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► **To cite this version:**

Elisa Frezza, Tina-Méryl Amans, Juliette Martin. Allosteric inhibition of adenylyl cyclase type 5 by G-protein: a molecular dynamics study. 2020. hal-02988423

HAL Id: hal-02988423

<https://hal.science/hal-02988423>

Preprint submitted on 5 Nov 2020

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2 **Allosteric inhibition of adenylyl cyclase type 5 by G-** 3 **protein: a molecular dynamics study**

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11 **Abstract**

12

13 Adenylyl cyclases (ACs) have a crucial role in many signal transduction pathways, in particular
14 in the intricate control of cyclic AMP (cAMP) generation from adenosine triphosphate (ATP).
15 Using homology models developed from existing structural data and docking experiments, we
16 have carried out all-atom, microsecond-scale molecular dynamics simulations on the AC5
17 isoform of adenylyl cyclase bound to the inhibitory G-protein subunit Gai in the presence and in
18 the absence of ATP. The results show that Gai have significant effects on the structure and
19 flexibility of adenylyl cyclase, as observed earlier for the binding of ATP and Gsa. New data on
20 Gai bound to the C1 domain of AC5 help to explain how Gai inhibits enzyme activity and to get
21 insight on its regulation. Simulations also suggest a crucial role of ATP in the regulation of
22 stimulation and inhibition of AC5.

23 **Author summary**

24 The neurons that compose the human brain are able to respond to multiple inputs from other
25 neurons. The chemical "integration" of these inputs then decides whether a given neuron
26 passes on a signal or not. External chemical messages act on neurons via proteins in their
27 membranes that trigger cascades of reactions within the cell. One key molecule in these
28 signaling cascades is cyclic adenosine monophosphate (cAMP) that is chemically synthesized
29 from adenosine triphosphate (ATP) by the enzyme adenylyl cyclase (AC). We are investigating
30 the mechanisms that control how much cAMP is produced as a function of the signals received
31 by the neuron. In particular, we have studied the inhibition effect of a key protein, termed Gai,
32 on AC, and we compare it with the stimulator effect of another key protein termed Gsa. Using
33 microsecond molecular simulations, we have been able to show how binding Gai to AC
34 changes its structure and its dynamics so that its enzymatic activity is quenched and that ATP
35 seems to have a crucial role in the regulation of stimulation and inhibition of AC5.

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45 **Introduction**

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47 One of the most studied signal transduction pathways is the intricate control of cyclic

48 AMP (cAMP) generation, a universal second messenger based on G-protein coupled receptors
49 (GPCR) in eukaryotes [1]. cAMP has a role in a vast number of biological systems, including but
50 not limited to hormone secretion [2], smooth muscle relaxation [3], olfaction [4], learning and
51 memory [5–7].

52 The family of enzymes responsible for cAMP synthesis is the adenylyl cyclases (also
53 commonly known as adenylate cyclases) which are highly regulated in order to tightly control
54 cAMP levels [8]. Nine mammalian transmembrane ACs are recognized, with a cytoplasmic
55 domain with catalytic properties (hereafter termed AC1-9) [8]. Each member of the family has
56 specific regulatory properties and tissue distributions [9,10]; however they all convert adenosine
57 triphosphate (ATP) into cAMP via a cyclization reaction.

58 Mammalian ACs share a similar topology of a variable N-terminus (NT) and two repeats
59 of a membrane-spanning domain followed by a cytoplasmic domain [11,12]. The two
60 cytoplasmic domains, called C1 and C2, contain a region of approximately 230 amino acid
61 residues that are roughly 40% identical, called C1a and C2a: This implies a pseudosymmetry in
62 ACs. Together the cytoplasmic domains form the catalytic moiety at the interface. The NT and
63 C-terminal portion of the C1 and C2 domains, called C1b and C2b, are the most variable
64 regions among the different isoforms and can differ among the species. The catalytic site of ACs
65 is located at the C1/C2 interface and binds a molecule of ATP accompanied by two magnesium
66 ions [13].

67 ACs' function is regulated by several modulators, either stimulators or inhibitors of cAMP
68 synthesis. These include the stimulatory G-protein subunit alpha (G_{α}) which is released from
69 its cognate receptor and binds to and activates the AC enzyme via the subunit interaction with
70 the C2 domain [10,14–16] upon GPCR activation [14,16,17], the inhibitory G-protein subunits
71 $G_{\alpha i}$ and $G_{\beta\gamma}$, calcium ions, calmodulin and a variety of kinases. AC isoforms integrate several
72 signals and they differ from each other for their modulators and for the different tissues where
73 they are more abundant [18–21]. Although all nine transmembrane ACs are expressed in the

74 brain, specific ACs are particularly abundant in specific brain regions, and AC5 is highly
75 expressed in the striatum, and therefore involved in signal transduction networks that are crucial
76 for synaptic plasticity in the two types of medium spiny neurons [22].

77 Structural information on AC cytoplasmic catalytic core [21] and on a complex containing
78 both AC catalytic domains bound to an active conformation of the stimulating G α , with or
79 without a bound ATP analog, is available [23]. However, the transmembrane regions contain six
80 predicted membrane-spanning helices each and their function, aside from membrane
81 localization, is unknown. Although the mechanism of stimulation of AC by G α is relatively well
82 understood, the mechanism of inhibition of AC activity is still debated: some mutational studies
83 suggest that G $\beta\gamma$ binds in an opposite binding site [24], but there are other hypotheses, like the
84 possibility of simultaneously binding of G $\beta\gamma$ and G α or a competition between the two G
85 proteins. However, there are no data on the enzyme bound to ATP (or an ATP analog) in the
86 absence of activating G α , on the enzyme in complex with G $\beta\gamma$ in the presence and absence of
87 ATP and on the possible trimeric form G $\beta\gamma$ +AC+G α in the presence or absence of ATP. Hence,
88 it is difficult to understand how G α subunits activate/inhibit adenylyl cyclase and what is the role
89 of ATP.

90 To gain insight into the functional mechanism of AC, some studies at the molecular level
91 have been conducted using all-atom molecular dynamics (MD) simulations. In our previous
92 work, we studied the stimulation mechanism of AC5, by performing MD simulations of AC5
93 alone, AC5+ATP, AC5+G α and AC5+ATP+G α [25]. We chose the mouse AC5 isoform
94 among the other isoforms, since this isoform notably plays a key role in a variety of neuronal
95 GPCRs-based signal cascades [19,26,27]. We extensively characterized the flexibility of the
96 four states, the protein-protein interfaces, the ATP mobility, the G α binding site and the G $\beta\gamma$
97 putative binding site on C1 and the effect of the ATP and G α on these properties. Our study
98 showed that both ATP and G α binding have significant effects on the structure and flexibility of
99 adenylyl cyclase. The comparison between the simulations of AC5+ATP and AC5+ATP+G α

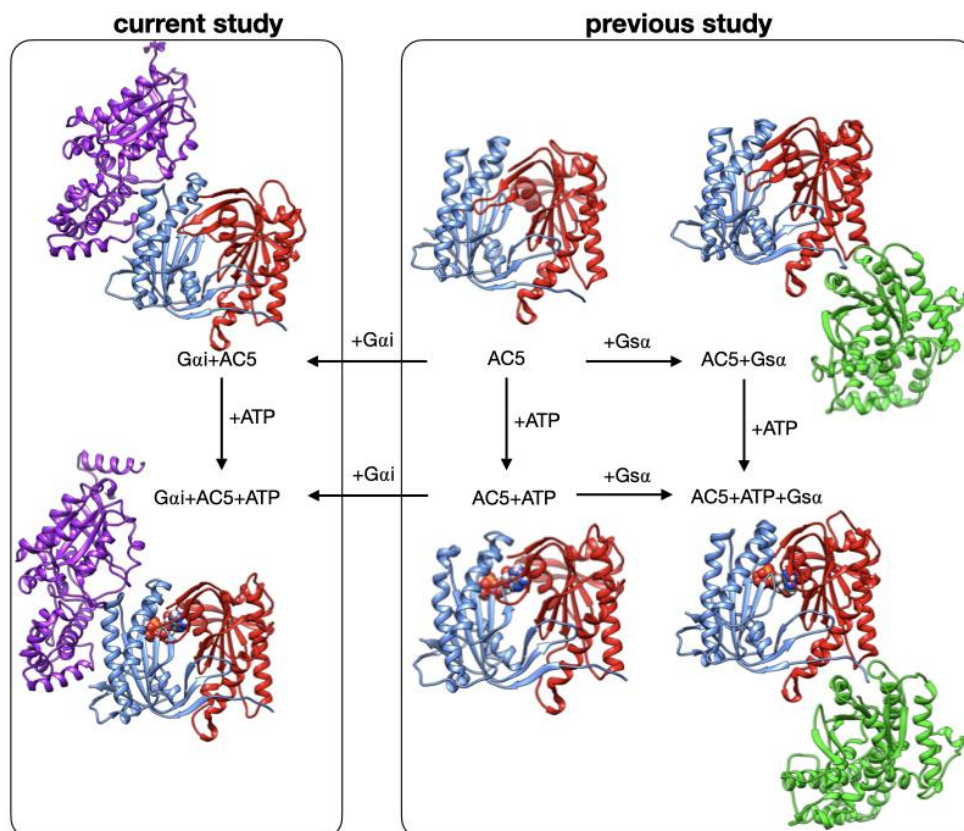
100 helped to explain how G α binding enhances enzyme activity and could therefore aid product
101 release. Our simulations also suggested that ATP binding could influence the binding of the
102 inhibitory G-protein subunit G $\beta\gamma$, if the potential binding site within domain C1 were to be
103 involved.

