

1 **Opening the side exit pores of ClpP by lowering the pH of proteolytic**
2 **chamber coupled with substrate hydrolysis**

3
4 Running Title: A product release mechanism of ClpP

5
6 Leehyeon Kim^{1,a}, Byung-Gil Lee^{1,a,b}, Min Kyung Kim^{1,b}, Do Hoon Kwon^{1,c}, Hyunmin Kim²,
7 Heike Brötz-Oesterhelt^{3,4}, Soung-Hun Roh^{2,*} and Hyun Kyu Song^{1,*}

8
9 ¹*Department of Life Sciences, Korea University, Seoul 02841, South Korea*

10 ²*School of Biological Sciences, Institute of Molecular Biology and Genetics, Seoul National*
11 *University, Seoul 08826, South Korea*

12 ³*Interfaculty Institute of Microbiology and Infection Medicine, Department of Microbial*
13 *Bioactive Compounds, University of Tuebingen, Tuebingen, Germany*

14 ⁴*Cluster of Excellence Controlling Microbes to Fight Infection, University of Tuebingen,*
15 *Tuebingen, Germany*

16

17 ^aThese authors contributed equally

18 ^bPresent address: Byung-Gil Lee and Min Kyung Kim, MRC Laboratory of Molecular
19 Biology, Cambridge, UK.

20 ^cPresent address: Do Hoon Kwon, Department of Biochemistry, Duke University School of
21 Medicine, Durham, NC, USA

22

23 *Correspondence

24

25 Hyun Kyu Song, Department of Life Sciences, Korea University, 145 Anam-ro, Seongbuk-gu,
26 Seoul 02841, South Korea; E-mail: hksong@korea.ac.kr

27

28 Soung-Hun Roh, School of Biological Sciences, Seoul National University, 1 Gwanak-ro,
29 Gwanak-gu, Seoul 08826, South Korea; E-mail: shroh@snu.ac.kr

30

31 **Abstract**

32 The ClpP serine peptidase is a tetradecameric degradation machine involved in many
33 physiological processes. It becomes a competent ATP-dependent protease with Clp-ATPases.
34 Small chemical compounds, acyldepsipeptides (ADEPs), are known to cause dysregulation
35 and activation of ClpP without ATPases, and have potential as novel antibiotics. Previously,
36 structural studies of ClpP from various species revealed the structural details, conformational
37 changes, and activation mechanism. Although product release by the side exit pores has been
38 proposed, the detailed driving force for product release remains elusive. Here, we report
39 crystal structures of ClpP from *Bacillus subtilis* (BsClpP) in unforeseen ADEP-bound states.
40 Cryo-electron microscopy structures revealed various conformational states at different pH
41 conditions. To understand the conformational change for product release, we investigated the
42 relationship between substrate hydrolysis and the pH lowering process. Our data, together
43 with previous findings, provide insight into the molecular mechanism of product release by
44 ClpP self-compartmentalizing protease.

45

46

47

48 **Keywords:** acyldepsipeptide; asymmetric binding; ClpP; conformational change; cryo-EM;
49 crystal; pH drop; protein degradation; proteolytic chamber

50

51

52 **Main**

53 Energy-dependent proteases are molecular machines conserved in all kingdoms of life^{1,2}.
54 They play an essential role in protein quality and quantity control by degrading misfolded,
55 damaged, short-lived or regulatory proteins using energy by ATP hydrolysis^{3,4}. Clp proteases
56 are an energy-dependent protease system in bacteria and mitochondria of eukaryotic cells⁵.
57 They contain two distinct functional components, a Clp-ATPase and the ClpP proteolytic core.
58 The energy-consuming hexameric AAA+ ATPase (ATPase associated with a variety of
59 cellular activities) is responsible for substrate selection, unfolding, and translocation into the
60 proteolytic core. Representatives include ClpA, ClpC, ClpE, and ClpX. The central
61 proteolytic machine ClpP is a barrel-like serine protease complex, composed of two stacked
62 heptameric rings that form an enclosed degradation chamber⁶. Both ClpP heptamers have a
63 central axial pore where the unfolded polypeptide chains are translocated.

64 The structures of ClpP from various species, from *E. coli* to humans, have been
65 solved, and information regarding folding, the axial entrance pore, and the catalytic triad of
66 ClpP is well defined⁶⁻¹³. The activation mechanism of ClpP by its Clp-ATPase partner was a
67 long-standing question in the field and partially revealed by structures of ClpP in complex
68 with acyldepsipeptides (ADEPs)^{11,14}, compounds that have antibiotic activity through
69 dysregulation of ClpP activity by mimicking the Clp-ATPase¹⁵⁻¹⁷. Very recently, more direct
70 evidence comes from the complex structures formed between the ClpX ATPase and ClpP
71 protease using cryo-electron microscopy (cryo-EM)¹⁸⁻²⁰. These studies showed how the
72 symmetry-mismatched ATPase activates the proteolytic machine by translocating substrate
73 molecules. The critical IGF/L (Ile-Gly-Phe in ClpX and Ile-Gly-Leu in ClpA) loops of the
74 Clp-ATPases bind to the hydrophobic pockets between the ClpP monomers, as shown in the
75 ADEP-bound structures. The asymmetric ClpAP complexes showed one or two empty IGL-

76 loop binding pockets during the engagement and disengagement cycle²¹, which had not been
77 clear in the previously determined structures of ClpP fully occupied with ADEP
78 compounds^{11,14,22}. The activation mechanism of ClpP by its cognate ATPases has now been
79 proposed. However, the detailed and coherent steps of ClpP motion, from substrate entry to
80 product release, are still elusive, although several conformational equilibria between active
81 and inactive states have been reported, such as pH-dependent switching, active site
82 perturbation, and regulation of the N-terminal loop²³⁻²⁶.

83 Once substrates reach the tetradecameric proteolytic chamber of ClpP, the degraded
84 products need to be eliminated for efficient processing. The equatorial pore for peptide
85 release was structurally revealed by the observation of major conformational changes of ClpP
86 within the same strains^{13,25,27,28}, corroborated by an elegant biophysical study using NMR²⁹.
87 Currently, several other factors have been proposed for the regulation of equatorial pore
88 opening^{23-26,30}. However, those studies could not fully explain the peptide product release
89 mechanism of ClpP coupled with its activators, AAA+ ATPases or ADEPs. Here, we present
90 the crystal and cryo-EM structures of ADEP-bound ClpP in various states and a plausible link
91 between a decrease in pH and peptide hydrolysis, which reveal conformational changes and
92 the peptide release mechanism of ClpP. These data, in combination with all available
93 structural information, show the complete processing of substrates, from recognition to
94 unfolding, translocation, degradation, and release.

95

96 **Results**

97 *Lowering the pH by the accumulation of hydrolyzed peptide products*

98 As described, there are significant conformational changes in the handle region of ClpP from

99 *Bacillus subtilis* (BsClpP), as shown in our previous studies^{11,27}, and those from other
100 species^{22-24,26,28}. It has been proposed that His145 interacting with the catalytic aspartate of an
101 adjacent protomer in *N. meningitidis* ClpP serves as a key determinant for a pH-dependent
102 conformational switch²³. However, the equivalent residue in BsClpP is alanine, and therefore,
103 there must be differences among species (Extended Data Fig. 1). In *M. tuberculosis* ClpP1P2,
104 peptide binding at the active site triggers the transition between active and inactive
105 conformations²⁴. Therefore, it is tempting to speculate that there must be a relationship
106 between pH change and peptide accumulation in the proteolytic chamber of ClpP.
107 Furthermore, there have been reports that the pH drop can be correlated with peptide bond
108 hydrolysis^{31,32}, and naturally, the numerous newly generated amino- and carboxy-terminal
109 groups contribute to the pH drop from neutral pH values. The average pI values of bacterial
110 proteomes are also slightly acidic³³. Therefore, we hypothesized that peptide bond hydrolysis
111 decreases the pH value of a protein solution. To test this hypothesis, we checked the pH
112 change during protein hydrolysis. The initial pH of 6.41 achieved using diluted PBS
113 (phosphate-buffered saline) with 80 μ M α -casein decreased to the terminal pH of 5.94 by the
114 addition of proteinase K. The drop of 0.47 pH units occurred very fast because of the high
115 enzymatic activity of proteinase K for the partially unfolded α -casein substrate (Fig. 1a and
116 Extended Data Fig. 2a). We observed a similar pH change with BsClpP protease in the
117 presence of ADEP1 (Fig. 1a). In contrast to monomeric proteinase K, the proteolytic activity
118 of BsClpP is lower, and thus, the pH drop was slower. Nonetheless, a substantial drop by 0.46
119 pH units was reached from the initial pH 6.58 to pH 6.12 after 300 min (Fig. 1a). The same
120 experiment performed with bovine serum albumin (BSA) showed an even stronger pH drop
121 of 0.76, from 6.80 to 6.04 (Fig. 1b). In the absence of a protease, the pH values were quite
122 stable (Fig. 1). In parallel to the pH measurement, protein degradation was followed by SDS-
123 PAGE (Extended Data Fig. 2). The pIs of intact α -casein and BSA are 4.91 and 5.32,

124 respectively. These values are derived from the summation of pK_a values of all side-chain
125 groups and amino- and carboxy-terminal groups. However, when proteases cleave numerous
126 peptide bonds in the proteins, many new α -amino and carboxylic groups are generated.
127 Subsequently, the pH value of the environment is mainly governed by the total summation of
128 all pK_a values of α -amino and carboxylic groups and is below 6.0, which is slightly acidic.
129 The cleaved peptide products must be highly concentrated in the proteolytic chamber of ClpP,
130 and therefore, the local pH of the handle region near the active site becomes acidic.
131 Consequently, we conclude that the conformation of BsClpP at low-pH conditions mimics the
132 state during peptide accumulation inside the proteolytic chamber.

133

134 *Asymmetric ADEP binding in crystal structures of the BsClpP-ADEP complex*

135 We solved two structures of BsClpP in complex with acyldepsipeptides (specifically ADEP2)
136 at 2.8 and 3.0 Å resolution (Fig. 2 and Supplementary Table 1). For molecular replacement,
137 previously compressed BsClpP (PDB ID: 3TT6) was used as a search model²⁷. Very
138 intriguingly, these two structures have two and five ADEP2 compounds, respectively, bound
139 to each heptameric ring (Fig. 2 and Extended Data Fig. 3), which is in stark contrast to the
140 previous 14 ADEP-bound BsClpP¹¹ as well as all other ADEP-ClpP complex structures of
141 from various organisms published to date^{22,34-36}. Our two new BsClpP tetradecamer structures
142 have a large difference in their degrees of compression as well as in their numbers of ADEP
143 molecules bound, four and ten (Fig. 2). Hereafter, 2ADEP and 5ADEP indicate 2 and 5
144 ADEPs for each heptameric ring (4 and 10 ADEPs for a BsClpP tetradecamer). Their axial
145 heights are approximately 86 Å (2 ADEPs) and 92 Å (5 ADEPs), and the state of BsClpP
146 bound to 4 ADEPs (2ADEP) has a similar height to the previous compressed apo-form
147 BsClpP structure²⁷. For consistency, the height was defined as the distance between the

148 outermost two atoms; therefore, it is slightly taller than that in previous reports, which is
149 usually the distance measured between the two most distant C α atoms. The height of BsClpP
150 in complex with 10 ADEPs (5ADEP) is in the middle of the extended and compressed
151 states²⁷, which is similar to the state called the ‘compact state’^{13,35,37}. Thus, these two
152 different states are referred to as ‘compressed’ for the 2ADEP structure and ‘compact’ for the
153 5ADEP structure. In both structures, ADEPs bind at the interface of two subunits in the order
154 of A-E-E-E-A-E-E (A: ADEP-bound and E: empty) in the compact state and A-A-A-E-A-A-E
155 in the compressed state (Fig. 2).

156

157 *Different compressions of BsClpP control the entrance and exit pores*

158 These two states differ in terms of the preservation of N-terminal residues in addition to
159 height. The N-terminal segments of compact 5ADEP are well structured and resolved,
160 whereas those of 2ADEP are disordered, so the pore sizes in the two states are markedly
161 different (Fig. 2). Due to the asymmetric binding of the activator ADEP, the entrance pore
162 showed distorted shapes in both states, and in particular, the compact state possessed even
163 more asymmetry, in that the diameter between empty subunits was nearly half that of ADEP-
164 bound subunits (Fig. 2b). More importantly, the pores in the lateral direction, known as the
165 product exit, were also different. This correlates with the flexibility or unwinding of the α 5-
166 helix, called the handle region^{13,27,28}. Since we obtained asymmetric ADEP-bound structures,
167 each monomer in different neighboring subunits was compared (Fig. 3 and Extended Data
168 Fig. 4). In both structures, each subunit has three different environments, and details of the
169 2ADEP and 5ADEP structures are different. In the orientation looking down from the
170 substrate entrance pore in the case of 2ADEP (Fig. 3a), there are three states: protomers
171 having ADEP on the right side (2 subunits), ADEP on the left side (2 subunits), and no

172 ADEP binding (3 subunits). The monomeric structures of protomers with ADEP bound at
173 either the right or the left sides are quite similar, whereas the unbound monomers are slightly
174 different, especially regarding the loop right before the handle region (Fig. 3b). The N-
175 terminal regions of all subunits are quite flexible and have an almost invisible electron
176 density; thus, the asymmetry of the entrance pore is only marginal (Fig. 2a).

