexes (K_D of ~15 nM and ~150 nM, respectively)(29) were pre-incubated at 37°C for 30 min
66 in a molar ratio of 1:1 to achieve >90% binding prior to each hydrogen-deuterium exchange
67 reaction. Deuterium labeling was performed for 1 min, 10 min and 100 min for isolated construct
68 of RBD, free ACE2, and RBD_{isolated}:ACE2 complex. For isolated S protein and S:ACE2 complex

69 1 min and 10 min labelling timescales were used. Pre-chilled quench solution 1.5 M GnHCl and
70 0.25 M Tris(2-carboxyethyl) phosphine-hydrochloride (TCEP-HCl) was added to deuterium
71 exchange reaction mixture to lower the pH_{read} to ~ 2.5 and lower temperature to $\sim 4^\circ\text{C}$. Next, the
72 quenched reaction was incubated at 4°C on ice for 1 min followed by pepsin digestion.

73 **Mass Spectrometry and peptide identification**

74 ~ 100 pmol quenched samples were injected onto chilled nanoUPLC HDX sample manager
75 (Waters, Milford, MA). The injected samples were subjected to online digestion using
76 immobilized Waters Enzymate BEH pepsin column (2.1×30 mm) in 0.1% formic acid in water
77 at $100 \mu\text{l}/\text{min}$. Simultaneously, the proteolyzed peptides were trapped in a 2.1×5 mm C18 trap
78 (ACQUITY BEH C18 VanGuard Pre-column, $1.7 \mu\text{m}$, Waters, Milford, MA). Following pepsin
79 digestion, the proteolyzed peptides were eluted using acetonitrile gradient of 8 to 40 % in 0.1 %
80 formic acid at a flow rate of $40 \mu\text{l min}^{-1}$ into reverse phase column (ACQUITY UPLC BEH C18
81 Column, 1.0×100 mm, $1.7 \mu\text{m}$, Waters) pumped by nanoACQUITY Binary Solvent Manager
82 (Waters, Milford, MA). Electrospray ionization mode was used to ionize peptides sprayed onto
83 SYNAPT G2-Si mass spectrometer (Waters, Milford, MA) acquired in HDMS^{E} mode of detection
84 and measurement. A flow rate of $5 \mu\text{l}/\text{min}$ was used to inject $200 \text{ fmol } \mu\text{l}^{-1}$ of $[\text{Glu}^1]$ -fibrinopeptide
85 B ($[\text{Glu}^1]$ -Fib) into mass spectrometer for lockspray correction.

86 Undeuterated protein samples were used to identify sequences from mass spectra data (in
87 HDMS^{E} mode) using Protein Lynx Global Server (PLGS) v3.0. Peptide identification search was
88 performed against a separate sequence database of each protein sequence along with its respective
89 affinity purification tag sequences. In the PLGS search parameters, i) no specific protease and ii)
90 no variable N-linked glycosylation modification options were selected for sequence identification.
91 The identified peptides were further filtered using a minimum intensity cutoff of 2500 for product
92 and precursor ions, minimum products per amino acids of 0.2 and a precursor ion mass tolerance
93 of <10 ppm using DynamX v.3.0 (Waters, Milford, MA) and tested for pepsin cleavage
94 specificity.⁽³⁰⁾ Peptides independently identified under the specified condition and present in at
95 least in two out of three undeuterated samples were retained for HDXMS analysis. S protein
96 contains 22 variable glycosylation sites⁽³¹⁾ out of which we identified peptides spanning 12
97 glycosylation sites in our sample (Fig. S2). For ACE2, we obtained 4 peptides overlapping the
98 glycosylation sites (Fig S7). Relative fractional deuterium uptake (RFU) is the ratio of number of
99 deuterons exchanged to the total number of exchangeable amides of the peptide. Centroid masses
100 of undeuterated reference spectra were subtracted from equivalent spectra of peptides showing
101 deuterium exchange to calculate the average deuterons exchanged with time for each peptide.
102 Deuterium exchange plots, relative deuterium exchange and difference plots were obtained from
103 DynamX 3.0. N-terminus and prolines were excluded for estimation of exchangeable amides per
104 peptide.⁽³²⁾ All deuterium exchange experiments were performed in triplicate and reported values
105 are not corrected for deuterium back exchange.