104 At the same time, another study by Van Keulen and co-workers has been published
105 where they investigated the mechanism of inhibition of AC5 by N-terminal myristoylated G $\beta\gamma$. In
106 their studies, they considered apo AC5 (i.e. without ATP) and they concluded that the
107 myristoylation seems to play a crucial role for the inhibition of AC5. By binding G $\beta\gamma$ to a
108 postulated C1 binding site, they found structural modifications that would disfavor both ATP and
109 G α [28]. Recently, they have also characterized the complex G $\beta\gamma$ +AC5+G α using N-terminal
110 myristoylated G $\beta\gamma$ [29,30] by comparing the different simulations in order to understand the
111 impact of the binding of both G α proteins. This comparison suggests that association of both
112 G $\beta\gamma$ and G α subunits results in an AC5 conformation similar to that sampled by the G $\beta\gamma$ +AC5
113 complex, indicating that the ternary complex mainly samples an inactive conformation.

114 Despite these recent studies, what impact G $\beta\gamma$ would have if ATP were already bound in
115 its AC5 pocket and also whether G α and G $\beta\gamma$ could nevertheless bind simultaneously to AC5 in
116 the presence of ATP is yet to be clarified. In the present study, we used the same approach
117 applied to investigate the stimulation mechanism in our previous work [25]. We have used all-
118 atom molecular dynamics simulations to study the impact of ATP and G $\beta\gamma$ on the structure and
119 flexibility of AC5. As in our previous study, we considered only the cytoplasmic domains of AC5,
120 since they are capable of reproducing many of the regulatory properties of the wild type enzyme
121 and therefore can be used as working models to investigate the regulation mechanisms of AC
122 [31,32]. Since no structural data are available for the complex AC5+G $\beta\gamma$, we computed docking
123 experiments using representative structures for G $\beta\gamma$ and AC5+ATP obtained from our MD
124 simulations and we considered two distinctive poses. The all-atom microsecond-scale
125 simulations of AC5 in complex with G $\beta\gamma$ with or without ATP studied here (see Figure 1) were

126 compared with our previous simulations of AC5, AC5+ATP, AC5+Gsa and AC5+ATP+Gsa in
127 order to help to explain how binding changes the properties of AC5 and notably to understand
128 the inhibition effect of Gai.

129



130

131

132 **Figure 1.** Structure of the cytoplasmic segment of the AC5 isoform of adenylyl cyclase and of its
133 complexes with ATP and the regulating G-proteins Gsa and Gai viewed from the side closest to
134 the cell membrane. Proteins are shown as backbone ribbons. The C1 and C2 subunits of AC5
135 are colored blue and red respectively, Gsa is colored green and Gai is colored purple. ATP is
136 shown in a CPK representation with standard chemical coloring. In each case, the structures
137 are averages taken from the molecular dynamics simulations. For the AC5 in complex with Gai
138 with and without ATP, we chose one of the docking poses we used in this work.

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142 **Results**

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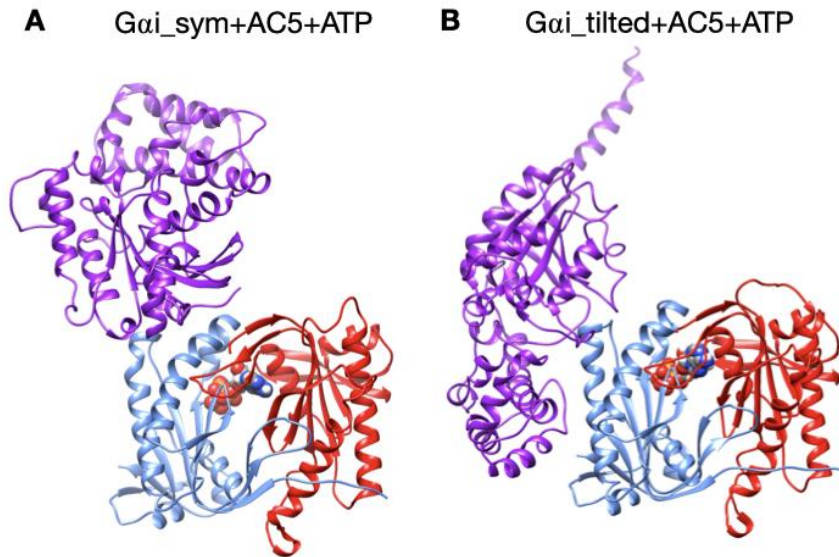
144 **Overview of simulations**

145 In the absence of any structural information on the catalytic domains of the enzyme with
146 the inhibiting G-protein subunit Gai, we use a combination of homology modelling, molecular
147 dynamics and protein-protein docking to get insight on the inhibition mechanism at molecular
148 level and the impact of the ligand or protein on the conformation and dynamics of AC5.

149 We have studied the behavior of two molecular species (see Figure 1): AC5 bound to
150 the inhibiting G-protein subunit Gai (Gai+AC5) and AC5 bound to both ATP and Gai
151 (Gai+AC5+ATP). For both complexes, we considered two different relative conformations: one
152 called Gai_sym+AC5 where the Gai protein has an orientation symmetrical to the Gsa protein in
153 the AC5+Gsa complex, presented in Figure 2A, and one called Gai_tilted+AC5, where the Gai
154 protein is tilted, presented in Figure 2B. For each of these species, we generated 1.5 μ s MD
155 trajectories in an aqueous environment with a physiological salt concentration (0.15 M KCl)
156 using the GROMACS 5 package [33–36] with the Amber 99SB-ILDN force field for proteins
157 [37,38]. The first 400 ns of each trajectory were treated as equilibration of the system and
158 analysis was carried out only on the remaining 1.1 μ s. We analyzed all-atom MD simulations
159 using average structures, time-averaged properties, angles and distances between helices (see
160 Figure 3), specific geometrical measurements to characterize protein-protein and protein-ligand
161 interface and residue-by-residue conformational and dynamic properties. In order to understand
162 the effect of Gai and ATP on AC5, we used the MD simulations for isolated AC5, AC5 with ATP
163 and two Mg²⁺ ions in its active site (AC5+ATP), AC5 bound to the activating G-protein subunit

164 G α (AC5+G α +GTP) and AC5 bound to both ATP and G α (AC5+ATP+G α +GTP) obtained in
165 our previous work [25,39]. Data are shown in Table S1-S3 and in Figure S3-S12.

166



167

168 **Figure 2.** The two different configurations of the Gai+AC5+ATP complex simulated in this study.

169 A: Gai_sym+AC5+ATP: Gai has an orientation symmetrical to the G α protein in the AC5+G α

170 complex. B: Gai_tilted+AC5+ATP: Gai protein is tilted with respect to AC5.