177 In the same orientation of 5ADEP (Fig. 3d), there are also three different states:
178 ADEP on both sides (3 subunits), ADEP on the right side (2 subunits), and ADEP on the left
179 side (2 subunits). Therefore, the main difference between 2ADEP and 5ADEP is that 2ADEP
180 has 3 monomeric subunits with ADEP on neither side and 5ADEP has 3 monomeric subunits
181 with ADEPs on both sides. In the case of 5ADEP, the monomeric structures with ADEP
182 bound on both sides are quite similar (Fig. 3f), whereas the monomers with a single ADEP on
183 either the right or the left side are different in the loop right before the handle region and in
184 the N-terminal regions (Fig. 3e). The superposition of all subunits in 5ADEP shows that the
185 N-terminal regions and the $\alpha 5$ handle region of all subunits are very dynamic, and thus, the
186 asymmetry of the entrance pore, as well as the side pores, is augmented (Figs. 2b, 3e, and
187 Extended Data Fig. 4).

188

189 *Diverse ADEP-bound states found in cryo-EM structures*

190 Although the 2ADEP and 5ADEP states in crystals must be intermediate structures in the
191 reaction cycle of ClpP, we performed cryo-EM experiments at the pH of the crystallization
192 buffer to rule out the possibility of crystal packing artifacts. We obtained 2ADEP and 5ADEP
193 structures with pH values of 4.2 and 5.6, respectively. Unfortunately, at pH 5.6, BsClpP
194 formed an aggregate, probably due to its pI of 5.2; therefore, we collected cryo-EM data at
195 pH 6.5 and 4.2. From datasets of the BsClpP particles, we were able to reconstruct 4 different

196 structures of BsClpP at 3.1, 3.2, 3.4, and 3.4 Å maps (Extended Data Fig. 5 and
197 Supplementary Table 2). The initial electron micrographs showed heterogeneous ADEP-
198 bound states. The 1:1 mixture between BsClpP and ADEP1 at a pH of 6.5 showed that the
199 majority of the particles were apo forms (Fig. 4a and Extended Data Fig. 5a), whereas in the
200 1:3 mixture (i.e., three-fold surplus of ADEP1) at the same pH, the majority were 14 ADEP-
201 bound forms (Fig. 4b and Extended Data Fig. 5b). The apo structure at pH 6.5 is similar to
202 that of the previous extended structure, and the ADEP-bound structure showed a very clear
203 N-terminal region (Extended Data Fig. 6). In the electron micrographs of the 1:3 mixture of
204 BsClpP and ADEP at pH 4.2, both apo and all ADEP-bound BsClpPs were shown as
205 significant populations (Extended Data Fig. 5c); thus, we determined the two structures with
206 one dataset (Fig. 4c,d, and Supplementary Table 2). However, as shown in Extended Data Fig.
207 5, other complex states also existed, and we believe that the 2ADEP and 5ADEP found in
208 crystals are intermediates that are energetically stable in the conformational transition from
209 extended to compressed.

210 The 14 ADEP-bound BsClpP cryo-EM structures at the two different pH values, 6.5
211 and 4.2, are markedly different in dimensions (Fig. 4b,c). The structure at pH 6.5 shows an
212 extended conformation, with a well-ordered N-terminal region slightly tilted to the outward
213 position, whereas the 14 ADEP-bound structure at pH 4.2 is similar to the compact structure,
214 with a relatively ordered N-terminal region to the inward position, suggesting that the N-
215 terminal region might be involved in the substrate feeding process. The N-terminal region of
216 ClpP acts as a gate for controlling substrate access with Clp-ATPases as well as ADEP
217 activators^{26,35,38,39}. This ADEP-bound structure at pH 4.2 is classified as a compact
218 conformation based on its dimension, and it is very similar to 5ADEP (Fig. 2b). However, the
219 entrance pore is symmetric because all subunits are virtually identical (Fig. 4c). The apo
220 BsClpP structure at pH 4.2 is structurally similar to 2ADEP (Fig. 2a), although details, such

221 as the entrance pore and handle regions, are subtly different.

222

223 ***Structural changes in the proteolytic chamber***

224 To understand more details about the various structural states at different pH conditions, we
225 focused on the interior of the proteolytic chamber. Although key determinants for the
226 maintenance of the active extended conformation of ClpP, including the β -sheet in the handle
227 regions of two subunits from each heptameric ring and critical salt-bridge pairs (Asp169–
228 Arg170':Asp169'–Arg170) at the tip of handle region, have been extensively studied, the
229 charge property of the chamber has not been examined thoroughly. We analyzed the
230 electrostatic potential surface of all different structural states from the perspective of
231 hydrolyzed substrates (Fig. 5). In general, the surface shows negatively charged features in all
232 states, and it can be an environment in which the negatively charged products are
233 energetically unfavorable, although they are just slightly acidic due to the summation of the
234 pK_1 ($-\text{COOH}$), pK_2 ($-\text{NH}_3^+$), and pK_R ($-\text{R}$: side chain) of all peptide fragments, as we
235 described in an earlier section (Lowering the pH by the accumulation of hydrolyzed peptide
236 products). To release this strained state derived from product generation, a conformational
237 change must be triggered. Usually, the side chain of the histidine residue ($pK_R = 6.0$) is
238 thought to be a sensor for the pH-dependent transition. Previously, His145 in *N. meningitidis*
239 ClpP was proposed to be a switch regulating the pH-dependent conformational change and it
240 forms an inter-subunit H-bond with the catalytic Asp178' residue from a neighboring ClpP
241 protomer²³. However, the equivalent residues in ClpPs from *B. subtilis*, *M. tuberculosis*, and
242 *S. aureus* are alanine and glutamine, which are not conserved (Extended Data Fig. 1).
243 Assuming the conservation of the molecular motion of ClpP among all species, the key
244 switch residues must be strictly conserved. Therefore, we thought that another histidine

245 residue, and particularly the strictly conserved catalytic His122, might be a candidate
246 (Extended Data Fig. 1). The catalytic triad Ser97-His122-Asp171 is an active configuration in
247 the extended conformation (Fig. 5a,b), whereas the compact and compressed conformation
248 possesses an inactive configuration (Fig. 5c-f). Naturally, the imidazole ring of His122 is
249 protonated at low pH, and subsequently, the catalytic triad is severely distorted. In the
250 compact and compressed structure, the catalytic triads in the upper and lower heptameric
251 rings close, and the previously identified key residues do not participate in the maintenance
252 of the extended structure. The generation of exit side pores is coupled with the transition from
253 an extended state to a compact state (Fig. 5c,d), and the pores for peptide release are
254 gradually enlarged to a compressed state (Fig. 5e,f).

255

256 *Comparison with ClpXP structures*

257 Recently, a long-awaited structure of the symmetry-mismatched ATP-dependent Clp protease
258 has been revealed by several independent groups^{18-21,26}. The complexes are between a
259 hexameric Clp-ATPase, ClpX or ClpA, and heptameric ClpP, and most of the structures show
260 that six IGF/L-loops of Clp-ATPase bind to six out of seven binding pockets of ClpP with a
261 10~16° tilt angle of each molecular axis. When we compared the ClpP structures of the
262 ClpXP complex with those of our BsClpP structures, the cryo-EM structures of the extended
263 ClpP at pH 6.5 were similar. More specifically, the monomeric subunit is very similar, while
264 in the oligomeric state of ClpP, the size of the entrance pore is larger in the ADEP complex
265 than in the ClpX complex (Extended Data Fig. 7), showing that ClpX does not induce pore
266 widening of ClpP¹⁹. The ADEP-binding site overlaps IGF/L-loop binding with an empty site,
267 as shown in ClpXP and ClpAP complexes¹⁸⁻²¹; however, it has been reported that when only 5
268 IGF/L loops are involved in the interaction with ClpP, the Clp-ATPase conformation has a

269 higher tilting angle (Extended Data Fig. 8)²¹, and this so-called ‘disengaged conformation’ is
270 important for the reaction cycle of the Clp-ATPase. Therefore, we compared our 5ADEP
271 structure with the disengaged conformation. As shown in Extended Data Fig. 8c, the two
272 empty sites in the disengaged conformation of ClpAP are consecutive, whereas the two
273 empty sites in 5ADEP are not (Fig. 2b). Considering the differences in activator binding, the
274 movement and diameter of the entrance pore, and the fact that ADEP cannot actively unfold a
275 protein, the substrate feeding processes realized by two different activators must be different.

276

277 **Discussion**

278 The major species of the ATP-dependent complex formed between the Clp-ATPases and ClpP
279 has been reported to be a 2:1 complex⁴⁰, and thus, how the proteolytic products are released
280 from the proteolytic chamber remained mysterious. The pioneering, elegant NMR study
281 coined the concept of the lateral exit pore²⁹, and subsequent reports established that the
282 compressed structure of ClpP is the state for product release^{13,27,28} and proposed several
283 factors for structural transitions from the extended state to the compressed state via the
284 compact state²³⁻²⁶. Since the first extended structure of ClpP was reported⁷, numerous crystal
285 structures of ClpP from different species and even in different conformational states have
286 been reported^{6,8-13,27}. When we analyzed the crystallization conditions of the reported
287 structures of ClpP, compressed or compact structures were not always but mainly reported for
288 crystals grown under low-pH conditions^{13,27}. Therefore, we speculated that there must be a
289 relationship between the physiological conditions and the low pH crystallization that yields
290 compressed structures. ClpP shows a cylindrical shape with doubly capped Clp-ATPases.
291 Thus, its proteolytic chamber is almost a closed compartment, and the proteolytic products
292 must accumulate in it. Therefore, the physicochemical properties of this compartmental

293 environment can be maintained locally. As we described earlier, the peptide products possess
294 millions of new amino- and carboxy-termini that govern the overall pH of this chamber. The
295 average pK_a value of all amino acid residues is below pH 6.0, and thus, although it depends
296 on the pI value of the substrates and the composition of amino acids, the local pH value must
297 be approximately pH 6.0. Due to the technical difficulty of direct measuring the pH value of
298 the proteolytic chamber of ClpP during protein hydrolysis, we set up a test experiment on
299 whether substrate hydrolysis indeed drops the pH of the system (Fig. 1). Instead of the
300 compartmentalizing chamber, we used minimal buffering conditions and used a high
301 concentration of the substrate to make a large amount of peptide products. Two model
302 proteins, α -casein and BSA, were degraded by proteinase K, and the pH of the system
303 dropped gradually (Fig. 1). Likewise, when the partially unfolded α -casein was degraded by
304 BsClpP in the presence of ADEP1 (Extended Data Fig. 2b), the pH of the system dropped
305 slowly as the hydrolyzed products were released. We speculate that the pH drop of the
306 proteolytic chamber must occur faster because the volume of the self-compartmentalizing
307 chamber is limited. Therefore, we propose that the main driving force for opening the side
308 exit pore of the Clp-ATPase/ClpP complex is the concentration of protons that regulates the
309 conformational change. The pH change controls countless biological processes, including
310 ATP synthesis, virus maturation, oligomerization, autophagy, and lysosomal activity⁴¹⁻⁴⁷.
311 Here, we add another example, in which the pH-dependent conformational change controls
312 the proteolytic activity of the self-compartmentalizing protease.

313 There are unique features of ClpP activation by the small ADEP molecules, which
314 are distinguished from those of Clp-ATPases. The ADEP-binding sites share the IGF/L
315 binding region; however, the numbers and positions are different. Previously, only structural
316 information about all 14 ADEP binding or no binding structures has been reported^{11,14,22,27},
317 and now, we present two other states, 5ADEP (10 ADEPs for a BsClpP tetradecamer) and

318 2ADEP (4 ADEPs for a BsClpP tetradecamer), which might be energetically stable
319 intermediates among heterogeneous binding states. However, as shown in recent ClpAP cryo-
320 EM structures²¹, depending on the conformational states of ClpA, 6 ADEP-binding sites in
321 the heptameric ClpP ring are occupied in the engaged conformation, and 5 ADEP-binding
322 sites are occupied in the disengaged conformation. The other difference is the entrance pore
323 formed by the N-terminal region of ClpP. Pore widening in the ClpXP complex is not
324 necessary because the ATPase fully unfolds the substrate and translocates it through the
325 narrow pore, whereas ADEP triggers pore opening for substrate translocation¹⁹. It is known
326 that stably folded model proteins are not degraded by the ADEP-ClpP complex, but partially
327 unfolded substrates such as casein and peptide substrates are well degraded^{11,16}. The
328 antibiotic mechanism of ADEP has been established, and the nascent polypeptide chains
329 emerging from the ribosome and, due to intrinsic instability, also the cell division protein
330 FtsZ are the target substrates of the ADEP-ClpP complex⁴⁸. Intriguingly, the intermediate
331 structures 5ADEP and 2ADEP show asymmetric pore shapes due to the molecular asymmetry
332 of ClpP (Fig. 2). In addition, the ClpXP and ClpAP are symmetry-mismatched complexes and
333 thus, the ClpP becomes asymmetric during the reaction cycle. Although the substrate feeding
334 steps realized by ADEP and Clp-ATPases are different, the remaining steps must be similar.
335 Once the substrate reaches the proteolytic chamber, hydrolyzed products are produced, and
336 subsequently, the local pH of the chamber must get lower in both cases. Indeed, negative
337 charges are distributed at the interior of the proteolytic chamber, and the acidic products must
338 be even worse in energetics. Except for the extended state, the strictly conserved catalytic
339 triad showed a distorted configuration, most likely due to the protonation state of His122. The
340 widening of side pores was observed gradually from the extended, fully occupied ADEP-
341 ClpP structure through 5ADEP and 2ADEP to the compressed apo-ClpP structure. In
342 combination with current results and previous reports, we proposed a model for product

343 release (Fig. 6). The product release mechanism of ClpP, initiated by substrate hydrolysis
344 followed by lowering pH, must be similar to both activators, small chemical compounds and
345 Clp-ATPases. However, the enlarged entrance pores of ClpP induced by ADEP binding might
346 be an additional exit site whereas ClpP engaged with processive Clp-ATPase utilizes only
347 side exit pores. Notwithstanding the differences, our current study suggests that the driving
348 force for opening the site exit pore of ClpP is the pH drops coupled with protein hydrolysis.
349 Furthermore, it can be a general concept for self-compartmentalizing proteases.