106

107 **Modelling and molecular dynamics (MD) simulations**

108 An integrative model of full-length SARS-CoV-2 S protein was built using Modeller v9.21.(33)
109 The cryo-EM structure of pre-fusion S ectodomain in the open conformation (PDB: 6VSB)(29)
110 was used as the template for the ectodomain (ECD) with missing loops on the NTD modelled
111 based on SARS S NTD crystal structure (PDB: 5X4S).(34) The NMR structure of SARS S HR2
112 domain (PDB: 2FXP)(35) was used as the template for the HR2 domain, while the TM domain
113 was modelled using the NMR structure of the HIV-1 gp-41 TM domain (PDB: 5JYN)(36). Ten
114 models were built and subjected to stereochemical assessment using the discreet optimized protein
115 energy (DOPE) score(37) and Ramachandran analysis.(38) The model with the lowest DOPE score
116 and the smallest number of Ramachandran outliers was chosen. Palmitoylation was added to three
117 cysteine residues (C1236, C1240 and C1243) on the CT domain based on a study showing its
118 importance in SARS S protein function.(39) The S protein model was then embedded into a model
119 membrane representing the endoplasmic reticulum-Golgi intermediate compartment
120 (ERGIC),(40) where coronaviruses are known to assemble in a bud form.(41, 42) The ERGIC
121 model membrane was built using CHARMM-GUI Membrane Builder.(43) All-atom MD
122 simulation was performed for 200 ns using GROMACS 2018(44) and the CHARMM36 force
123 field.(45) The systems were solvated with TIP3P water molecules and 0.15 M NaCl salt.
124 Minimization and equilibration were performed following standard CHARMM-GUI
125 protocols.(46) Temperature was maintained at 310 K using the Nosé-Hoover thermostat(47, 48)
126 and the pressure was maintained at 1 atm using the Parrinello-Rahman barostat.(49) Electrostatics
127 were calculated using the smooth particle mesh Ewald (PME) method(50) with a real space cut-
128 off of 1.2 nm and the van der Waals were truncated at 1.2 nm with force switch smoothing between
129 1.0 to 1.2 nm. Constraints were applied to covalent bonds with hydrogen atoms using the LINCS
130 algorithm(51) and a 2 fs integration time step was employed. For simulations of the ACE2
131 receptor, the cryo-EM structure of ACE2-B⁰AT1 complex in the open conformation (PDB:
132 6M1D)(52) was used. The ACE2-B⁰AT1 complex was embedded into a model membrane
133 representing the epithelial cell membrane.(53, 54) All-atom MD simulation was performed for 200
134 ns using the protocols described above. Principal component analysis (PCA) and root means
135 square fluctuation (RMSF) analyses were performed using GROMACS, and simulations were
136 visualized in VMD.(55)

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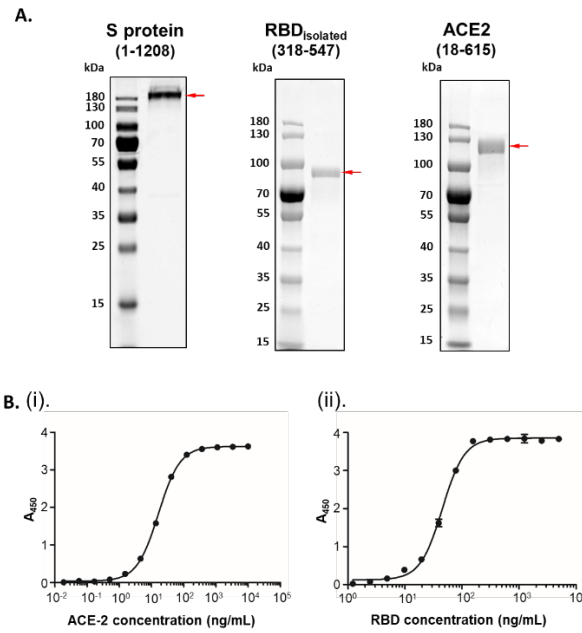
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199 **Supplementary Figures**