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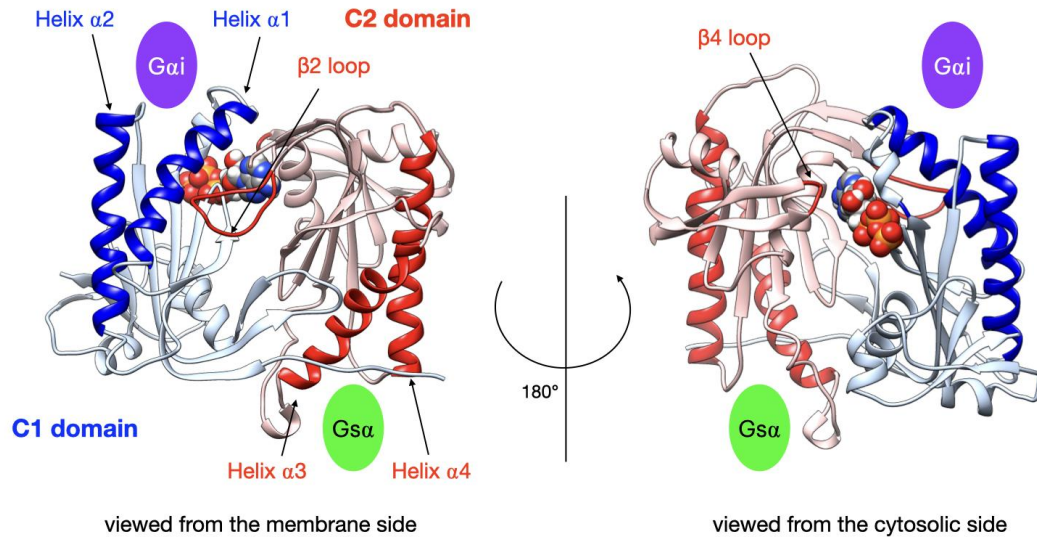
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179 **Figure 3.** Illustration of the key regions of AC5 catalytic domain structure, with bound ATP. The
180 C1 domain is colored blue and the C2 domain in red, with relevant parts in darker color: the
181 helices of C2 involved in binding of the stimulatory protein Gs α , the helices of C1 involved in
182 binding of the inhibitory protein Gai, the $\beta 2$ loop of C2 (left side) and the $\beta 4$ loop of C2 (right
183 side) which bears the catalytic Lysine residue. The green oval indicates the binding site of Gs α
184 and the purple oval indicates the binding site of Gai.

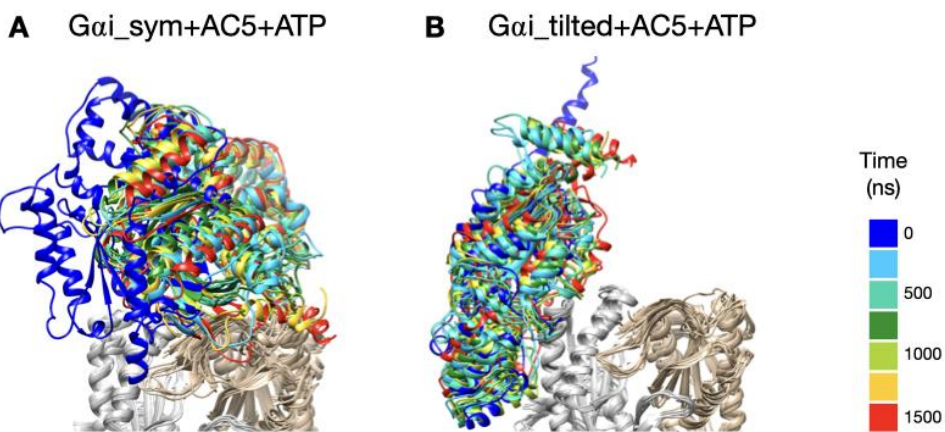
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186 **Stability of Gai+AC5 complexes in the presence and in the absence of ATP**

187 The two types of complexes behave differently in the presence of ATP. In the
188 Gai_{sym}+AC5+ATP simulation, the Gai protein reallocates significantly with respect to AC5
189 toward the C2 domain, ending in a configuration where it is in contact with the C2 domain, see
190 Figure 4A. The peculiarity of this system is also apparent in the rest of the study and will be
191 commented later. On the contrary, in the Gai_{tilted}+AC5+ATP system, the Gai protein
192 fluctuates around its initial position, without significant reallocation, indicating that this complex
193 is very stable, see Figure 4B. We quantified the fraction of interface contacts between Gai and
194 AC5 that are conserved throughout the simulation time, and the total number of interface
195 contacts as a proxy of the interface size. For Gai_{sym}+AC5+ATP, the fraction of conserved

196 contacts at the Gai/AC5 interface drops rapidly below 25%, while the total number of contacts
197 increases significantly (see Figure S1). In Gai_tilted+AC5+ATP, 50 to 75% of the initial contacts
198 are conserved during the simulation time, and the total number of contacts also tends to
199 increase, although less dramatically (Figure S1). In the absence of ATP, both systems maintain
200 between 50 and 75% of their initial contacts, with more moderate reallocation and variation in
201 terms of contact number (see Figure S1 and S2).

202



203

204 Figure 4. Snapshots of the Gai+AC5+ATP complexes observed during the simulations, viewed
205 from the membrane side. Gai structures extracted every 250 ns are colored on a rainbow scale
206 from blue to red. The C1 domain of AC5 is colored in grey and the C2 domain in beige.

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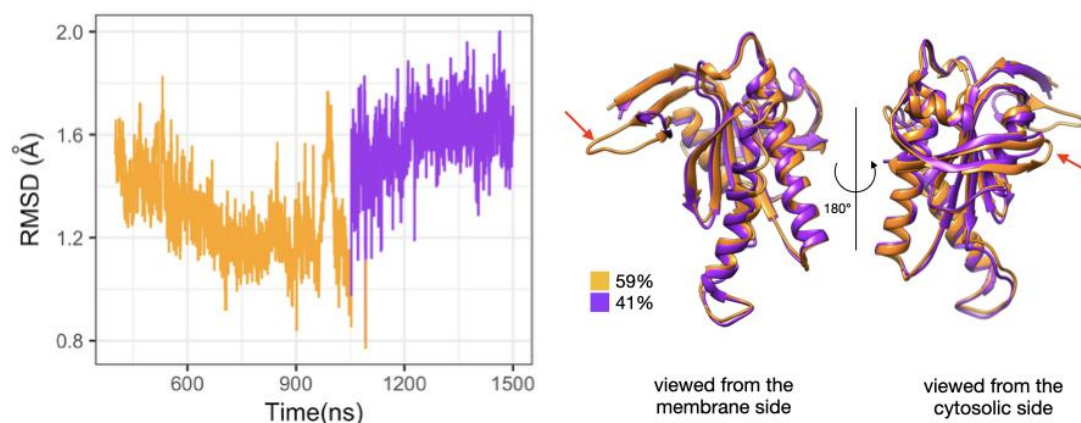
213 **Impact of Gai on AC5+ATP**

214

215 We begin by considering the global impact of Gai on AC5+ATP by computing the RMSD
216 on backbone atoms separately on each AC5 domain. RMSD calculations with respect to the
217 average MD structure of each AC5 domain show that Gai binding has a significant effect on
218 both the structure and the dynamics of the enzyme (see Table S1, and Figure S3 where in order
219 to allow comparison with the results obtained in our previous study [25] values for AC5,
220 AC5+ATP, AC5+Gsa, AC5+ATP+Gsa were also included). On the one hand, the domain C1 is
221 slightly rigidified by the binding of Gai. On the other hand, the C2 domain visits several
222 conformational substates involving the ATP binding pocket (β 2 loop and β 4 loops) in
223 Gai_sym+AC5 complex (see Figure 5). These two substates also lead to two different substates
224 for ATP (see Figure S4) which is more mobile, increasing the average RMSD from 0.6 Å for
225 AC5+ATP to 0.9 Å for Gai_sym+AC5+ATP (see Table S3 and Figure S5). In the case of
226 Gai_tilted+AC5+ATP, the C2 domain visits a specific substate close to the one sampled in
227 AC5+ATP where the mobility of ATP is unchanged due to the presence of Gai.

228 The presence of several substates upon binding of Gai is in contrast with the
229 stabilization of a specific substate upon binding of ATP and/or Gsa. Indeed, in our previous
230 work, we observed that the C2 domain can investigate several substates when AC5 is isolated,
231 and the presence of either ATP alone or ATP and Gsa stabilizes two distinct substates. In the
232 former case, a single substate for the β 2 loop is selected (the longest-lived substate in isolated
233 AC5) in a close conformation. In the latter, an opening of loop β 2 away from the active site is
234 observed. The selection of a specific substate is correlated to the mobility of ATP and its
235 reactivity [25].