350

351 **Methods**

352 **Sample preparation.** BsClpP protein was purified as previously described^{11,27}. For cryo-EM
353 experiments, we further purified the proteins using a SuperoseTM 6 Increase 10/300 GL (GE
354 Healthcare, 29-0915-96) size-exclusion column pre-equilibrated with 50 mM sodium acetate
355 pH 4.2, 500 mM KCl, and 5% (w/v) glycerol [or 50 mM N-(2-acetamido)iminodiacetic acid
356 pH 6.5, 200 mM NaCl, and 5% (w/v) glycerol]. SDS-PAGE and negative stain electron
357 microscopy were used to assess protein purity and quality. All biological materials are
358 available upon request.

359

360 **pH measurement during protein hydrolysis.** For *in vitro* pH change measurement, bovine
361 milk α -casein (Sigma, C6780) and bovine serum albumin (Sigma, A7030) were used as
362 substrates. Reactions were carried out with either 100 μ g/ml proteinase K (Sigma, P2308) in
363 diluted PBS (1.37 mM NaCl, 0.27 mM KCl, 0.1 mM Na₂HPO₄, and 0.18 mM KH₂PO₄)
364 containing 1 mM CaCl₂ or with 0.4 μ g/ml BsClpP₁₄ in the presence of 11 μ M ADEP1
365 (Cayman Chemical, A-54556A, ~2-fold molar ratio)¹¹ in the same PBS at 40 °C. The final

366 concentration of substrate was 80–100 μ M, and the final assay volume was 20 ml. The pH
367 was measured every 30 sec for 10 min, every 1 min for 20 min, every 5 min for 210 min, and
368 every 10 min for 60 min, reaching a total of 300 minutes, using a pH meter (Thermo
369 Scientific, 13-644-928).

370

371 **Crystallography.** A cocrystallization method was used to generate crystals of BsClpP-ADEP
372 complexes by mixing BsClpP and ADEP2 at a 1:1 molar ratio. Crystals of the compressed
373 and compact form of the BsClpP-ADEP complex were grown in hanging drops at 22 °C
374 using 100 mM sodium acetate pH 4.6, 500 mM potassium thiocyanate (for the compressed
375 state), 100 mM sodium citrate pH 5.6, 100 mM Li_2SO_4 , and 9~11% (w/v) polyethylene glycol
376 6,000 (for the compact state). Diffraction data were processed with the program HKL2000
377 (ref. ⁴⁹). The phases of 2ADEP and 5ADEP structures were obtained by molecular
378 replacement using a previously solved compressed BsClpP structure²⁷ as a search model with
379 the program MOLREP in the CCP4 suite⁵⁰. Model building and refinement were performed
380 using the programs COOT⁵¹ and PHENIX⁵², respectively.

381

382 **Cryo-EM data collection and processing.** The fraction eluted from size-exclusion
383 chromatography was isolated and incubated with 100 μ M ADEP1 (Cayman Chemical, A-
384 54556A). In particular, the BsClpP pH 6.5 sample was also treated with 4 mg ml⁻¹ amphipol.
385 After 10 min of incubation, a 5 μ l drop was applied to a graphene-treated and glow-
386 discharged holey carbon grid (R 1.2/1.3 Quantifoil) for the BsClpP pH 4.2 sample and
387 negatively glow-discharged holey carbon grid (R 1.2/1.3 Quantifoil) for the BsClpP pH 6.5
388 sample. They were then blotted for 3 sec at 4 °C and 100% humidity with Whatman no. 595
389 filter paper before being plunge frozen in liquid ethane using a Vitrobot Mark IV system

390 (Thermo Fisher Scientific Inc., USA). Cryo-EM images of frozen-hydrated BsClpP particles
391 were collected at Korea Basic Science Institute with a Titan Krios TEM system (Thermo
392 Fisher Scientific Inc., USA) operated at 300 keV using electron counting mode and automatic
393 data acquisition software (EPU, Thermo Fisher Scientific Inc., USA). Detailed data
394 acquisition conditions and parameters are given in **Supplementary Table 2**.

395 Cryo-EM images for BsClpP pH 4.2 were processed using RELION 3.0 (ref. ⁵³) and
396 CryoSPARC v2 (ref. ⁵⁴). Beam-induced motion correction and dose weighting were
397 performed using MotionCor2 v1.2.1 (ref. ⁵⁵), and CTF estimation was performed using Gctf
398 v1.06 (ref. ⁵⁶). Then, 341,414 particle images were selected from 964 micrographs after
399 reference-free 2D class averages. The 3D initial model was generated by CryoSPARC-
400 implemented Ab initio reconstruction. Multiple rounds of successive 3D classifications were
401 performed, and 179,322 homogeneous particles were selected for further processing.
402 Homogeneous refinement with D7 symmetry then yielded a consensus map at 3.6 Å. Of note,
403 particles displayed a significant preferred orientation, showing a dominant top view. Selected
404 particles with angular information were transferred to RELION for further 3D classification
405 without an angular orientation search. To evaluate the possible heterogeneity of ADEP
406 binding, we performed focused 3D classification around ADEP binding sites with expanded
407 particles with C7 symmetry. We only observed the all-ADEP and apo states from the analysis,
408 and thus, we clustered particles into apo or all-ADEP states. Finally, each selected particle set
409 was imported to CryoSPARC and refined at 3.4 Å.

410 The images of BsClpP pH 6.5 were processed using CryoSPARC v2 (ref. ⁵⁴). We
411 originally analyzed ADEP binding heterogeneity in the dataset of a 1:3 mixture of BsClpP
412 and ADEP at pH 6.5 using the same procedure as described above in BsClpP pH 4.2.
413 However, particles were homogeneous, as all ADEP binding sites were occupied in the

414 dataset. Therefore, we obtained two separate imaging sessions for apo (1,130 movies) and
415 ADEP-bound structures (1,182 movies) at pH 6.5. The recorded movies were subjected to
416 motion correction and CTF estimation using patch-motion correction and Gctf v1.06 in
417 CryoSPARC. For the apo map at pH 6.5, a total of 108,389 particle images were selected
418 after 2D class averages. After subsequent heterogeneous refinement for suitable particles,
419 78,413 particles were finally used for 3D reconstruction at 3.2 Å resolution. For the ADEP
420 bound map at pH 6.5, a total of 153,078 particle images were selected after 2D class averages.
421 After subsequent heterogeneous refinement, 138,976 particles yielded the map at 3.1 Å
422 resolution. All cryo-EM images were processed using computing resources at the Center for
423 Macromolecular and Cell Imaging, Seoul National University.

424

425 **Molecular modeling.** Both BsClpP pH 4.2 and pH 6.5 structural models were built manually
426 in COOT⁵¹ by referring to the BsClpP crystallography structure (PDB ID: 3TT6 for the pH
427 4.2 model and 3KTJ for the pH 6.5 model) and refined using phenix.real_space_refine in the
428 PHENIX software suite⁵⁷. Additionally, the quality of the final models was evaluated using
429 the comprehensive model validation section and MolProbity in PHENIX.

430

431 **Acknowledgments**

432 We thank the staff at beamlines 5C and 11C at the Pohang Accelerator Laboratory in South
433 Korea and beamline NW12 at the Photon Factory in Japan for their help with X-ray data
434 collection. We also thank the staff of the cryo-TEM facility at the Korea Basic Science
435 Institute and the Center for Macromolecular and Cell Imaging, Seoul National University.
436 This study was supported by National Research Foundation of Korea (NRF) grants from the

437 Korean government (grant Nos. 2020R1A2C3008285, 2020R1A5A1019023, and
438 2021M3A9I4030068 for HKS; 2021M3A9I4021220, 2019M3E5D6063871, and
439 2020R1A5A1018081 for SHR) and the Deutsche Forschungsgemeinschaft (German Research
440 Foundation, DFG) TRR 261, project-ID 398967434.

441

442

443 **Author contributions**

444 L.K. and B.-G.L. performed sample preparation for biochemical experiments; B.-G.L. and
445 M.K.K. prepared the crystals and solved the X-ray structures; L.K., M.K.K, D.H.K. and S.-
446 H.R. performed the cryo-EM studies; L.K., B.-G.L., H.K., H.B.-O., S.-H.R., and H.K.S.
447 analyzed the data; H.B.-O. discussed results and edited the manuscript; and L.K., B.-G.L.,
448 and H.K.S. designed the experiments and wrote the manuscript.

449

450 **Competing interests**

451 The authors declare no competing interests.

452

453 **Data availability**

454 The cryo-EM maps have been deposited in the EMDB: ADEP1-BsClpP complex at pH 4.2
455 (EMD-31561), apo-BsClpP at pH 4.2 (EMD-31562), ADEP1-BsClpP complex at pH 6.5
456 (EMD-31559), and apo-BsClpP at pH 6.5 (EMD-31560). The coordinates have been
457 deposited in the PDB: crystal structures – 2ADEP (7P80) and 5ADEP (7P81); cryo-EM

458 structures – ADEP1-BsClpP complex at pH 4.2 (7FER), apo-BsClpP at pH 4.2
459 (7FES), ADEP1-BsClpP complex at pH 6.5 (7FEP), and apo-BsClpP at pH 6.5 (7FEQ).

460

461 **Code availability**

462 Not applicable.

463

464 **References**

- 465 1. Goldberg, A.L. ATP-dependent proteases in prokaryotic and eukaryotic cells. *Semin*
466 *Cell Biol* **1**, 423-32 (1990).
- 467 2. Groll, M., Bochtler, M., Brandstetter, H., Clausen, T. & Huber, R. Molecular
468 machines for protein degradation. *Chembiochem* **6**, 222-56 (2005).
- 469 3. Sauer, R.T. & Baker, T.A. AAA+ proteases: ATP-fueled machines of protein
470 destruction. *Annu Rev Biochem* **80**, 587-612 (2011).
- 471 4. Gottesman, S. *Annual Review of Cell and Developmental Biology* **19**, 565-587 (2003).
- 472 5. Baker, T.A. & Sauer, R.T. ClpXP, an ATP-powered unfolding and protein-degradation
473 machine. *Biochim Biophys Acta* **1823**, 15-28 (2012).
- 474 6. Yu, A.Y. & Houry, W.A. ClpP: a distinctive family of cylindrical energy-dependent
475 serine proteases. *FEBS Lett* **581**, 3749-57 (2007).
- 476 7. Wang, J., Hartling, J.A. & Flanagan, J.M. The structure of ClpP at 2.3 Å resolution
477 suggests a model for ATP-dependent proteolysis. *Cell* **91**, 447-56 (1997).
- 478 8. Kang, S.G., Maurizi, M.R., Thompson, M., Mueser, T. & Ahvazi, B. Crystallography
479 and mutagenesis point to an essential role for the N-terminus of human mitochondrial
480 ClpP. *J Struct Biol* **148**, 338-52 (2004).