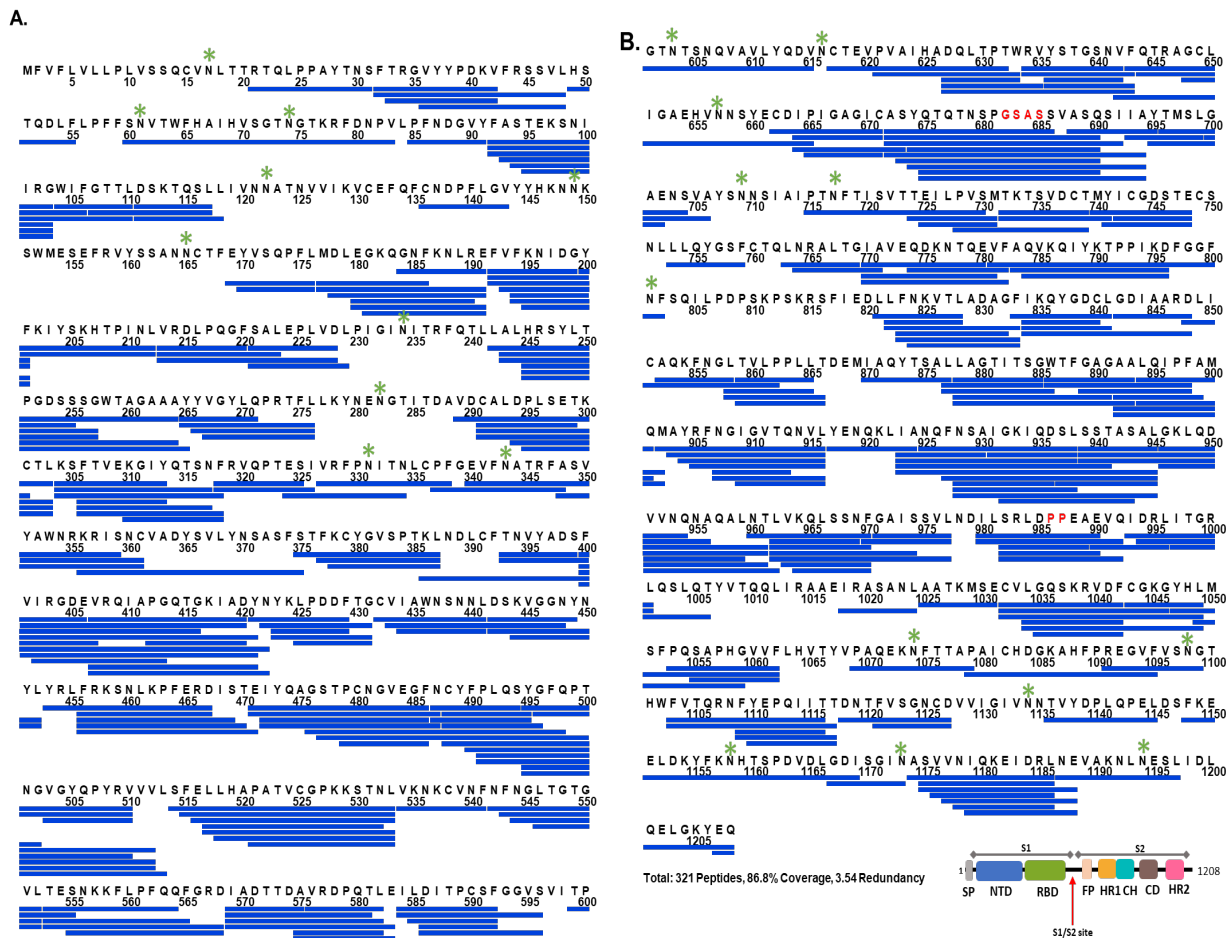
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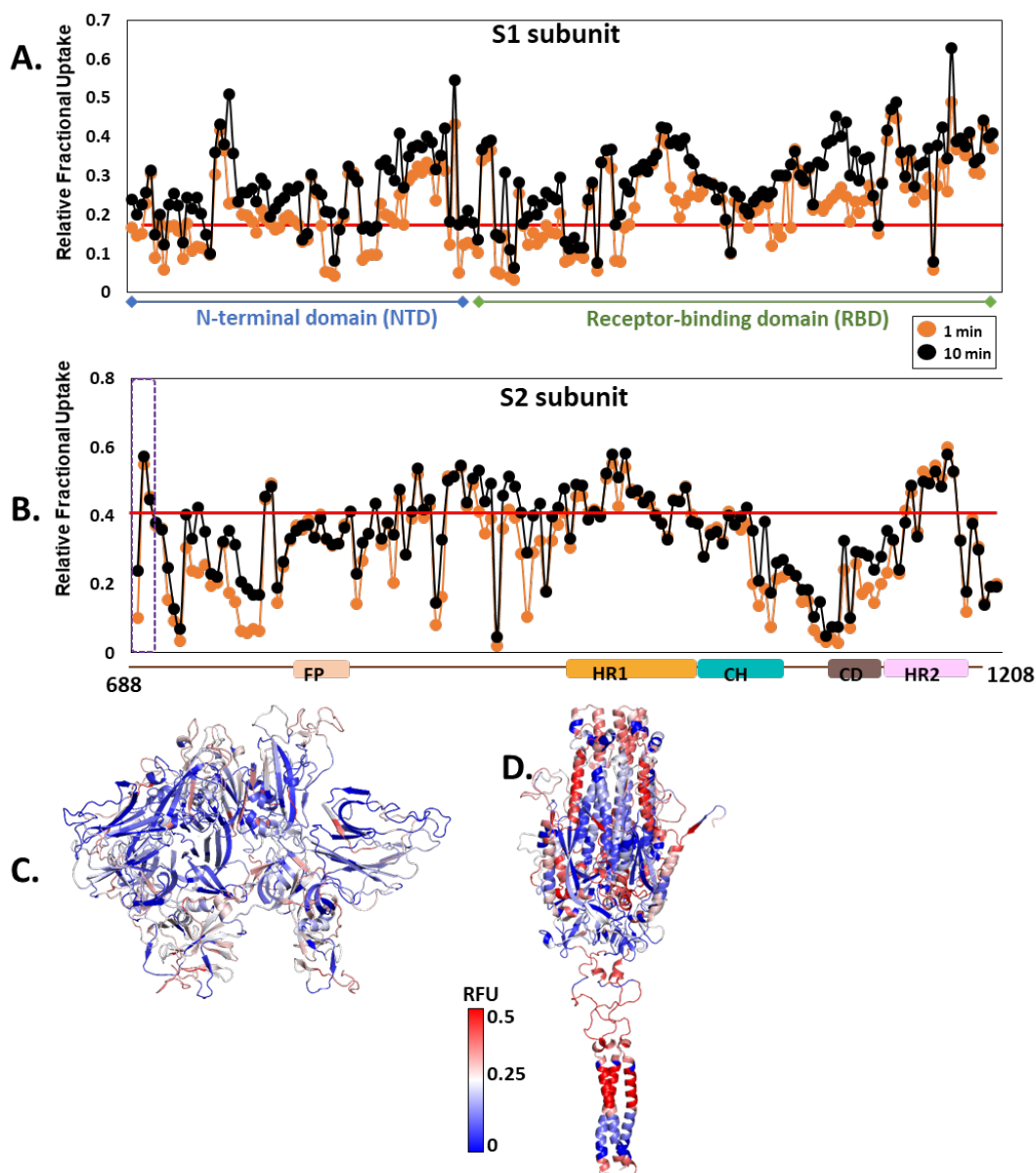
202 **Figure S1:**