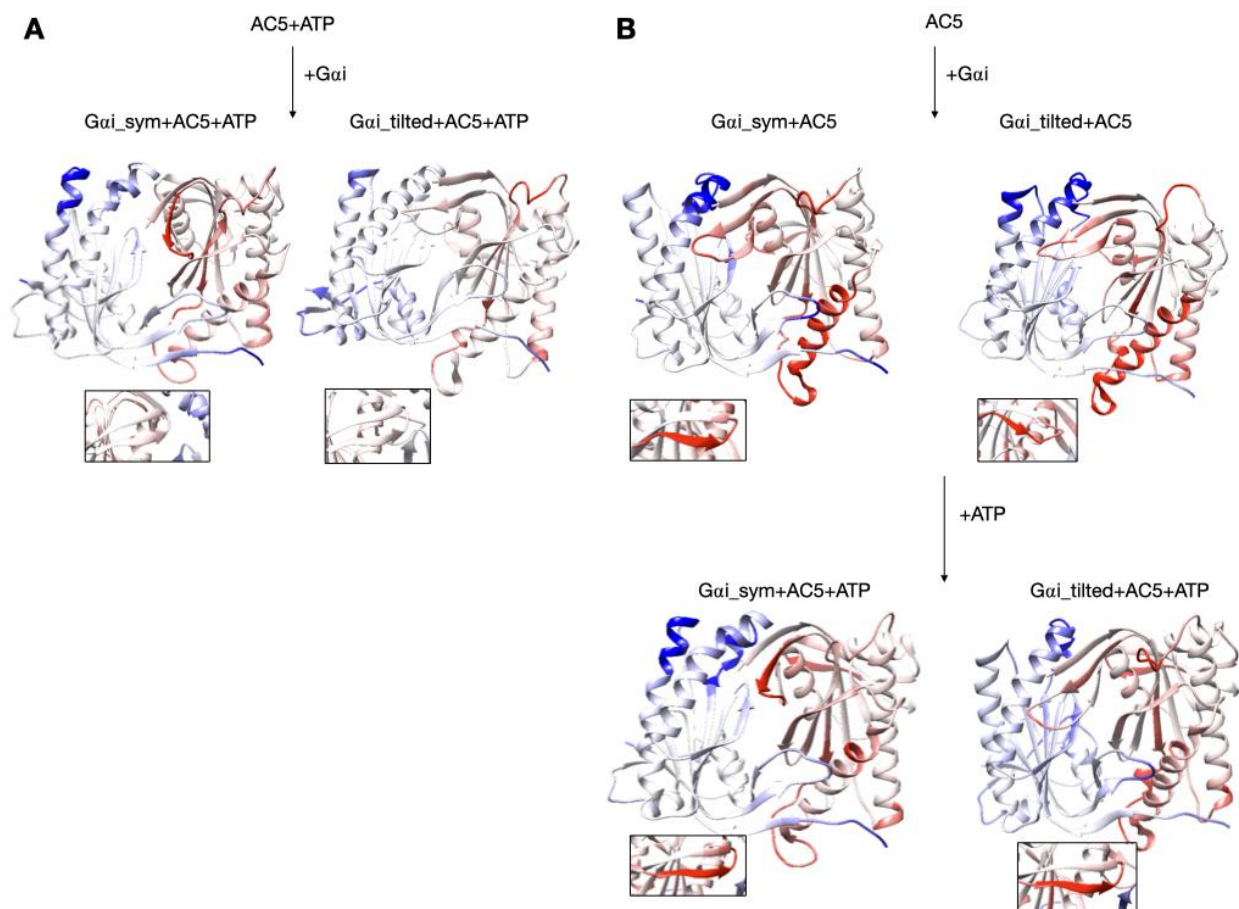
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237
238 Figure 5. Sub-states of domain C2 observed during the simulation of Gai_sym+AC5+ATP
239 complex. Left side: RMSD time series for the C2 domain, colored according to cluster
240 membership. Right: structures closest to the center of each cluster, and relative size of each
241 cluster as percentages. Prominent structural changes are indicated by red arrows.

242
243 Despite the decrease in flexibility of the C2 domain, also observed when Gas is bound to
244 AC5+ATP, in the presence of Gai, ATP is still rather mobile (see Table S3) and for
245 Gai_sym+AC5+ATP an increase in mobility is observed: this impact is opposite to the one
246 observed in AC5+ATP+Gsa, where a higher stability of ATP is observed (average RMSD equal
247 to 0.3 Å). In both simulations in the presence of ATP, the interactions between the terminal
248 phosphate group of ATP and Lys 1065 (belonging to loop β 2) and the interactions between the
249 penultimate phosphate and Arg 1029, a key functional residue, are absent (see Figure S6): the
250 arginine side chain is separated from its target oxygen atom by roughly 9 Å. Moreover, it is
251 known that ATP has stronger interactions with the C1 domain via its associated two Mg²⁺ ions,
252 notably with residues Asp 396 and Asp 440. For Gai_tilted+AC5+ATP, these interactions are
253 stable and are not affected by the presence of Gai. On the contrary, for Gai_sym+AC5+ATP,
254 these interactions are absent justifying the increase of ATP mobility (see Table S3).

255 Gai binding also turns out to have more global effects on AC5+ATP. First, the angles
256 and the distance between the pairs of α -helices in both AC5 domains are modified. In
257 Gai_sym+AC5+ATP, the angle between the helices α_1 and α_2 in domain C1 is significantly
258 reduced (by 9° , see Table S1) and the angle the helices α_3 and α_4 in domain C2 is slightly
259 increased (by 4°). In Gai_titled+AC5+ATP, an opposite effect is observed: the angle between
260 the helices α_1 and α_2 in domain C1 is slightly affected (increased by 1° , see Table S1 and
261 Figure S7) and the angle between the helices α_3 and α_4 in domain C2 is slightly decreased (by
262 3°). In addition, the distance between the C2 helices in Gai_titled+AC5+ATP complexes is
263 maintained around 13 Å, whereas our earlier results indicate that it is around 16 Å in the
264 AC5+ATP+Gs α complex (see Table S1 and Figure S8). In both simulations, the C1/C2 interface
265 remains mostly as tight as in isolated AC5+ATP (gap index from 2.8 Å for AC5+ATP to 2.9 Å
266 once Gai is bound, Table S1 and Figure S9) involving a movement of helix α_3 (see Figure 6). In
267 terms of flexibility, Gai binding mainly flexibilizes the binding site region of ATP in AC5, although
268 it also decreases the flexibility of the C-terminals of helices α_1 and α_3 (see Figure 7A).
269



270
271 Figure 6: Changes in conformation induced by Gai protein. A: scenario where ATP is already
272 bound to AC5 when Gai interacts, B: scenario where ATP is not yet bound to AC5 when Gai
273 interacts. More intense colors (blue for domain C1 and red for domain C2) correspond to larger
274 movements compared to the preceding structure (i.e. AC5+ATP for A and AC5 and AC5+Gai for
275 B) on a scale of 0 to 4 Å. The insets display the β 4 loop.

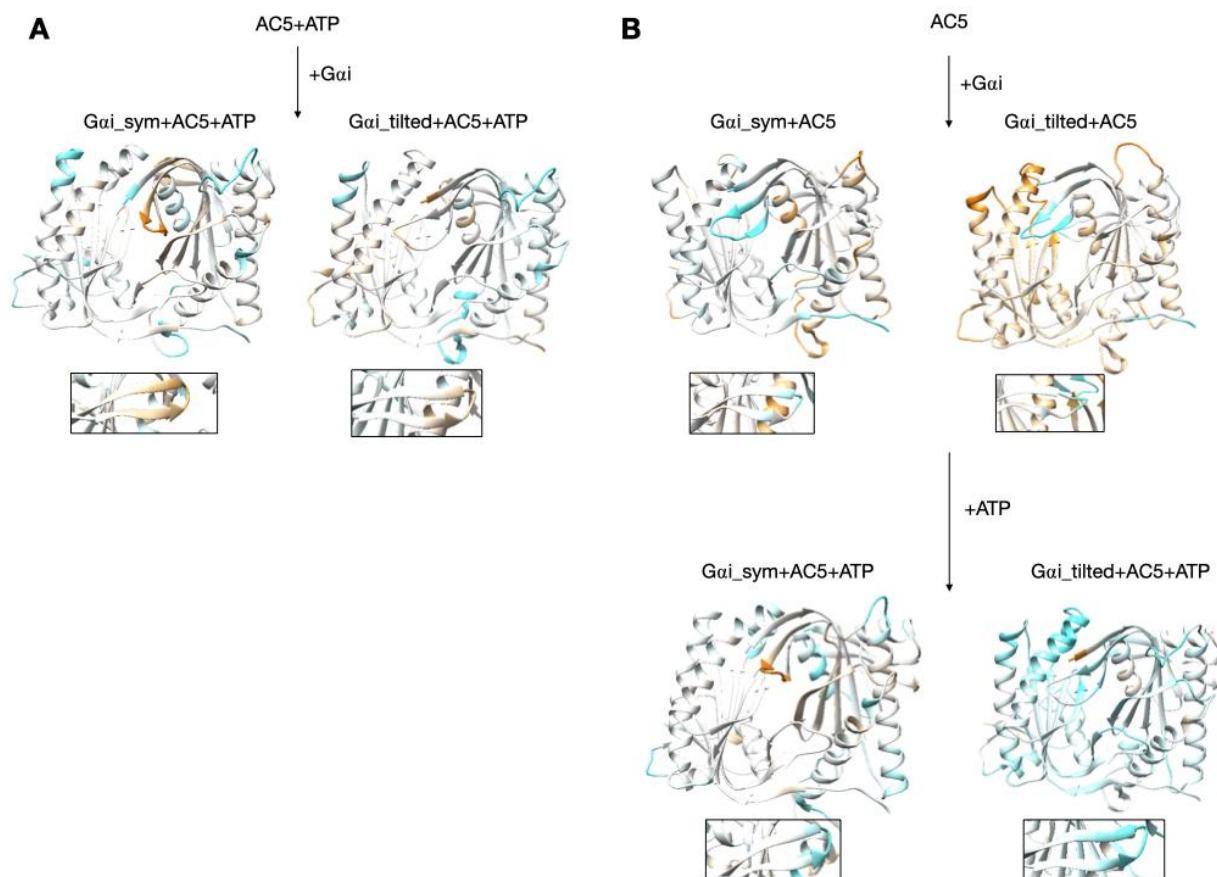
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281
282 Figure 7: Changes in flexibility induced by G proteins. A: scenario where ATP is already bound
283 to AC5 when Gai interacts, B: scenario where ATP is not yet bound to AC5 when Gai interacts.
284 More intense colors (orange for increased flexibility and cyan for decreased flexibility)
285 correspond to differences with respect to the preceding structure on a scale of -1.2 to +1.2 Å.
286 The insets display the β 4 loop.