- 481 9. Gribun, A. et al. The ClpP double ring tetradecameric protease exhibits plastic ring-
482 ring interactions, and the N termini of its subunits form flexible loops that are
483 essential for ClpXP and ClpAP complex formation. *J Biol Chem* **280**, 16185-96
484 (2005).
- 485 10. Kim, D.Y. & Kim, K.K. The structural basis for the activation and peptide recognition
486 of bacterial ClpP. *J Mol Biol* **379**, 760-71 (2008).
- 487 11. Lee, B.G. et al. Structures of ClpP in complex with acyldepsipeptide antibiotics reveal
488 its activation mechanism. *Nat Struct Mol Biol* **17**, 471-8 (2010).
- 489 12. El Bakkouri, M. et al. The Clp chaperones and proteases of the human malaria
490 parasite *Plasmodium falciparum*. *J Mol Biol* **404**, 456-77 (2010).
- 491 13. Geiger, S.R., Bottcher, T., Sieber, S.A. & Cramer, P. A conformational switch
492 underlies ClpP protease function. *Angew Chem Int Ed Engl* **50**, 5749-52 (2011).
- 493 14. Li, D.H. et al. Acyldepsipeptide antibiotics induce the formation of a structured axial
494 channel in ClpP: A model for the ClpX/ClpA-bound state of ClpP. *Chem Biol* **17**, 959-
495 69 (2010).
- 496 15. Brotz-Oesterhelt, H. et al. Dysregulation of bacterial proteolytic machinery by a new
497 class of antibiotics. *Nat Med* **11**, 1082-7 (2005).
- 498 16. Kirstein, J. et al. The antibiotic ADEP reprogrammes ClpP, switching it from a
499 regulated to an uncontrolled protease. *EMBO Mol Med* **1**, 37-49 (2009).
- 500 17. Sass, P. et al. Antibiotic acyldepsipeptides activate ClpP peptidase to degrade the cell
501 division protein FtsZ. *Proc Natl Acad Sci U S A* **108**, 17474-9 (2011).
- 502 18. Fei, X. et al. Structures of the ATP-fueled ClpXP proteolytic machine bound to
503 protein substrate. *Elife* **9**, e52774 (2020).
- 504 19. Gatsogiannis, C., Balogh, D., Merino, F., Sieber, S.A. & Raunser, S. Cryo-EM
505 structure of the ClpXP protein degradation machinery. *Nat Struct Mol Biol* **26**, 946-

- 506 954 (2019).
- 507 20. Ripstein, Z.A., Vahidi, S., Houry, W.A., Rubinstein, J.L. & Kay, L.E. A processive
508 rotary mechanism couples substrate unfolding and proteolysis in the ClpXP
509 degradation machinery. *Elife* **9**, e52158 (2020).
- 510 21. Lopez, K.E. et al. Conformational plasticity of the ClpAP AAA+ protease couples
511 protein unfolding and proteolysis. *Nat Struct Mol Biol* **27**, 406-416 (2020).
- 512 22. Gersch, M. et al. AAA+ chaperones and acyldepsipeptides activate the ClpP protease
513 via conformational control. *Nat Commun* **6**, 6320 (2015).
- 514 23. Ripstein, Z.A., Vahidi, S., Rubinstein, J.L. & Kay, L.E. A pH-Dependent
515 Conformational Switch Controls N. meningitidis ClpP Protease Function. *J Am Chem*
516 *Soc* **142**, 20519-20523 (2020).
- 517 24. Vahidi, S. et al. Reversible inhibition of the ClpP protease via an N-terminal
518 conformational switch. *Proc Natl Acad Sci U S A* **115**, E6447-E6456 (2018).
- 519 25. Gersch, M., List, A., Groll, M. & Sieber, S.A. Insights into structural network
520 responsible for oligomerization and activity of bacterial virulence regulator
521 caseinolytic protease P (ClpP) protein. *J Biol Chem* **287**, 9484-94 (2012).
- 522 26. Vahidi, S. et al. An allosteric switch regulates Mycobacterium tuberculosis ClpP1P2
523 protease function as established by cryo-EM and methyl-TROSY NMR. *Proc Natl*
524 *Acad Sci U S A* **117**, 5895-5906 (2020).
- 525 27. Lee, B.G., Kim, M.K. & Song, H.K. Structural insights into the conformational
526 diversity of ClpP from Bacillus subtilis. *Mol Cells* **32**, 589-95 (2011).
- 527 28. Zhang, J. et al. Structural switching of Staphylococcus aureus Clp protease: a key to
528 understanding protease dynamics. *J Biol Chem* **286**, 37590-601 (2011).
- 529 29. Sprangers, R., Gribun, A., Hwang, P.M., Houry, W.A. & Kay, L.E. Quantitative NMR
530 spectroscopy of supramolecular complexes: dynamic side pores in ClpP are important

- 531 for product release. *Proc Natl Acad Sci U S A* **102**, 16678-83 (2005).
- 532 30. Li, M. et al. Structure and Functional Properties of the Active Form of the Proteolytic
533 Complex, ClpP1P2, from *Mycobacterium tuberculosis*. *J Biol Chem* **291**, 7465-76
534 (2016).
- 535 31. Ge, S.J. & Zhang, L.X. Control of the Degree of Hydrolysis of a Protein Modification
536 with Immobilized Protease by the Ph-Drop Method. *Acta Biotechnologica* **13**, 151-
537 160 (1993).
- 538 32. Mozersky, S.M. & Panettieri, R.A. Is Ph Drop a Valid Measure of Extent of Protein
539 Hydrolysis. *Journal of Agricultural and Food Chemistry* **31**, 1313-1316 (1983).
- 540 33. Kozlowski, L.P. Proteome-pI: proteome isoelectric point database. *Nucleic Acids Res*
541 **45**, D1112-D1116 (2017).
- 542 34. Wong, K.S. et al. Acyldepsipeptide Analogs Dysregulate Human Mitochondrial ClpP
543 Protease Activity and Cause Apoptotic Cell Death. *Cell Chem Biol* **25**, 1017-1030 e9
544 (2018).
- 545 35. Brotz-Oesterhelt, H. & Vorbach, A. Reprogramming of the Caseinolytic Protease by
546 ADEP Antibiotics: Molecular Mechanism, Cellular Consequences, Therapeutic
547 Potential. *Front Mol Biosci* **8**, 690902 (2021).
- 548 36. Yang, T. et al. Dysregulation of ClpP by Small-Molecule Activators Used Against
549 *Xanthomonas oryzae* pv. *oryzae* Infections. *J Agric Food Chem* **69**, 7545-7553 (2021).
- 550 37. Liu, K., Ologbenla, A. & Houry, W.A. Dynamics of the ClpP serine protease: a model
551 for self-compartmentalized proteases. *Crit Rev Biochem Mol Biol* **49**, 400-12 (2014).
- 552 38. Jennings, L.D., Bohon, J., Chance, M.R. & Licht, S. The ClpP N-terminus coordinates
553 substrate access with protease active site reactivity. *Biochemistry* **47**, 11031-40 (2008).
- 554 39. Malik, I.T. et al. Functional Characterisation of ClpP Mutations Conferring Resistance
555 to Acyldepsipeptide Antibiotics in Firmicutes. *Chembiochem* **21**, 1997-2012 (2020).

- 556 40. Ortega, J., Lee, H.S., Maurizi, M.R. & Steven, A.C. ClpA and ClpX ATPases bind
557 simultaneously to opposite ends of ClpP peptidase to form active hybrid complexes. *J*
558 *Struct Biol* **146**, 217-26 (2004).
- 559 41. Stock, D., Gibbons, C., Arechaga, I., Leslie, A.G. & Walker, J.E. The rotary
560 mechanism of ATP synthase. *Curr Opin Struct Biol* **10**, 672-9 (2000).
- 561 42. Roh, S.H. et al. Cryo-EM and MD infer water-mediated proton transport and
562 autoinhibition mechanisms of Vo complex. *Sci Adv* **6**, eabb9605 (2020).
- 563 43. Carr, C.M., Chaudhry, C. & Kim, P.S. Influenza hemagglutinin is spring-loaded by a
564 metastable native conformation. *Proc Natl Acad Sci U S A* **94**, 14306-13 (1997).
- 565 44. Burkard, C. et al. Coronavirus cell entry occurs through the endo-/lysosomal pathway
566 in a proteolysis-dependent manner. *PLoS Pathog* **10**, e1004502 (2014).
- 567 45. Kim, J.H. et al. Insights into autophagosome maturation revealed by the structures of
568 ATG5 with its interacting partners. *Autophagy* **11**, 75-87 (2015).
- 569 46. Kwon, D.H. et al. Insights into degradation mechanism of N-end rule substrates by
570 p62/SQSTM1 autophagy adapter. *Nat Commun* **9**, 3291 (2018).
- 571 47. Banerjee, S. & Kane, P.M. Regulation of V-ATPase Activity and Organelle pH by
572 Phosphatidylinositol Phosphate Lipids. *Front Cell Dev Biol* **8**, 510 (2020).
- 573 48. Silber, N. et al. Cell Division Protein FtsZ Is Unfolded for N-Terminal Degradation by
574 Antibiotic-Activated ClpP. *mBio* **11**(2020).
- 575 49. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in
576 oscillation mode. *Methods Enzymol.* **276**, 307-326 (1997).
- 577 50. Winn, M.D. et al. Overview of the CCP4 suite and current developments. *Acta*
578 *Crystallogr D Biol Crystallogr* **67**, 235-42 (2011).
- 579 51. Casanal, A., Lohkamp, B. & Emsley, P. Current developments in Coot for
580 macromolecular model building of Electron Cryo-microscopy and Crystallographic

- 581 Data. *Protein Sci* **29**, 1069-1078 (2020).
- 582 52. Afonine, P.V. et al. Real-space refinement in PHENIX for cryo-EM and
583 crystallography. *Acta Crystallogr D Struct Biol* **74**, 531-544 (2018).
- 584 53. Zivanov, J. et al. New tools for automated high-resolution cryo-EM structure
585 determination in RELION-3. *Elife* **7**, e42166 (2018).
- 586 54. Punjani, A., Rubinstein, J.L., Fleet, D.J. & Brubaker, M.A. cryoSPARC: algorithms
587 for rapid unsupervised cryo-EM structure determination. *Nat Methods* **14**, 290-296
588 (2017).
- 589 55. Zheng, S.Q. et al. MotionCor2: anisotropic correction of beam-induced motion for
590 improved cryo-electron microscopy. *Nat Methods* **14**, 331-332 (2017).
- 591 56. Zhang, K. Gctf: Real-time CTF determination and correction. *J Struct Biol* **193**, 1-12
592 (2016).
- 593 57. Liebschner, D. et al. Macromolecular structure determination using X-rays, neutrons
594 and electrons: recent developments in Phenix. *Acta Crystallogr D Struct Biol* **75**, 861-
595 877 (2019).
- 596
- 597
- 598
- 599

600 **Figure Legends**

601

602 **Fig. 1: pH change during protein hydrolysis.** **a**, Monitoring of the pH change during
603 degradation of α -casein by BsClpP in the presence of ADEP1 and proteinase K. Blue (square)
604 and red (triangle) symbols represent α -casein with BsClpP+ADEP1 and proteinase K,
605 respectively. **b**, Monitoring of the pH change during degradation of bovine serum albumin
606 (BSA) by proteinase K (red triangle). Black circles represent the control, only substrates
607 without proteases (**a,b**). Data points represent the mean value of three measurements and the
608 error bars show standard deviations.

609

610 **Fig. 2: X-ray structures of ADEP-bound BsClpP.** **a**, Ribbon diagram of 2ADEP-bound
611 BsClpP (cyan) with a transparent molecular surface and one monomer in each heptameric
612 ring, colored darker for clarity (2ADEP). **b**, Ribbon diagram of 5ADEP-bound BsClpP (green)
613 with a transparent molecular surface and one monomer in each heptameric ring, colored
614 darker for clarity (5ADEP). Tetradecameric BsClpP is viewed along a 7-fold molecular
615 symmetry axis (upper), and the 2-fold side view is observed by rotating 90° (lower). The
616 bound ADEP molecules colored red are shown as a stick model. Dimensions of the models
617 are indicated. The two diameters are noted due to the asymmetric shape of the entrance pore.

618

619 **Fig. 3: Structural analyses of the compressed 2ADEP and the compact 5ADEP.** **a**, Three
620 different subunit environments of 2ADEP viewed along a 7-fold axis: two cyanish subunits
621 (1 and 1') with the bound ADEP (pink molecular surface) on the right side, two orangish
622 subunits (2 and 2') with the bound ADEP on the left side, and three yellowish subunits (3, 3'

623 and 3'') with no ADEP molecule. **b**, Superposition of all 7 subunits in the heptameric ring of
624 2ADEP viewed by rotating the panel (a) 45° about the horizontal axis. The invisible N-
625 terminal region, due to flexibility, is marked with a transparent oval, and the structurally
626 dynamic handle region is marked with a dashed circle. **c**, Superposition of cyanish subunits (1,
627 1'), orangish subunits (2, 2'), and yellowish subunits (3, 3', 3''). The view is the same as that
628 of panel (b). **d**, Three different subunit environments of 5ADEP viewed along a 7-fold axis:
629 three greenish subunits (1, 1' and 1'') with the bound ADEP (pink molecular surface) on both
630 the left and right sides, two bluish subunits (2 and 2') with the bound ADEP on the left side,
631 and two reddish subunits (3 and 3') with ADEP on the right side. **e**, Superposition of all 7
632 subunits in the heptameric ring of 5ADEP viewed by rotating the panel (d) 45° about the
633 horizontal axis. The very flexible N-terminal region and handle region are marked with
634 dashed circles. **f**, Superposition of greenish subunits (1, 1', 1''), bluish subunits (2, 2'), and
635 reddish subunits (3, 3'). The view is the same as that of panel (e).

636

637 **Fig 4: Cryo-EM structures of the BsClpP-ADEP1 complex.** **a**, Ribbon diagram, with the
638 transparent molecular surface of the extended apo-BsClpP structure at pH 6.5 viewed along a
639 7-fold molecular symmetry axis (upper row) and viewed with 90° rotation to display the side
640 view of the 2-fold symmetry axis (lower row). One monomer is shown in darker orange color
641 for clarity. **b**, Same representation as that of panel (a) showing the 14 ADEP1-bound BsClpP
642 at pH 6.5. The bound ADEP1 molecules are shown as stick models colored red. **c**, Same
643 representation of the compressed BsClpP with 14 ADEP1 molecules at pH 4.2. One
644 monomer is shown in darker yellow color for clarity. **d**, Same representation of the
645 compressed apo BsClpP at pH 4.2. Dimensions of the models are indicated.