203 **Homogeneity of protein samples.** (A) Images of denaturing polyacrylamide gel electrophoresis
204 of purified proteins of the S protein (mutant), isolated RBD and ACE2 are shown, and their
205 molecular sizes are highlighted with red arrow, alongside protein standards. Domain organization
206 is shown for reference. (B) Interactions between ACE2 and RBD represented by the binding
207 curves.

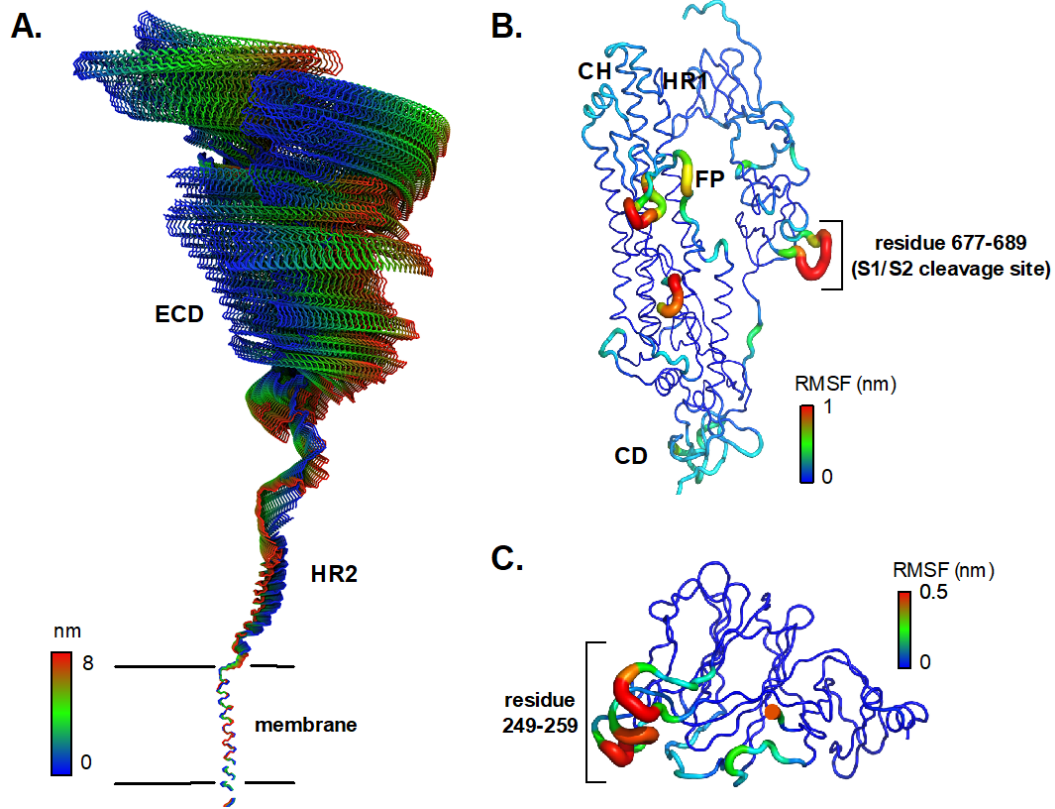


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210 **Figure S2:**
211 **Primary sequence coverage map of pepsin proteolysed peptides of the S protein.** Coverage
212 map showing 321 peptides spanning 87% of the S protein: (A) 1 – 600 and (B) 601 – 1208, with
213 the mutations highlighted in red. Glycosylation sites are indicated by asterisks (*) and peptide
214 coverage for C-terminal twin strep-tag is not shown. The domain organization for S protein
215 construct 1-1208 is shown.
216