287
288 **Impact of Gai on apo AC5 and further impact on ATP on AC5+Gai**

289 Although it seems probable that ATP is already bound to AC5 based on our previous
290 study [25], we also consider the scenario where ATP is not already present when Gai binds on
291 AC5. We begin by considering the global impact of Gai on AC5. Gai_sym and Gai_tilted have
292 different effects on the C1 domain of AC5: Gai_sym slightly rigidifies it as attested by the RMSD

293 calculation (Table S1 and Figure S3), whereas Gai_tilted flexibilizes it, and this flexibility
294 concerns the binding helices $\alpha 1$ and $\alpha 2$ (Figure 7B). For the C2 domain, in both Gai_sym+AC5
295 and Gai_tilted+AC5 complexes, the $\beta 2$ loop and the $\beta 4$ loop are rigidified upon addition of Gai
296 (see Figure 7B). Their conformations slightly differ in both complexes: the $\beta 2$ loop is more
297 closed with Gai_tilted than with Gai_sym whereas the $\beta 4$ loop, at the back of the structure, is
298 half open with Gai_tilted compared to with Gai_sym (see Figure S10). In Gai_sym+AC5, the C2
299 domain visits several conformational substates involving the helices $\alpha 3$ and $\alpha 4$ (see
300 Figure S11).

301 The conformation of the binding helices in both AC5 domains is significantly altered by
302 Gai. Gai notably displaces helix $\alpha 3$ (Figure 6B). In both Gai_sym+AC5 and Gai_tilted+AC5, the
303 angle between the helices $\alpha 1$ and $\alpha 2$ in domain C1 is significantly increased (by 24° and 19° ;
304 see Table S1 and Figure S7). On the contrary, the angle between the helices $\alpha 3$ and $\alpha 4$ in
305 domain C2 is significantly decreased (by 7° and 12°), and the helices are also closer to each
306 other (Table S1 and Figure S8). The C1/C2 interface remains as tight as in isolated AC5, in
307 contrast with what we observed previously with the binding of Gsa, which resulted in a looser
308 C1/C2 interface (gap index equal to 3.8 Å, see Table S2 and Figure S9).

309 In the scenario where ATP is not already bound to AC5 when Gai interacts, we can also
310 analyze the effect of ATP addition on the pre-formed Gai+AC5 complex. As shown in Figure 7B,
311 the addition of ATP notably rigidifies AC5. The interface at the Gai/AC5 interface is quite loose
312 in the Gai_tilted+AC5 complex (gap index equal to 4.6 Å, see Table S2) and the addition of ATP
313 tends to tighten this interface (gap index equal to 4.2 Å). On the contrary, in the Gai_sym+AC5
314 complex, the initial Gai/AC5 interface is made more loose by the addition of ATP (gap index
315 from 5.4 Å in Gai+AC5 to 3.2 Å when ATP is bound), probably due to the change of interface as
316 observed by the losing of 75% of the native contacts by adding ATP (see Figure S1). By
317 comparison, in the AC5+Gsa complex, the gap index decreased from 3.2 Å to 2.7 Å upon ATP

318 addition (Table S2). Despite the variation of the values of gap index for the Gai/AC5 interface
319 upon binding of ATP, all the values are typical of obligate protein-protein interfaces [40].

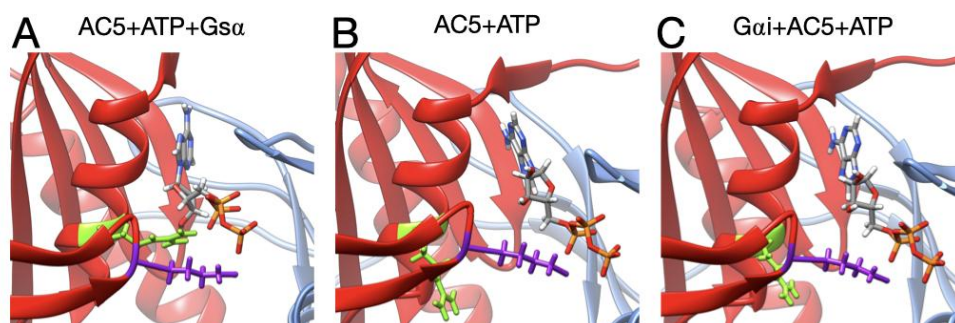
320

321 **Discussion**

322 As already observed in our previous work [25], microsecond-scale simulations are
323 necessary to investigate the allosteric coupling existing within AC5 and the effect of the binding
324 of Gai. As van Keulen and Rothlisberger [28], we studied the scenario where Gai binds to AC5
325 in the absence of ATP, but we could not exclude the possibility that ATP is bound on AC5 when
326 Gai binds, based on our previous work [25]. For the lack of structural information on the
327 complex Gai and AC5, we considered two different docking poses in our study: Gai_sym and
328 Gai_tilted. In the former, the Gai protein is bound to AC5 in a symmetrical fashion compared to
329 what is known for the AC5+ATP+Gsa complexes (Figure 2A). In the latter, the Gai protein is
330 rotated and tilted onto the C1 domain (Figure 2B). Both complexes stay bound during the
331 simulations, but a greater stability is obtained for the Gai_tilted configuration, suggesting more
332 biological relevance. In the case where Gai binds on AC5+ATP in a symmetrical fashion, an
333 allosteric effect is observed: a closure on the Gai site is coupled with an opening on the Gsa site
334 and an opening of the $\beta 2$ loop of C2, as observed for Gsa. Despite that, this conformation
335 seems to be less likely because the complementarity is very low and the interface is unstable,
336 as already mentioned above. On the contrary, the Gai_tilted configuration is also very similar to
337 the one reported by van Keulen and Rothlisberger in their recent work where they studied the
338 complex between myristoylated Gai and AC5 in the absence of ATP [28], although the starting
339 conformation of AC5 and Gai are quite different. The binding of Gai slightly rigidifies the C2
340 domain in all simulations and an opening of Gai binding site is coupled with a closure of the Gsa
341 binding site in particular in the Gai_tilted+AC5 complexes with and without ATP, as observed in

342 van Keulen and Roethlisberger's simulation [28]. As in the case of AC5+G α complex, ATP
343 stabilizes the G α i/AC5 interface when G α i is tilted, whereas the latter is less complementary. All
344 these changes involve coupling through AC5 over distances of tens of angstroms.

345 Turning now to the enzymatic function of AC5, it is known that specific hydrogen bonds
346 between AC5 and ATP play an important role in the production of cAMP from ATP as already
347 shown in hybrid QM/MM free energy calculations, notably the hydrogen bond between the
348 highly conserved Arg 1029 and the primary phosphate group of ATP [41]. The present
349 simulations show that this interaction is not formed upon binding of G α i and other ones are lost
350 (for example between Lys 1065 and ATP, see Figure S11), when on the contrary it is formed
351 upon binding of G α , see Figure 8.



352
353 Figure 8: Interactions between ATP and key residues in different complexes. A: active
354 AC5+ATP+G α , B: inactive AC5+ATP complex, C: inactive G α i-tilted+AC5+ATP complex. L of
355 C2 are shown as sticks and colored in purple (Lys 1065) and green (Arg 1029). For clarity, the
356 region 394-428 of C1 has been omitted from the representation.

357
358 The binding of G α i inhibits AC5 by increasing the flexibility of the active site allowing a
359 high mobility of ATP without changing the complementarity of the C1/C2 interface. The further
360 inactivation of AC5+ATP by G α i does not allow to exclude the possibility that ATP is bound to
361 AC5 during the inhibition process and it may have a role in the tight regulation of the enzyme.