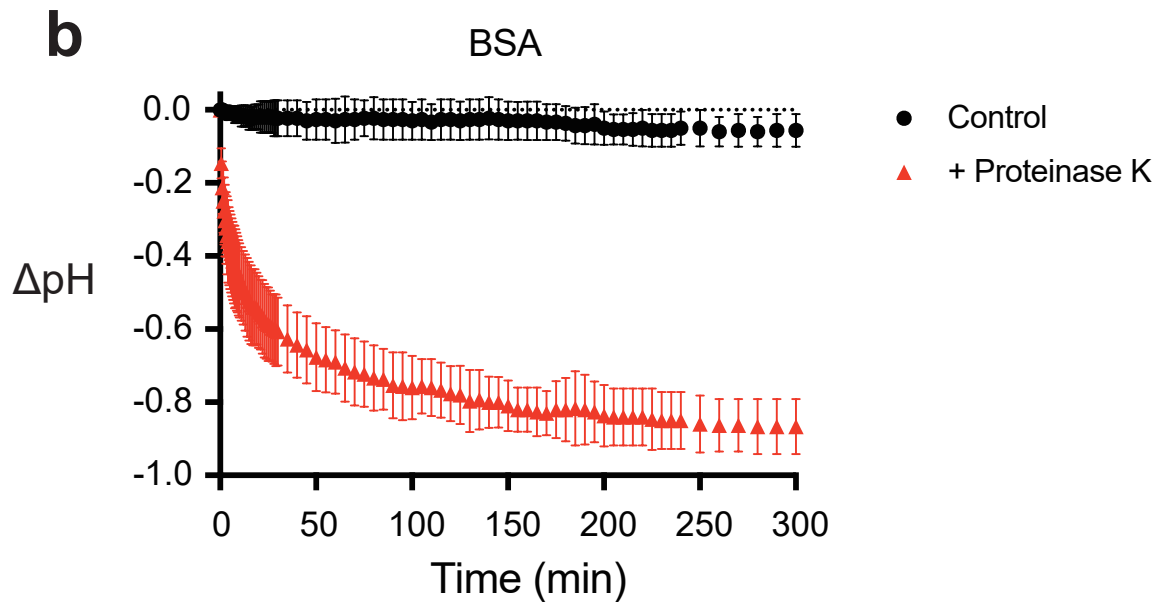
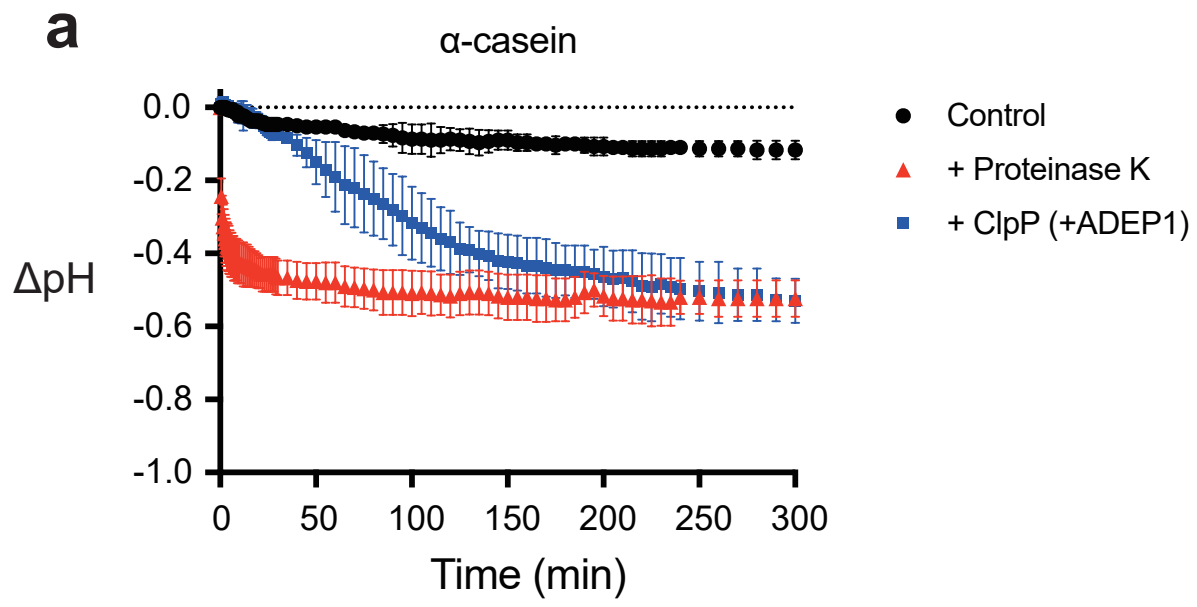
646

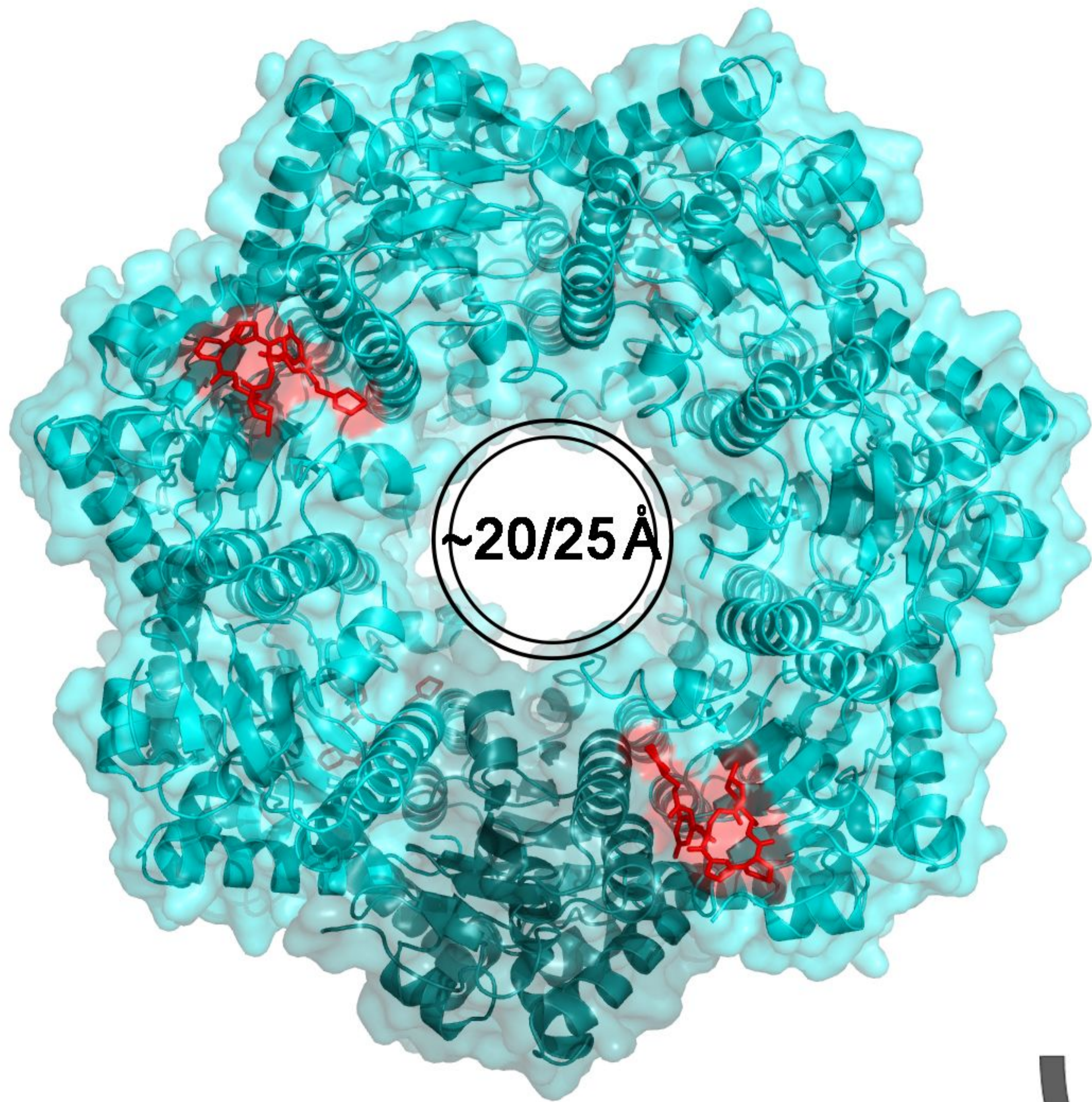
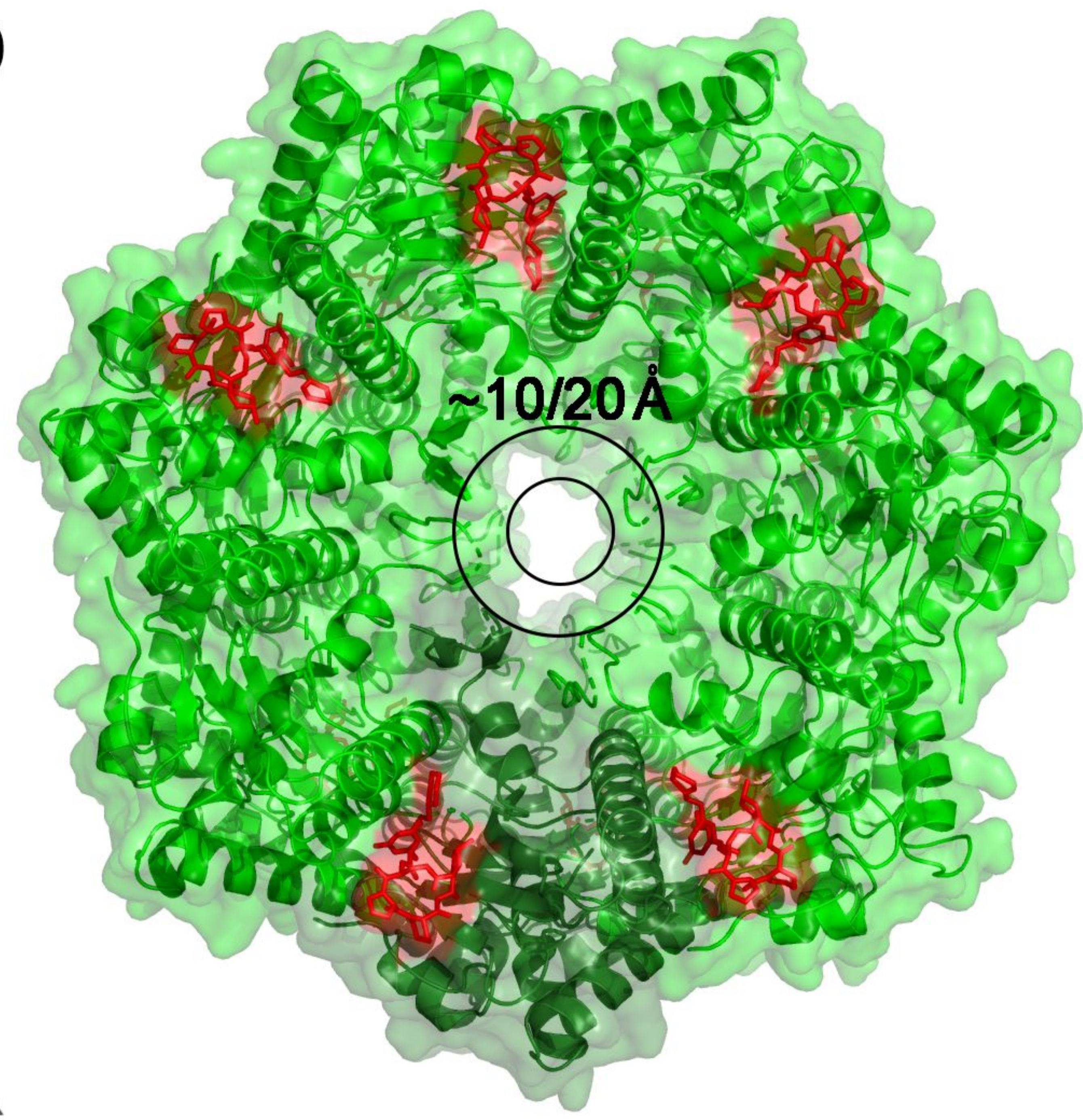
647 **Fig. 5: Electrostatic potential of the proteolytic chamber of various structural states. a,**
648 Ribbon diagram, with the transparent electrostatic potential surface of extended BsClpP
649 viewed from the center of the proteolytic chamber. The catalytic triad (S97-H122-D171)
650 shows the fully active configuration. **b,** Same representation of the 14 ADEP-bound BsClpP
651 cryo-EM structure at pH 6.5. The residues maintaining the extended conformation are now
652 slightly loosened, and the catalytic triad is slightly distorted. **c,** Same representation of 14-
653 ADEP bound BsClpP at pH 4.2. The conformation is now compact, and side exit pores (black
654 arrows) start to appear. **d,** Same representation of 5ADEP at pH 5.6. The conformation of
655 BsClpP is compact, and the catalytic triads at the upper and lower heptameric rings become
656 closer and exhibit an inactive configuration. **e,** Same representation of 2ADEP at pH 4.2. The
657 conformation of BsClpP is compressed, and the catalytic triads at the upper and lower
658 heptameric rings are very close. **f,** Same representation of compressed apo BsClpP. The exit
659 pores are wide open for product release. In all panels, the residues maintaining the extended
660 conformation of ClpP are shown as a stick model and labeled. For clarity, the residues in the
661 neighboring subunit of the different heptameric rings are marked with prime (') after the
662 residue number, and those in the neighboring subunit of the same heptameric ring are marked
663 with prime (') before a single letter code used to represent the amino acid. Positively and
664 negatively charged surfaces are colored blue and red, respectively. The inner surface of the
665 proteolytic chamber is virtually negatively charged (red color), and the acidic proteolytic
666 products must be energetically very unfavorable.