217
218 **Figure S3:**
219 **Time dependent changes in deuterium exchange for free S protein.** Deuterium uptake of each
220 pepsin proteolysed peptide listed from N-to C-terminus (X-axis) spanning S1 subunit (A) and
221 S2 subunit (B) at deuterium labelling times 1 min and 10 min are represented as relative fractional
222 uptake (RFU, Y-axis) values. Red line indicates the average RFU value. RFU values at 1 min of
223 deuterium labelling time mapped on to the structures of the S1 (C) and S2 (D) subunits. High and
224 low exchanging regions are as per key.
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Figure S4:

228

Dynamics of the S protein trimer from all-atom MD simulation. (A) The first principal motion

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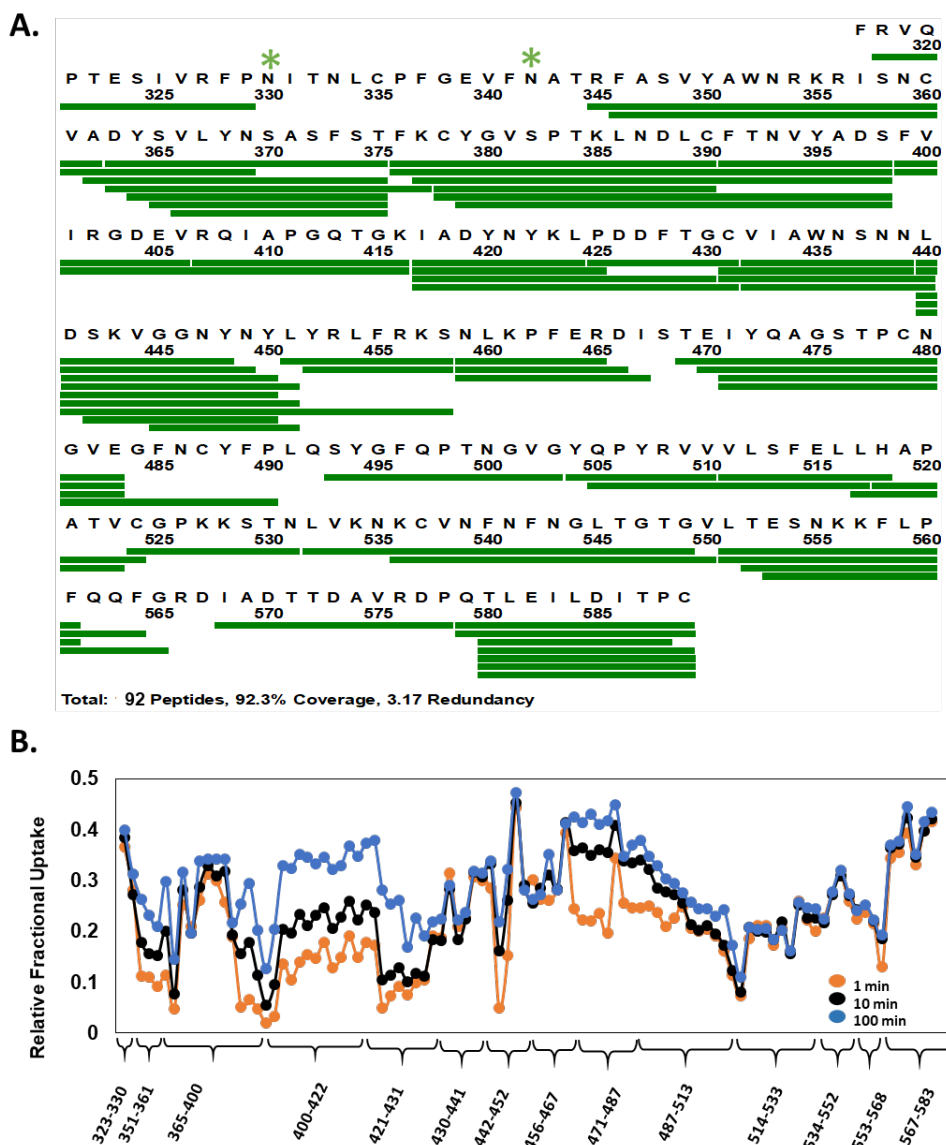
of all backbone atoms for the full-length S protein during all-atom MD simulations as determined