362 Based on our previous study [25] and the current one, we can speculate on the
363 regulation mechanism of AC5 by considering only the Gai_tilted configuration. In the absence of
364 ATP, based on our simulations, only G α could interact with AC5, due to the close conformation
365 of the binding site on the C1 domain. However, we cannot exclude the existence of Gai+AC5
366 based on our study. The binding of ATP induces an opening of the angle between the pair of α -
367 helices of domain C1 (α_{C1}) and a closure of the pair of α -helices of domain C2 (α_{C2}), which have
368 a similar value close to the one observed upon binding of G α . If Gai binds first AC5+ATP, the
369 closure of the angle α_{C2} does not allow the binding of G α and the ATP pocket remains close.
370 When Gai dissociates from AC5+ATP, AC5 undergoes a conformational change that allows the
371 binding of G α . On the contrary, if G α binds first AC5, the enzyme is active thanks to the
372 stabilization of ATP in its pocket and the formation of specific hydrogen bonds and cycles
373 between AC5+ATP+Gs (favourable to Gi binding) and AC5+Gs (unfavourable to Gi binding). If
374 G α s dissociates, after cAMP release, AC5 is an apo conformation and there is no need for
375 further inhibition. If G α s dissociates from AC5+ATP, then the conformation of AC5 becomes
376 accessible to Gai for the inhibition. Another possibility is that due to the open conformation of
377 the binding site on the C1 domain, Gai can bind to the AC5+ATP+G α s complex to form a
378 ternary complex, whose existence is still unknown.

379

380

381

382 **Conclusions**

383

384 We perform all-atom molecular dynamics simulations in an attempt to better understand
385 the regulation of adenylyl cyclase, a key enzymatic player in cellular signalling cascades.
386 Microsecond-scale simulations of the G-protein subunit Gai bound to adenylyl cyclase in the

387 presence and in the absence of ATP in two different conformations help to better understand
388 some features of this important signal transmission protein since no structural information on
389 this complex is available. They notably provide information on a single, non-chimeric adenylyl
390 cyclase isoform, AC5, bound to the inhibitory G-protein in the presence and in the absence of
391 ATP.

392 The simulations show that protein binding creates significant changes in the structure
393 and in flexibility, throughout AC5 and due to a strong allosteric coupling existing within AC5 in a
394 different fashion than the stimulatory G-protein and ATP. They provide data that help to explain
395 the inhibition action of G α i, whose binding increases the conformational and positional
396 fluctuations of ATP in the active site of AC5 and its flexibility by moving away the key residues
397 involved in the enzymatic reactions.

398 Our results also show that G α i binding to the C1 domain does not impact C1/C2
399 interface complementarity, flexibilizes C1 domain and significantly closes the angle between the
400 C2 α -helices that cannot bind G α s when G α i is in tilted conformation. The simultaneous binding
401 of ATP and G α i in a titled conformation at the AC5 interface results in a rigidification of the C2
402 domain, without affecting the C1/C2 interface complementarity, and a slight increase of the
403 angle between the C2 α -helices. Hence, G α i has an important impact on AC5 dynamics and its
404 effects are enhanced when ATP binds, by increasing the conformational freedom of the bound
405 ligand, thus putting it in an unfavourable configuration within its binding site and not allowing to
406 establish key interactions between ATP and AC5 that leave therefore AC5 completely inactive.

407 Our simulations also show that ATP has a crucial role in the regulation of AC5 and we
408 cannot exclude the presence of ATP during the inhibition. Our previous simulations already
409 showed that ATP binding could influence the binding of the inhibitory G-protein subunit α i at the
410 domain C1. Here, we propose that the presence of ATP is needed to induce the competition
411 between G α s and G α i to tightly regulate AC5. However, based on our results, we cannot
412 exclude the existence of the G α i+AC5+ATP+G α s complex and the G α i+AC5+G α s complex. For

413 the latter, other molecular dynamic studies of this hypothetical ternary complex concluded that if
414 existing it would be inactive [29,30]. Further studies have to be conducted to shed lights on this
415 point.

416

417 **Methods**

418 **Models**

419 Models of the cytoplasmic domains of AC5 and Gai protein were built by homology to known
420 proteins using Modeller v9.12 [42]. In each case, 100 homology models were generated and the
421 model with the lowest DOPE score [43] was selected. For AC5, a homology model for the
422 mouse sequence with bound ATP was generated using with structure 1CJK [13] as template.
423 The identity percentage between template and model is equal to 98% for the C1 domain and
424 57% for the C2 domain. Since we observed in our earlier study [25] that the conformation of
425 AC5 is affected by the binding of G proteins, we used the structure closest to the centre of the
426 largest cluster of the last 500 ns of the MD simulation of AC5+ATP [25], in the absence of G
427 proteins.

428 For Gai, a homology model for the mouse sequence bound with GSP and Mg ion was
429 generated using Modeller with three templates: 1CJK [13] (bovine G α 38% sequence identity),
430 1AS3 [44] (rat Gai, 81% identity), and 1AGR [45] (rat Gai, 87% sequence identity). This model
431 was used in docking (see below). In addition, we considered a model of Gai sampled from MD
432 simulation: a simulation of 1 μ s was run starting from the homology model. The structure closest
433 to the center of the largest cluster observed during the simulation was used for docking.

434 Models of AC5 and Gai were docked using CLUSPRO [46–48] to generate
435 Gai+AC5+ATP complexes shown in Figure 2, using known interface residues on both proteins
436 as restraints (see Figure S12). A first complex was built by docking the model of AC5 sampled

437 from simulation with the homology model of Gai. The resulting complex locates Gai in an
438 orientation similar to Gsa with respect to AC5 in the 1CJK complex, with the G protein binding in
439 the groove formed by the two α -helices (see Figure 2A). This orientation is called the
440 symmetrical orientation. Resulting complex is denoted Gai_sym+AC5+ATP.

441 A second complex was built by docking the model of AC5 sampled from MD simulation
442 with the model of Gai sampled from MD simulation. In the resulting complex, Gai is tilted
443 compared to the Gsa orientation with respect to AC5: the G protein is in contact not only with
444 the helix groove, but also with residues on the side of the C1 domain (see Figure 2B). Resulting
445 complex is denoted Gai_tilted+AC5+ATP.

446 Available structures of Gai display different conformations of the N-terminal helix: either
447 protruding from the structure (in 1AGR) or packed onto the structure core (in 1AS3). In the initial
448 model, this helix was packed. During the simulation of Gai, this helix appeared very mobile. In
449 order to minimize possible bias and to avoid Periodic Boundary Condition problems in the
450 simulations of Gai+AC5 complexes, we manually unpacked the N-terminal helix from the
451 structure core after the docking step.

452 Systems without ATP were also simulated, starting from the same systems after ATP
453 removal. Throughout the study, we compared our results with those obtained in our previous
454 study of AC5 alone and in complex with the activating protein Gsa [25] (see Figure 1, right
455 box).

456

457 **All-atom molecular dynamics simulations**

458 Molecular dynamics simulations were performed with the GROMACS 5 package [33–36,49]
459 using the Amber99SB-ILDN force field for proteins that has been shown to yield an accurate
460 description of many structural and dynamical properties of proteins [38,50–52]. Side chain
461 protonation states of titratable amino acids were assigned using a value of pH = 7.4 with the
462 help of the pdb2pqr software [53]. Capping acetyl and methyl-amino groups were added to the

463 N and C termini of both AC5 domains and Gai. The four states we study (Gai_sym+AC5,
464 Gai_sym+AC5+ATP, Gai_tilted+AC5, Gai_tilted+AC5+ATP) were each placed in a truncated
465 octahedral box and solvated with TIP3P water molecules [54] to a depth of at least 11 Å. The
466 solute was neutralized with potassium cations and then K+Cl⁻ ion pairs [55] were added to
467 reach a physiological salt concentration of 0.15 M. Parameters for ATP and GTP were taken
468 from [56]. The parameters for Mg²⁺ came from [57]. This new set of parameters was developed
469 to improve the kinetic properties of Mg²⁺ ions with water and with the phosphate ion and it was
470 implemented in Amber99. This new set of parameters also provided a better description of the
471 structure of Mg²⁺-phosphate binding than previous sets (these interactions are naturally
472 important in our simulations in the presence of ATP) [57]. Hence, the combination of Amber
473 99SB-ILDN and the new set of parameters of Mg²⁺ ions is currently the best choice to reproduce
474 the dynamics of AC5 and Gα, and to properly describe the interactions of Mg²⁺ with AC5 and
475 ATP.