667

668 **Fig. 6: Model proposed for product release of ClpP.** Before and immediately after
669 substrate binding, ClpP is in an active extended conformation, and ADEP widens the entrance
670 pore for better substrate feeding. Our previous crystal structure of the 14 ADEP complex

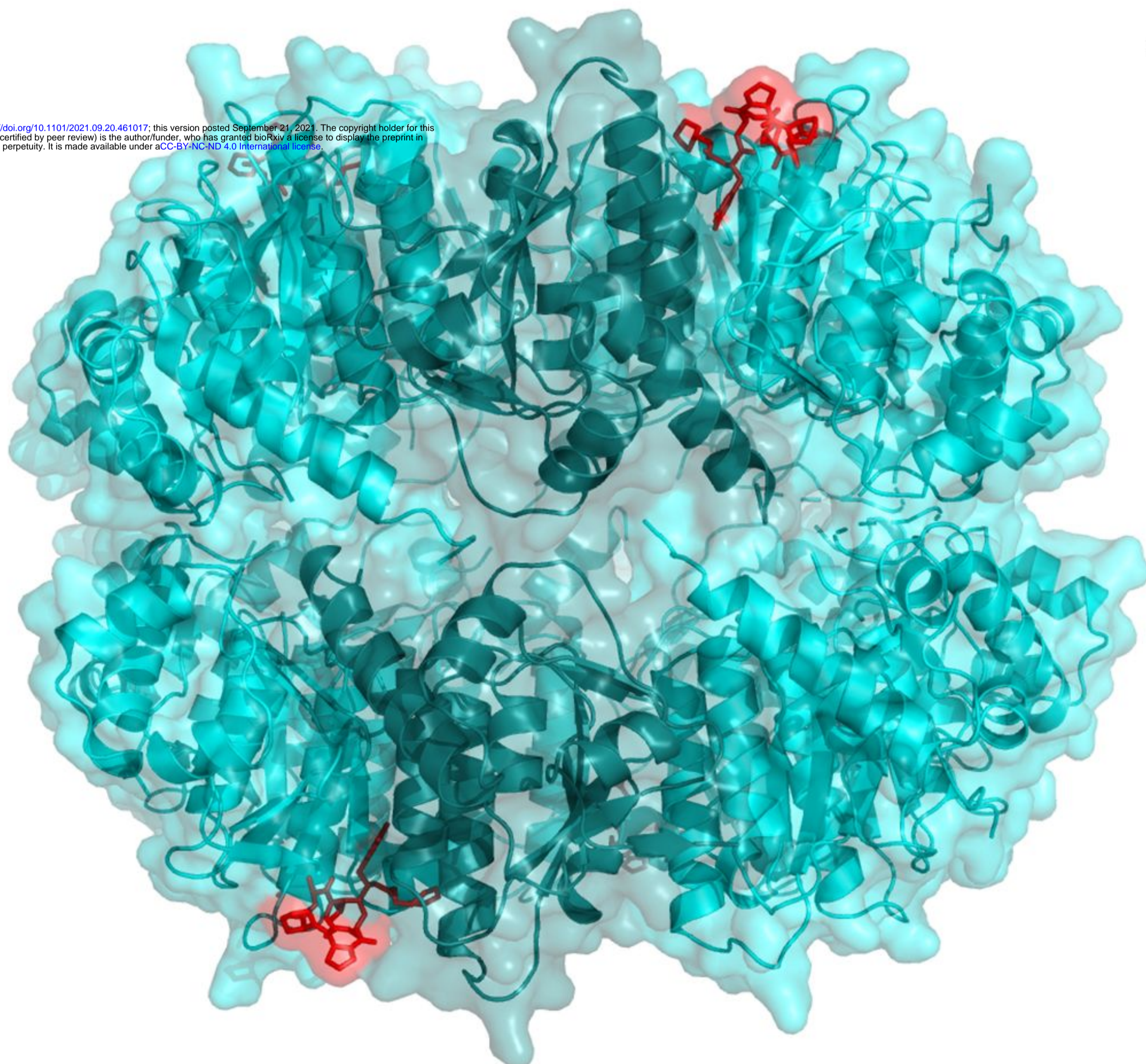
671 showed a disordered N-terminal region¹¹ and our current cryo-EM structure of the same
672 complex demonstrates an ordered N-terminal region. In both cases, the entrance pore is
673 enlarged. From the extended structure to the compressed structure, intermediate
674 conformations were observed. Based on the solution cryo-EM analysis, these intermediate
675 conformations represented heterogeneous complex species of BsClpP with different numbers
676 of ADEP molecules bound. Two major species among the intermediate states, 5ADEP and
677 2ADEP, were crystallized and showed dimensions similar to those of previously known
678 ‘compact’ and ‘compressed’ structures. Both possess asymmetric entrance pores, especially
679 5ADEP, which is more distorted. 5ADEP is less compressed, and thus, the number and size of
680 the side pores are fewer and smaller than those of a fully compressed structure, respectively.
681 The apo BsClpP at pH 4.2 exhibits compression and larger side exit pores. In the model, the
682 red color of the proteolytic chamber represents the lower pH condition derived from substrate
683 hydrolysis.