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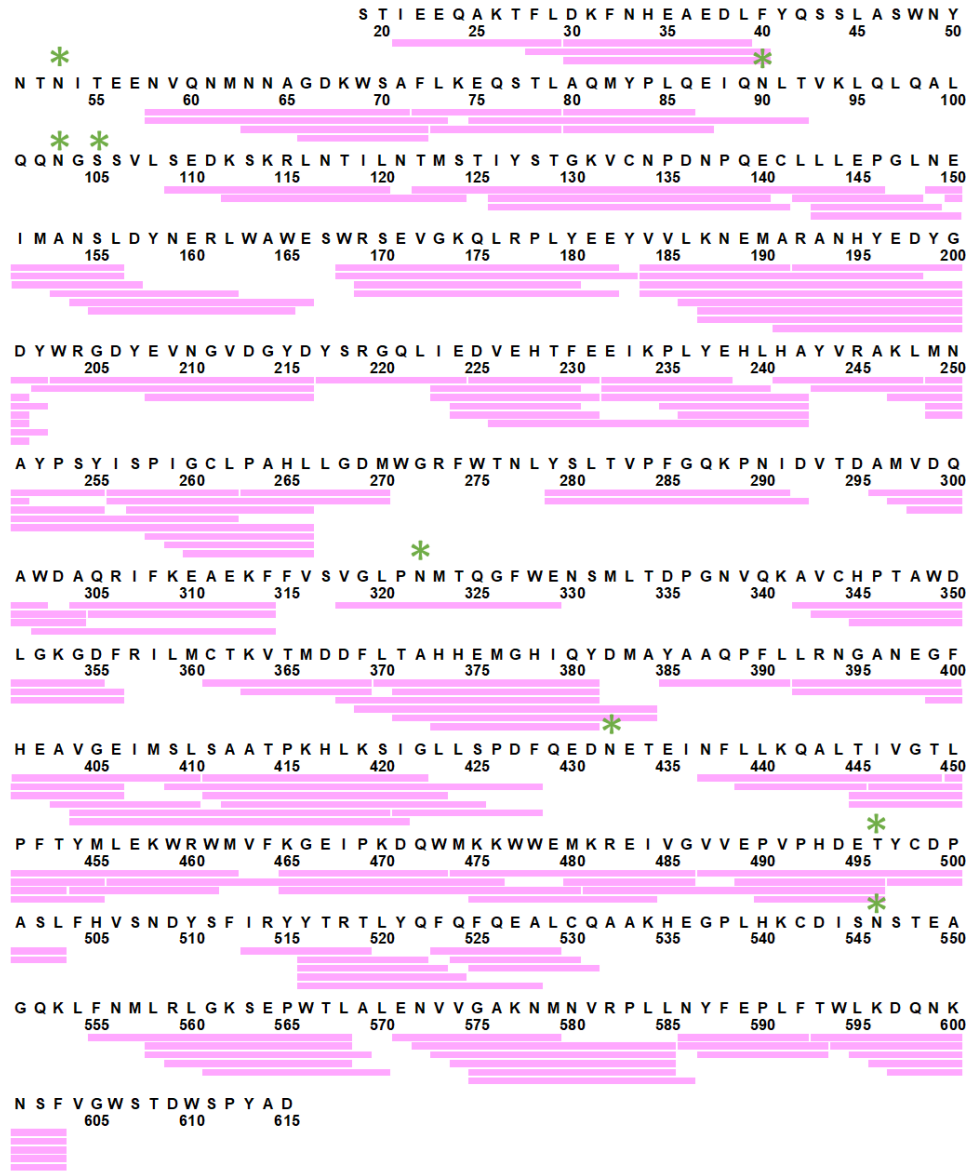
by principal component analysis (PCA). (B-C) RMSF values of backbone atoms on the S2 subunits

231

and NTD. Residues with high RMSF are labelled.



232
 233 **Figure S5:**
 234 **Primary sequence coverage and deuterium exchange profile of RBD_{isolated}.** (A) Coverage map
 235 showing 92 peptides (green bar) spanning ~92% sequence of MBP-RBD_{isolated} (318-589) fusion
 236 protein. N-terminal maltose-binding protein (MBP) affinity-tag is not shown. Glycosylation sites
 237 are indicated by green asterisk. (B) RFU plot of pepsin proteolyzed peptides of RBD_{isolated} listed
 238 N-to C-terminus for deuterium labelling times as per key.