476 Long-range electrostatic interactions were treated using the particle mesh Ewald
477 method [58,59] with a real-space cutoff of 10 Å. We used virtual interaction sites for the
478 hydrogens and bond lengths were restrained using P-LINCS [36,60], allowing a time step of 4
479 fs [61]. Translational movement of the solute was removed every 1000 steps to avoid any
480 kinetic energy build-up [62]. After energy minimization of the solvent and equilibration of the
481 solvated system for 10 ns using a Berendsen thermostat ($t_T = 1$ ps) and Berendsen pressure
482 coupling ($t_P = 4$ ps) [63], the simulations were carried out in an NTP ensemble at a temperature
483 of 310 K and a pressure of 1 bar using a Bussi velocity-rescaling thermostat [64] ($t_T = 1$ ps) and
484 a Parrinello-Rahman barostat ($t_P = 1$ ps) [65]. Simulations were carried out using typically
485 between 72 and 120 computer cores depending on the system size, which allowed a production
486 rate of about 100 ns/ day. Analysis was carried out on a 1.1 μs production segment for each
487 simulation, following a 400 ns equilibration period as in our previous study [25].

488

489 **Analysis of all-atom MD simulations**

490 We analyzed our all-atom MD simulations using average structures, time-averaged properties
491 such as RMSD (Root-Mean-Square-Deviation), angle between helices, distance between helix
492 axes, distance between the ATP/Mg²⁺ ion and some key residues, and specific geometrical
493 measurements described below, protein-protein and protein-ligand interface characteristics and,
494 in some cases, residue-by-residue conformational and dynamic properties.

495 When RMSD distributions indicated the existence of distinct conformations, a cluster
496 analysis was carried out using the gromos algorithm of GROMACS [66], using a RMSD cutoff
497 equal to 1.5 Å on backbone atoms, on the conformations collected in the production phase.
498 Clusters accounting for less than 100 ns were discarded.

499 The C1/C2 interface was characterized using three quantities: the gap volume, the
500 change of accessible surface area upon binding (Δ ASA), and the Gap index [67,68]. The Gap
501 index, defined by the gap volume between two protein chains divided by the interface area,
502 measures the shape complementarity at protein-protein interfaces [68]. The gap volume was
503 computed by the SURFNET software [67], and the interface area was calculated using a local
504 implementation of the Lee and Richards algorithm [69] and the same radii.

505 In order to characterize G protein binding sites, as in our previous work [25], we
506 computed the angle α_{C2} between the pairs of α -helices within domain C2 that bind G α (termed
507 α_3 and α_4 in Figure 3) and also the angle α_{C1} between the quasi-symmetric pair of helices within
508 domain C1 (termed α_1 and α_2 in Figure 3) that binds G α_i in the present study. The angles were
509 measured using helical axes derived from the residues that remain in stable α -helical
510 conformations throughout the simulations (C1: 408–420 and 468–475, C2: 910–918 and 978–
511 988) as defined in [25]. We also computed the distances between the center of the helices in
512 each domain (d_{C1} and d_{C2} , respectively).

513 To characterize protein-protein interfaces, we computed the interface contacts with the
514 python/C code available at <https://github.com/MMSB-MOBI/ccmap>, using a fixed cutoff of 5 Å
515 between heavy atoms.

516 To characterize the ATP binding site, we computed two distances between ATP and two
517 key residues for AC5 activity (distance between O₂γ and Lys 1065 and between O₂α and Arg
518 1029) and the distance between Asp 460 and Asp396 and the two Mg²⁺ ions.

519 **Supporting Information**

520 Supporting Information including 3 tables and 12 figures.

521 **Acknowledgements**

522 This project/research has received funding from the European Union's Horizon 2020 Framework
523 Programme for Research and Innovation under the Specific Grant Agreement No. 720270
524 (Human Brain Project SGA1) and the Specific Grant Agreement No. 785907 (Human Brain
525 Project SGA2). We thank Alexis Michon and Samuel Bosquin (UMS 3760, Institut de Biologie et
526 Chimie des Protéines, Lyon, France) for technical assistance and hardware support. We wish to
527 acknowledge GENCI for a generous allocation of computer time on the CINES supercomputer
528 OCCIGEN (Grant 2016 - c201607758, Grant 2017 - A0020707585, Grant 2018 -
529 A0040710357).

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720 Supporting information captions

721 Figure S1. Number of contacts at the AC5/Gai interface. Contacts are defined using a 5 Å cut-
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729

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739 Figure S4: Substates of ATP and C2 observed in the Gai_sym+AC5+ATP simulation. A:
740 structural clusters obtained using gromos (cutoff=1.5 Å on backbone atoms for C2 and cutoff=1
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744

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761

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783 Figure S10. Local comparison of C2 loops in average structures without ATP. White: AC5,
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787 without ATP. Left: RMSD time series for the C2 domain, colored according to cluster

788 membership. Right: structures closest to the center of each cluster, and relative size of each
789 cluster as percentages. Prominent structural changes are indicated by red arrows.

790

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792 Figure S12: Gai+AC5 complexes, viewed from the membrane side. Gai is colored in purple,
793 AC5 in blue (C1 domain) and red (C2 domain) and interface residues used as restraints for
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795

796 Table S1. Average and standard deviation of backbone RMSD for the C1 and C2 domains of
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798 index for the interface C1/C2. ^a: data in italic are from our previous study (Frezza E, Martin J,
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803

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806

807 Table S3. Distance between Mg ions and the arginine residues (Asp 396 and Asp 460 in C1
808 domain).

SUPPLEMENTARY MATERIAL

Allosteric inhibition of adenylyl cyclase type 5 by G-protein: a molecular dynamics study