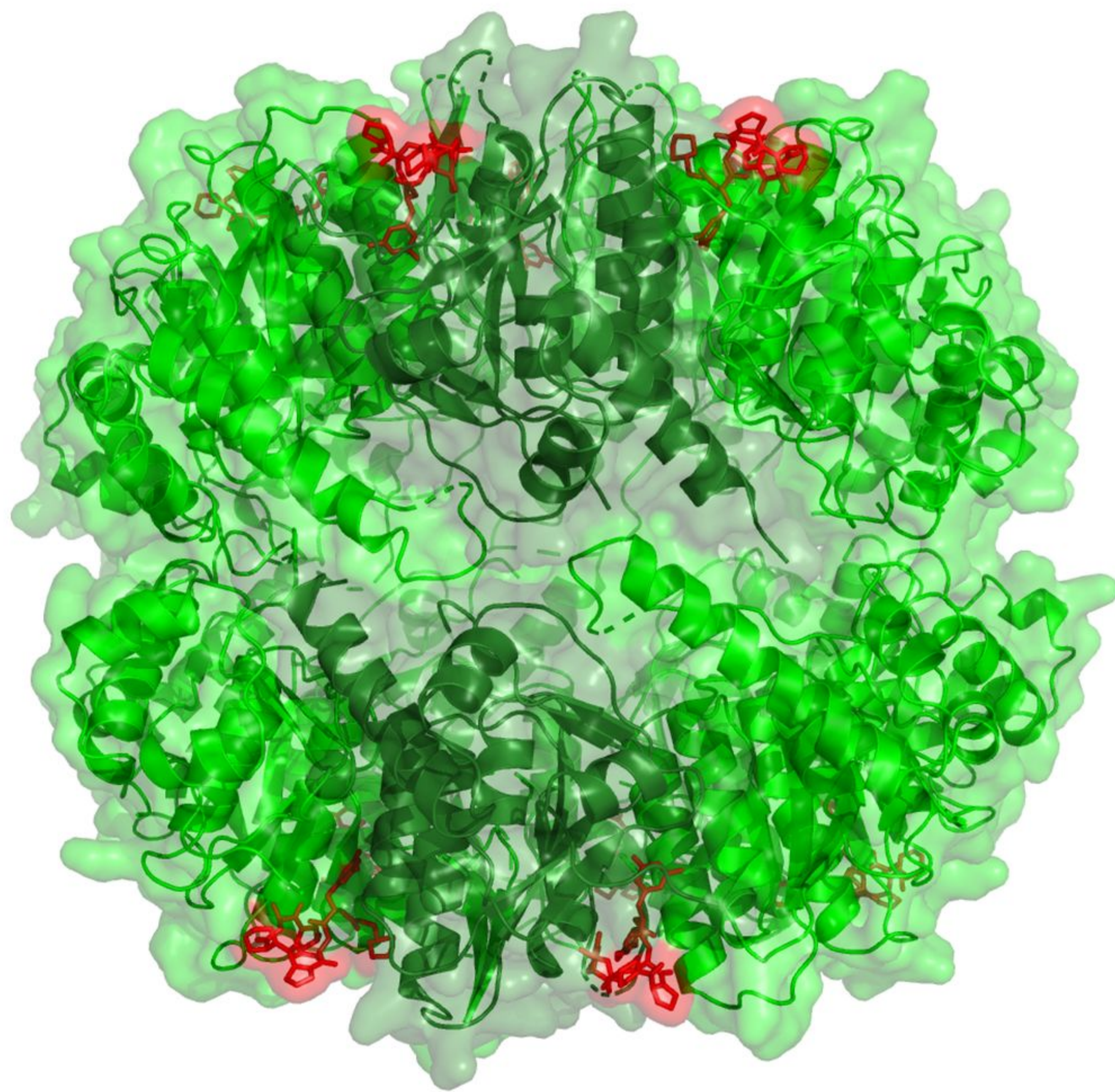
a**b**

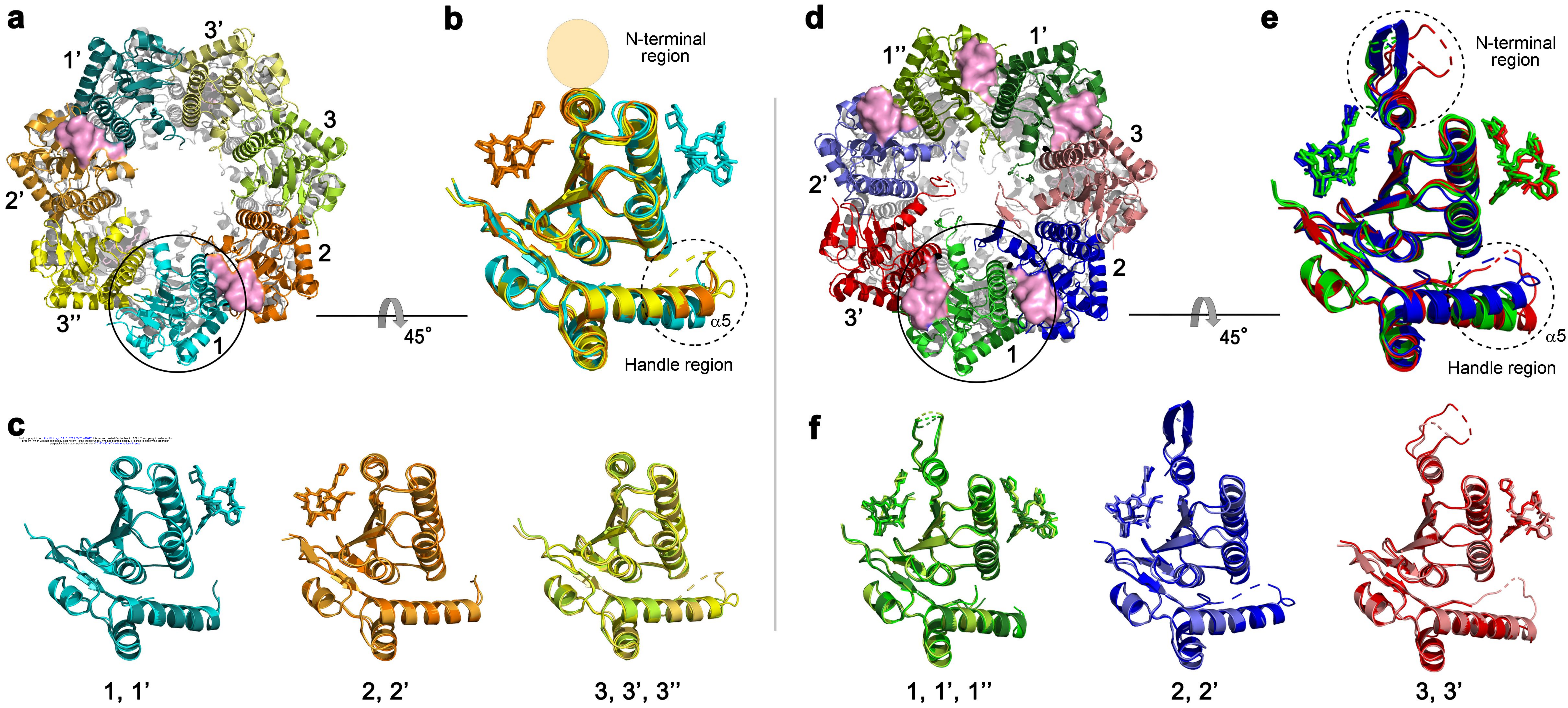
90°

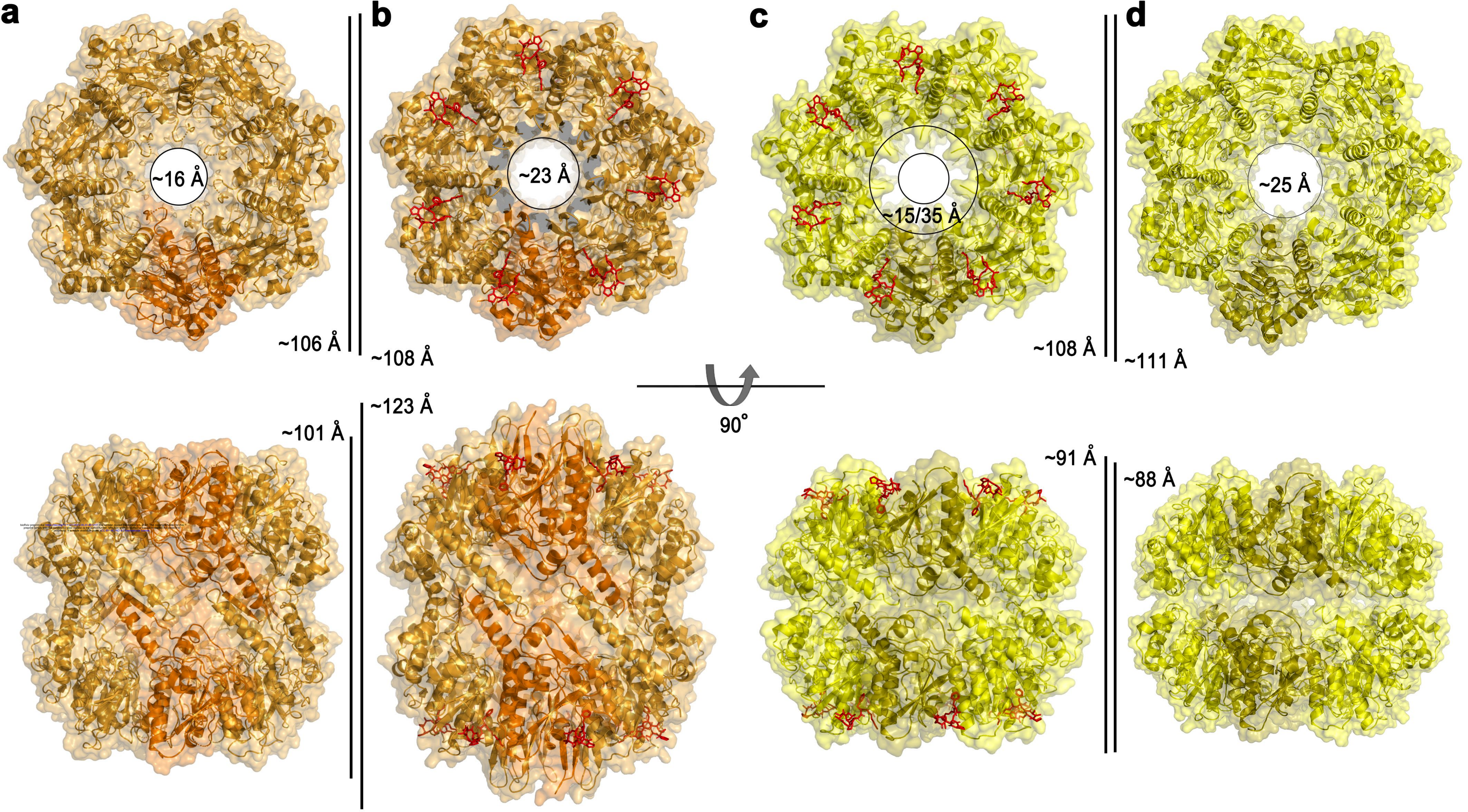
~86 Å

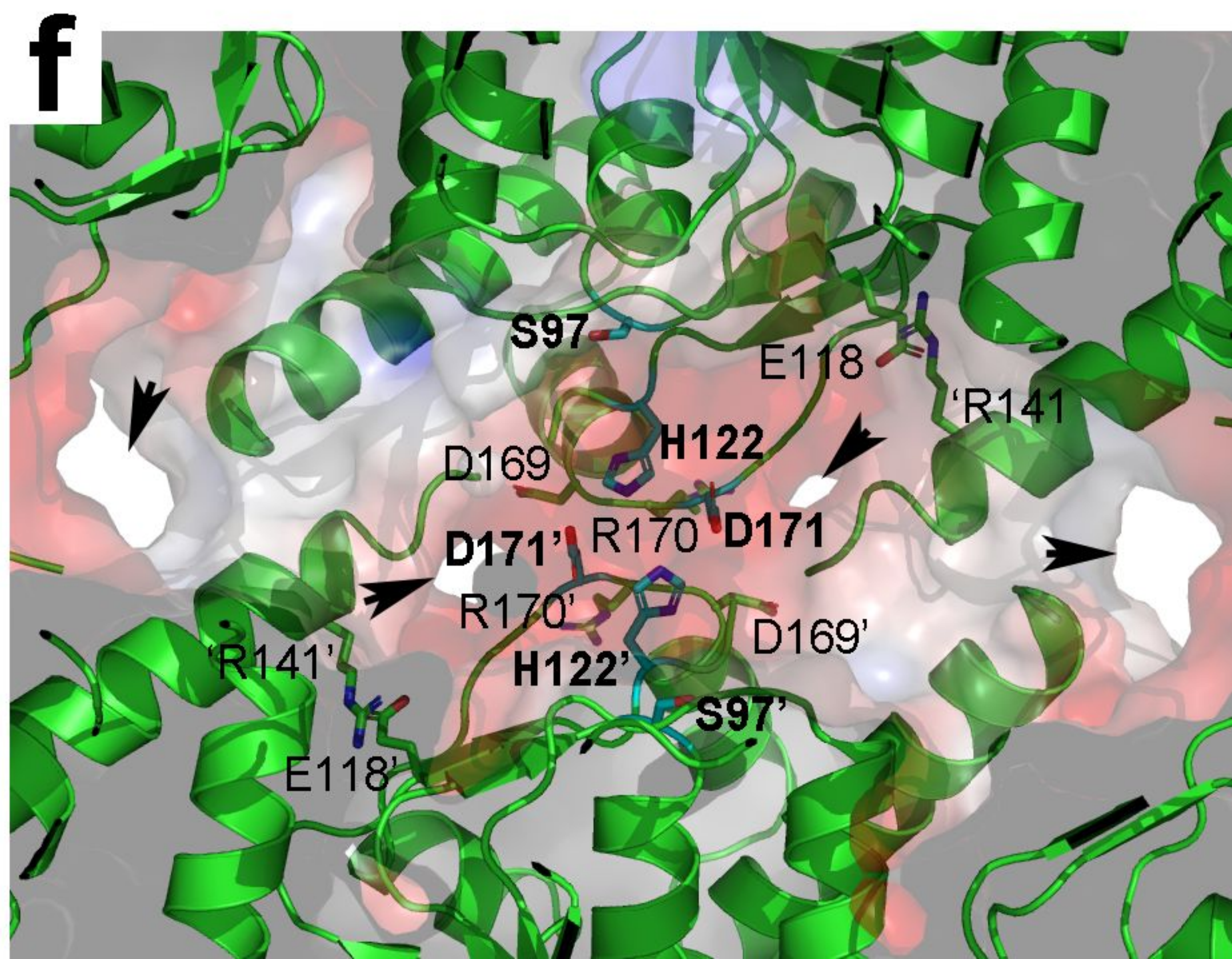
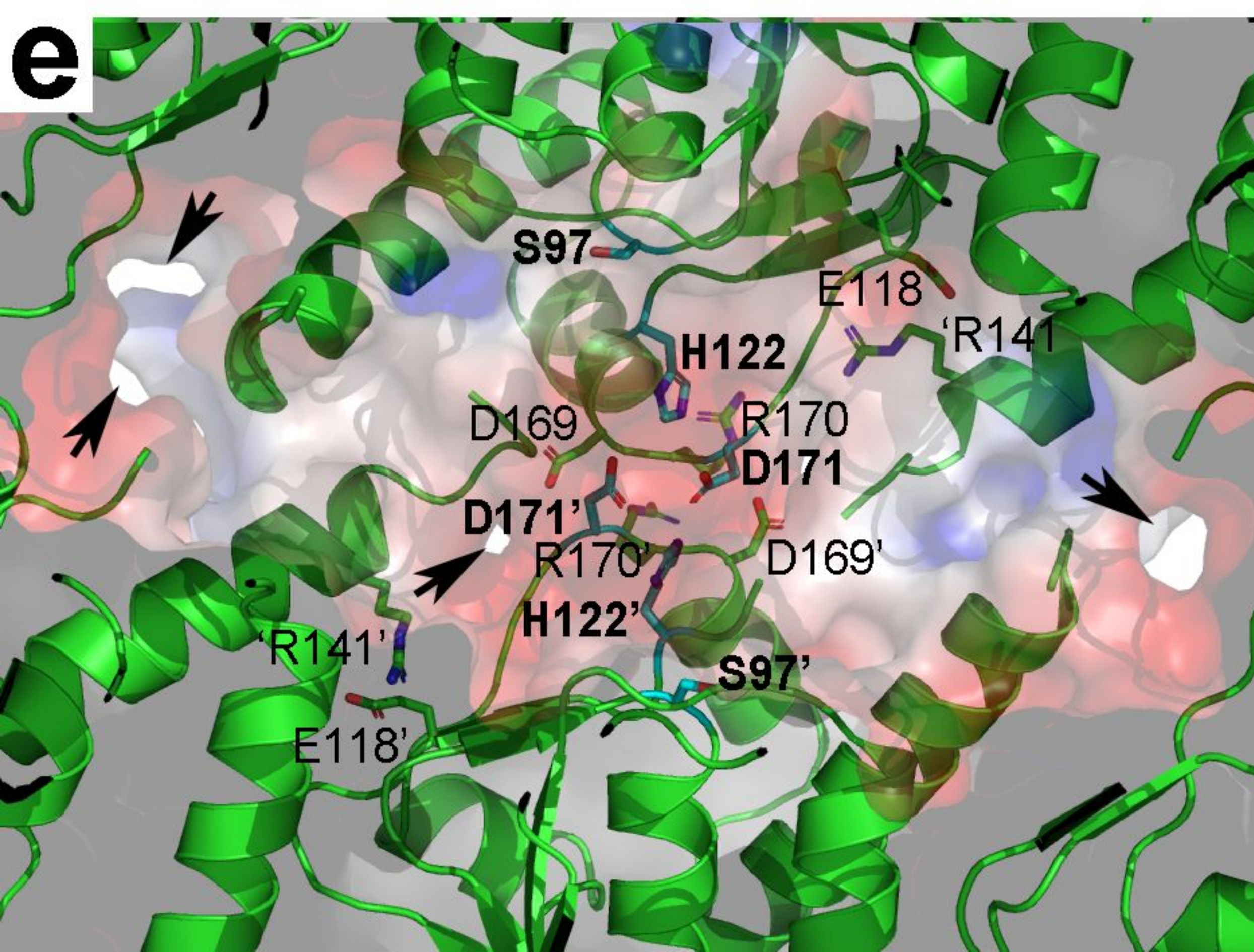