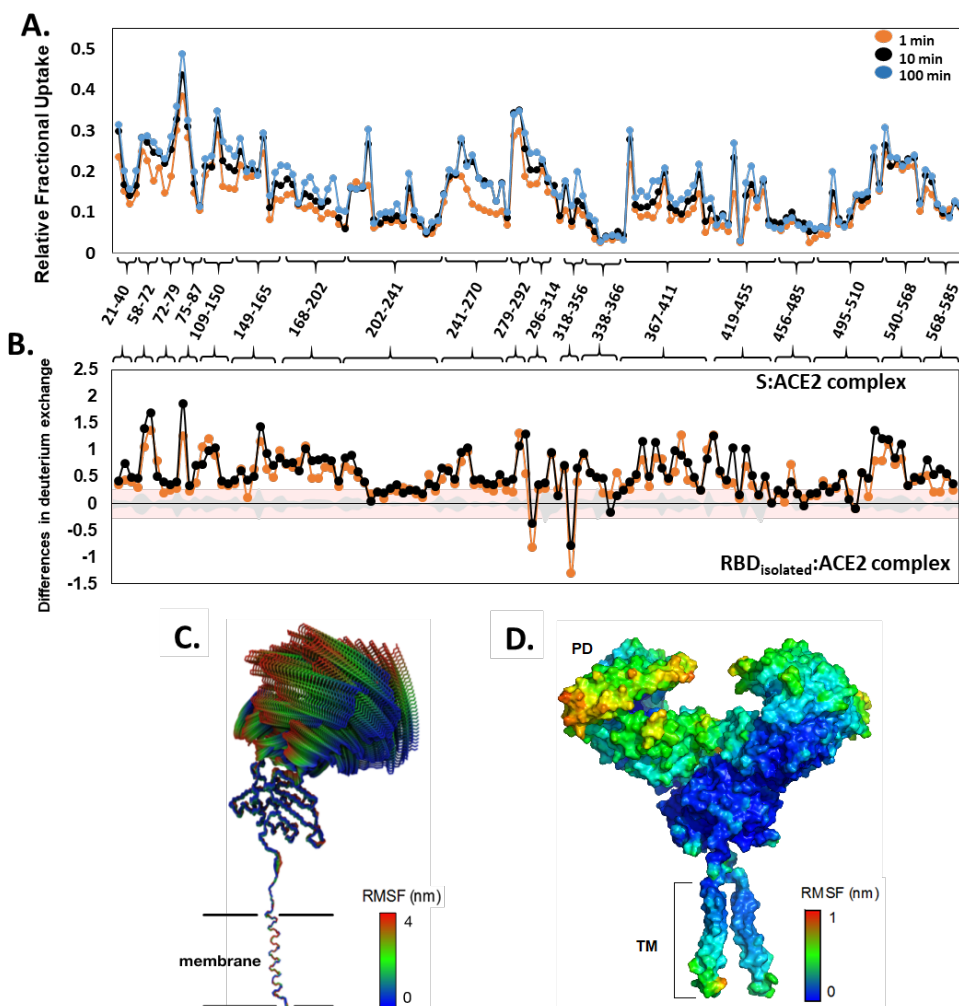
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241 **Figure S6: Pepsin digest map and sequence coverage ACE2.**

242 (A) Coverage map showing 140 peptides (pink horizontal bars) covering ~80% sequence of ACE2

243 (18-615). Sequence of FC-tag is not shown. Glycosylation sites are represented by green asterisk.