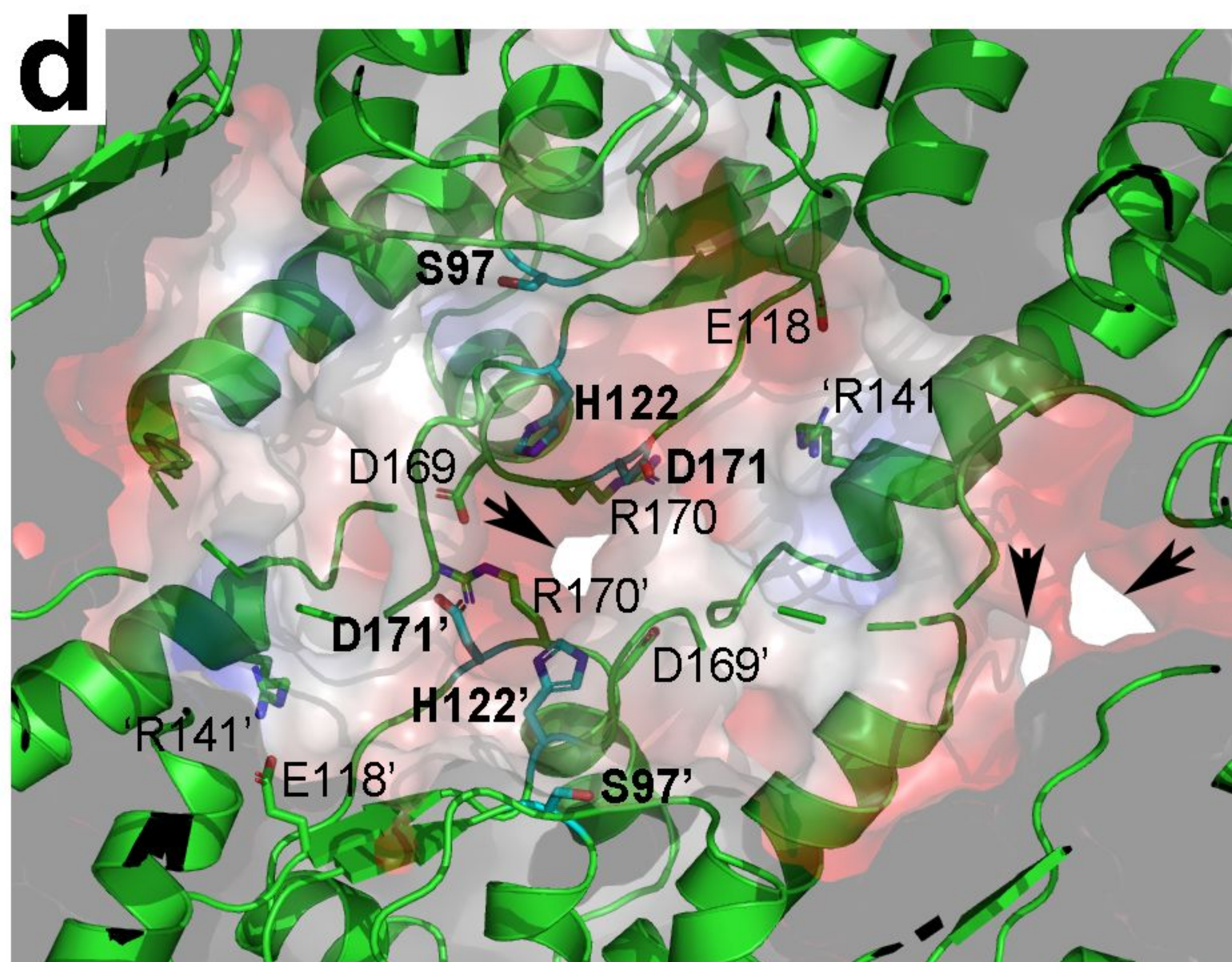
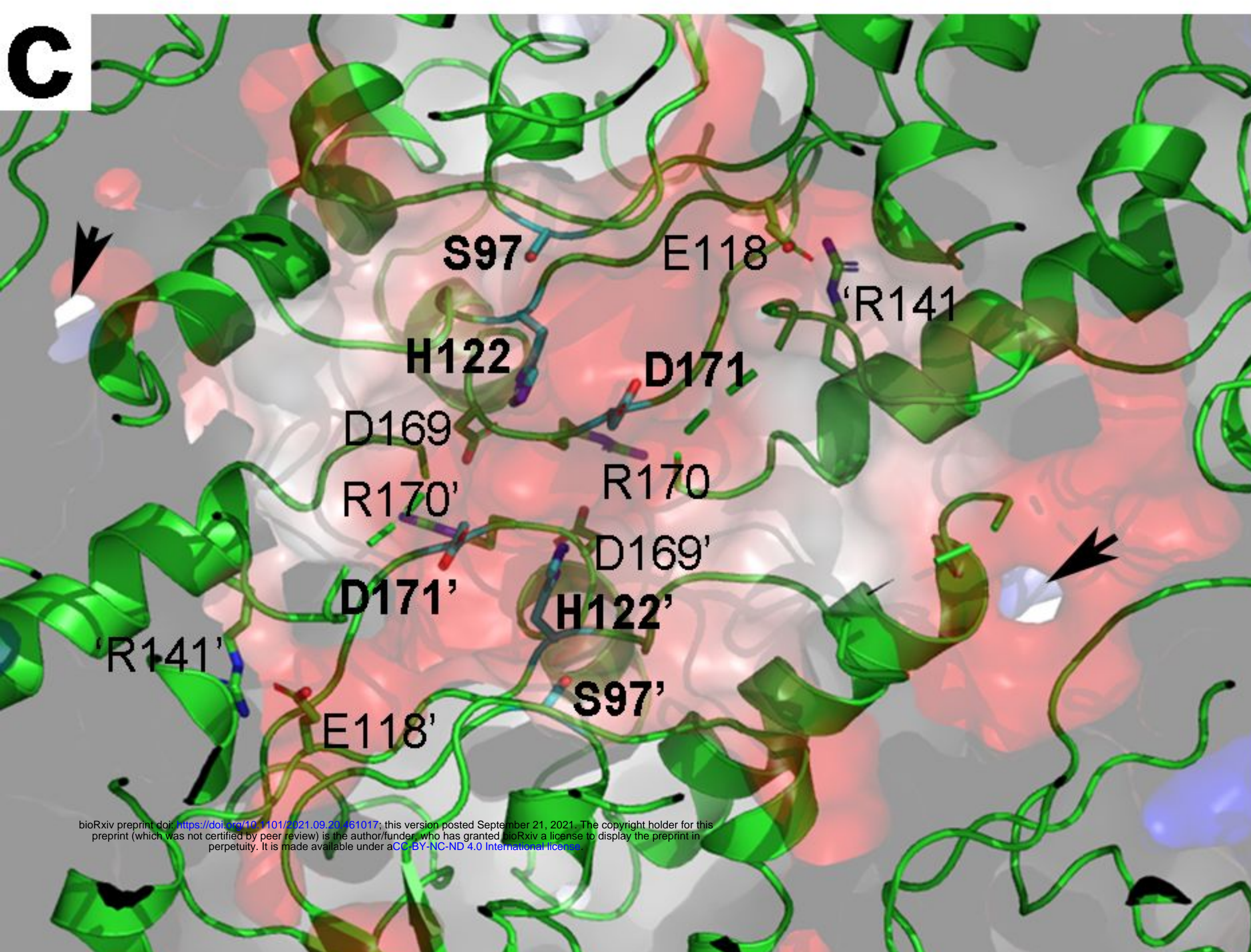
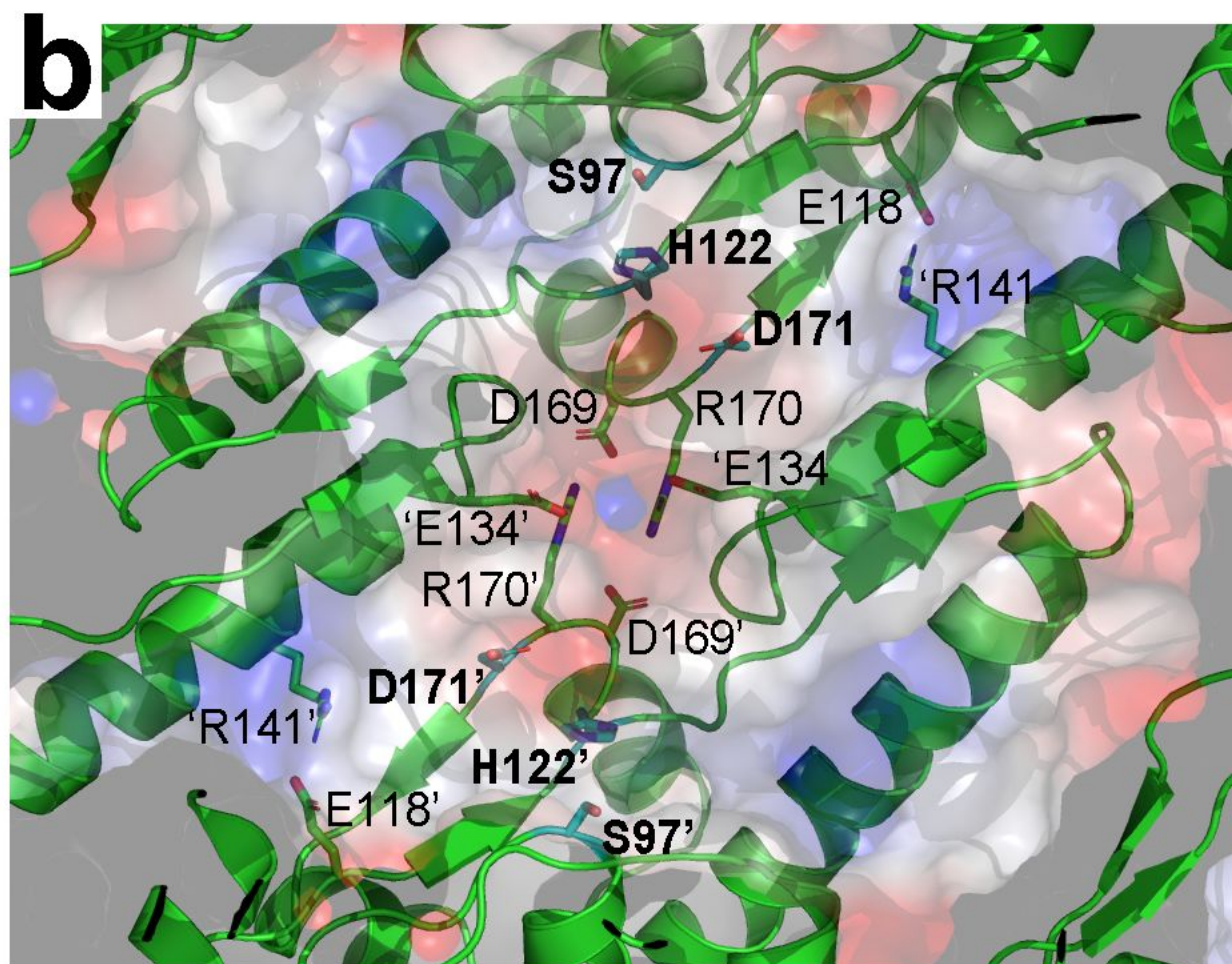
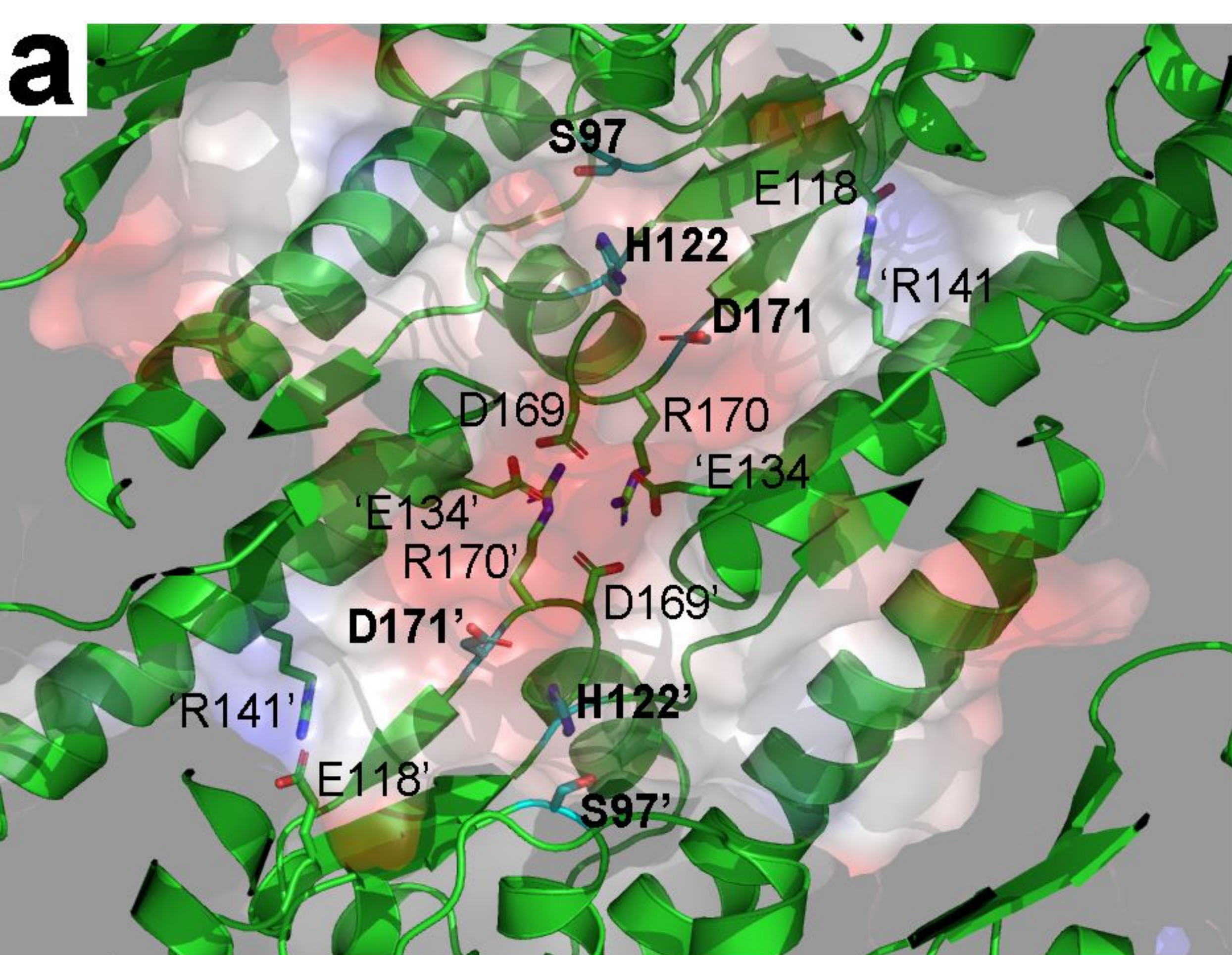


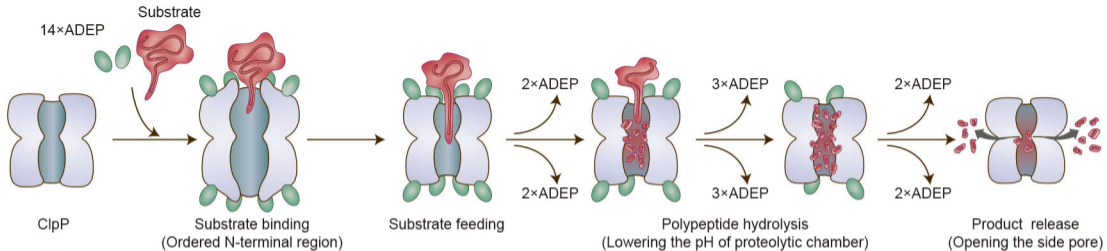
~92 Å











90°



Asymmetric pore