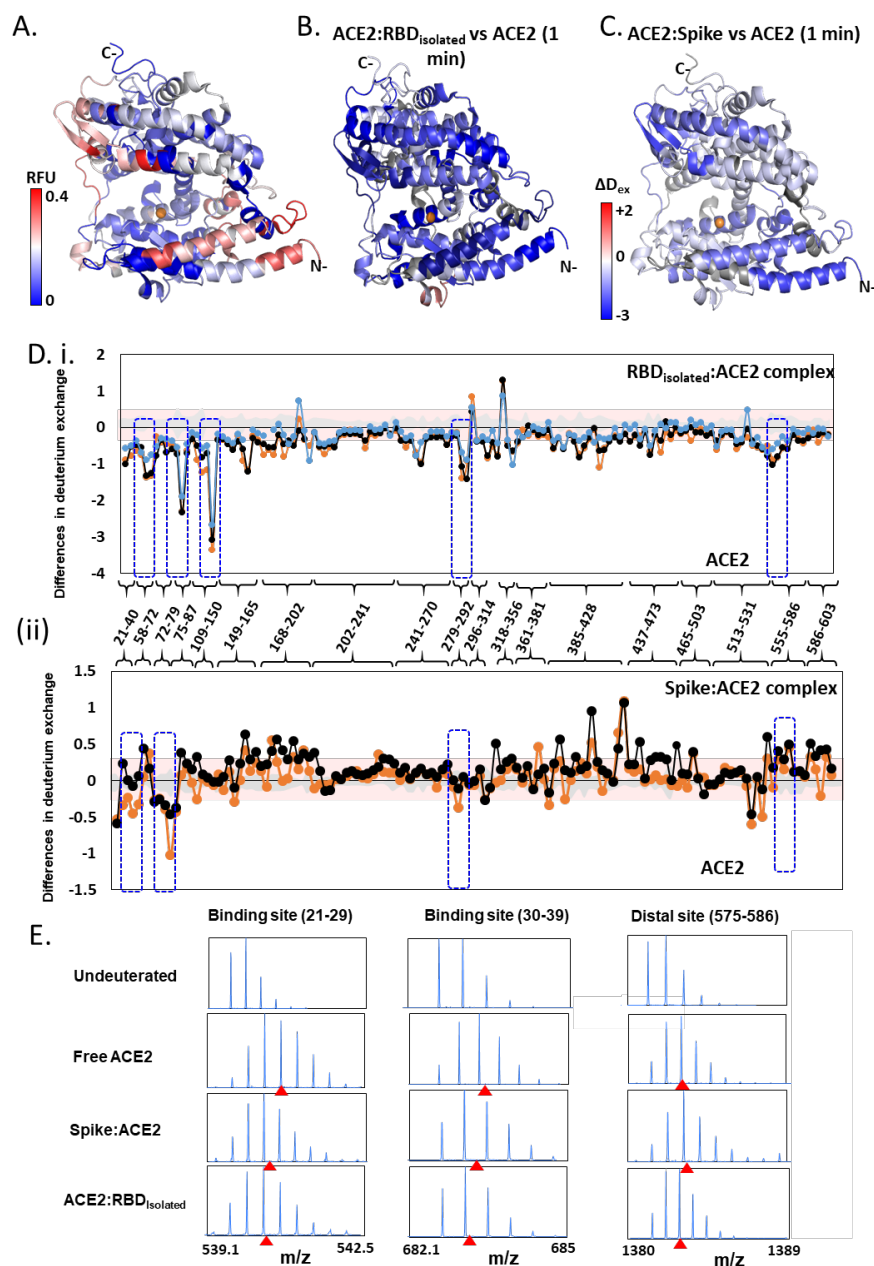
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246 **Figure S7: Deuterium uptake profile for ACE2 receptor and all-atom MD simulation of the**
247 **ACE2-B0AT1 complex.**

248 (A) RFU values of pepsin proteolysed peptides listed in N-to C-terminus of ACE2 (peptide 18-
249 615) for deuterium labelling times are shown. (B) Differences in deuterium exchange (Y-axis) of
250 ACE2 peptides listed from N-to C-terminus (X-axis) between S:ACE2 complex and
251 RBD_{isolated}:ACE2 complex. Deuterium exchange significance threshold of ± 0.3 D is indicated
252 in red and standard errors in gray. (C) The first principal motion of the all backbone atoms of the
253 ACE2 monomer as determined by PCA. (D) The RMSF values of the ACE2 receptor mapped onto
254 the surface of the ACE2.

255



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257

258 **Figure S8: Effect of RBD_{isolated} and RBDs complexes on ACE2 dynamics.**

259 (A) Structure of extracellular domain of ACE2 receptor (PDB ID: 1R42) depicting the RFU at t
260 $= 1$ min. (B) Differences in deuterium exchange of RBD_{isolated}:ACE2 complex and free ACE2 at t
261 $= 1$ min is mapped onto the structure of ACE2, predominantly showing decreased deuterium
262 exchange in ACE2 (shades of blue). (C) Heat map of differences in deuterium exchange ($t = 1$
263 min) of S:ACE2 complex and free ACE2. (D) Plot showing differences in deuterium exchange
264 between ACE2 and complexes with RBD (i) and S (ii) at different labeling times. Pepsin digest
265 fragments are indicated by their residue numbers. Cutoff ± 0.3 D is the deuterium exchange
266 significance threshold, indicated by pink shaded box, and standard errors are in gray. Positive

267 differences denote increased deuterium exchange in (i) RBD:ACE2 or (ii) S:ACE2 compared to
268 free ACE2, while negative differences denote decreased deuterium exchange. Peptides spanning
269 the sites of interaction with RBD and two distal sites (278-292, 574-585) are highlighted. (E)
270 Stacked mass spectra showing isotopic distribution for select peptides spanning the binding sites
271 (21-29, 30-39) and a distal allosteric site (575-586) for ACE2, S:ACE2 and RBDisolated:ACE2
272 are shown at 1 min deuterium labeling time. Centroids indicated by red arrow-heads.
273

274 **Data S1 to S3**

275 **Table S1:**

276 Relative Fraction uptake values at various deuterium labeling times for Spike and S:ACE2
277 complex
278

279 **Table S2:**

280 Relative Fractional Uptake values at various deuterium labeling times for free and ACE2-bound
281 RBD (isolated)
282

283 **Table S3:**

284 Relative Fractional Uptake values at various deuterium labeling times for free ACE2 and its
285 complexes with isolated RBD and Spike protein
286