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S1. Figures

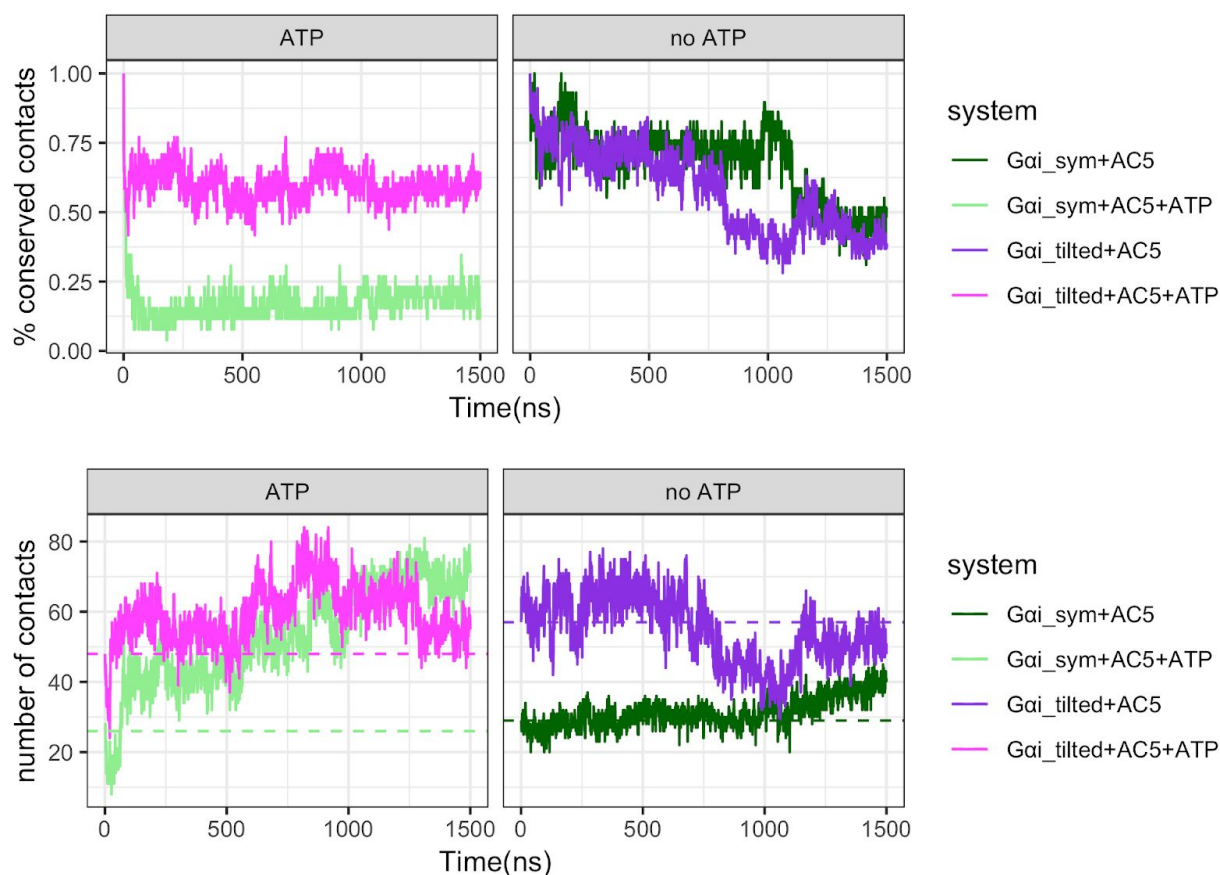


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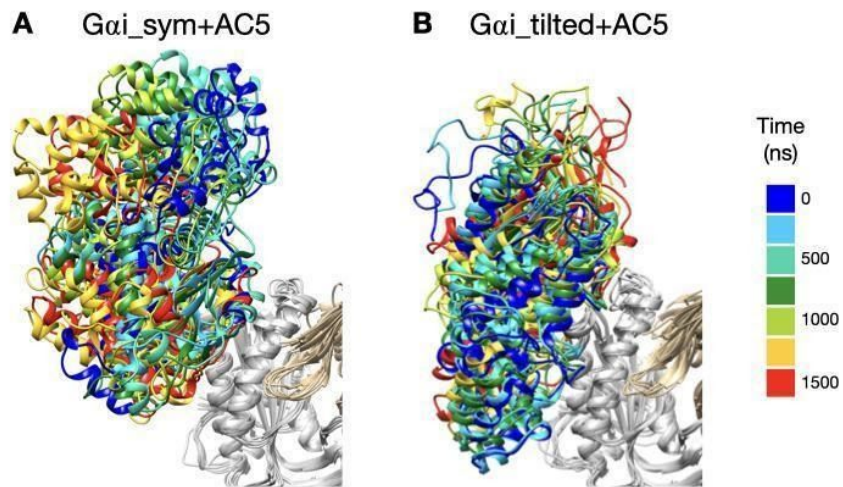


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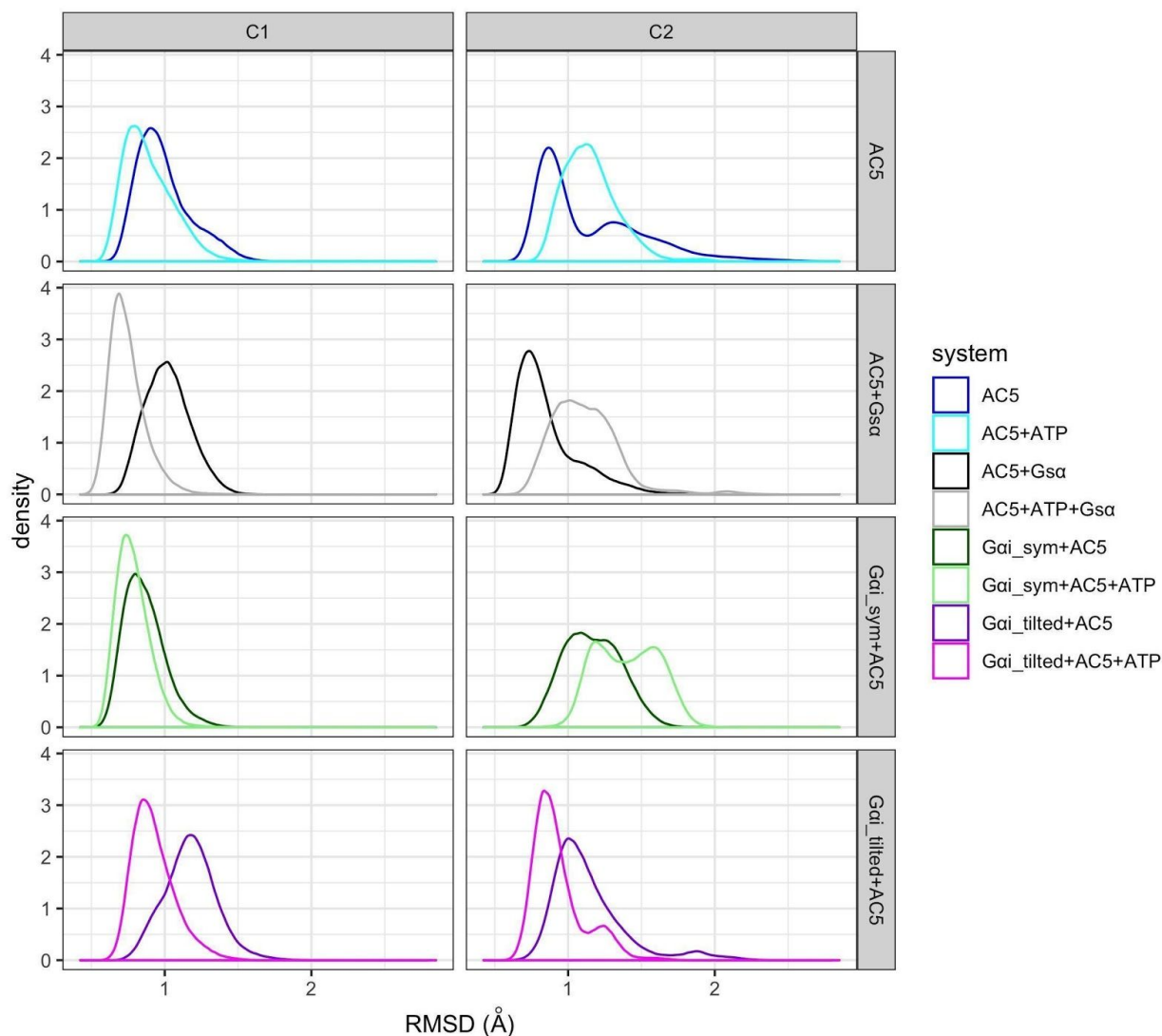


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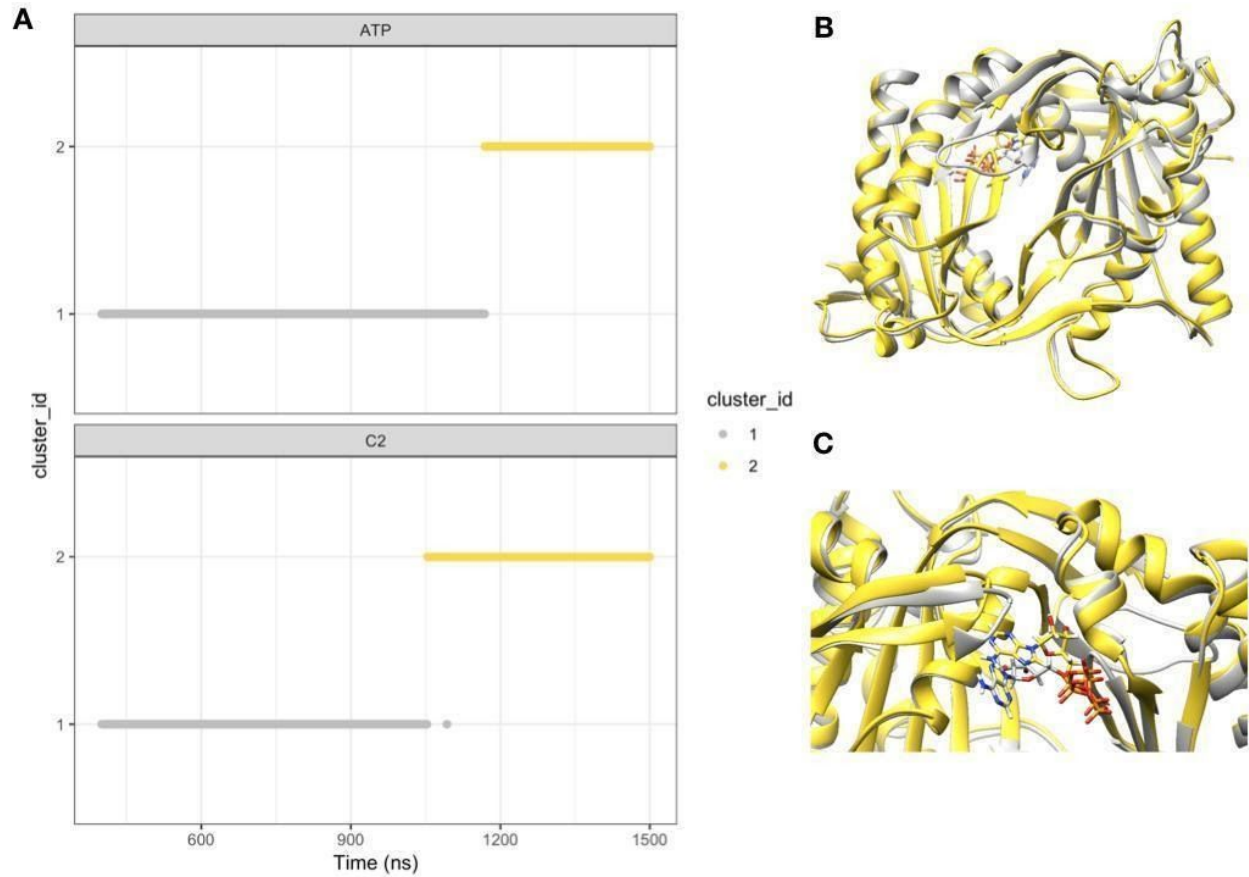


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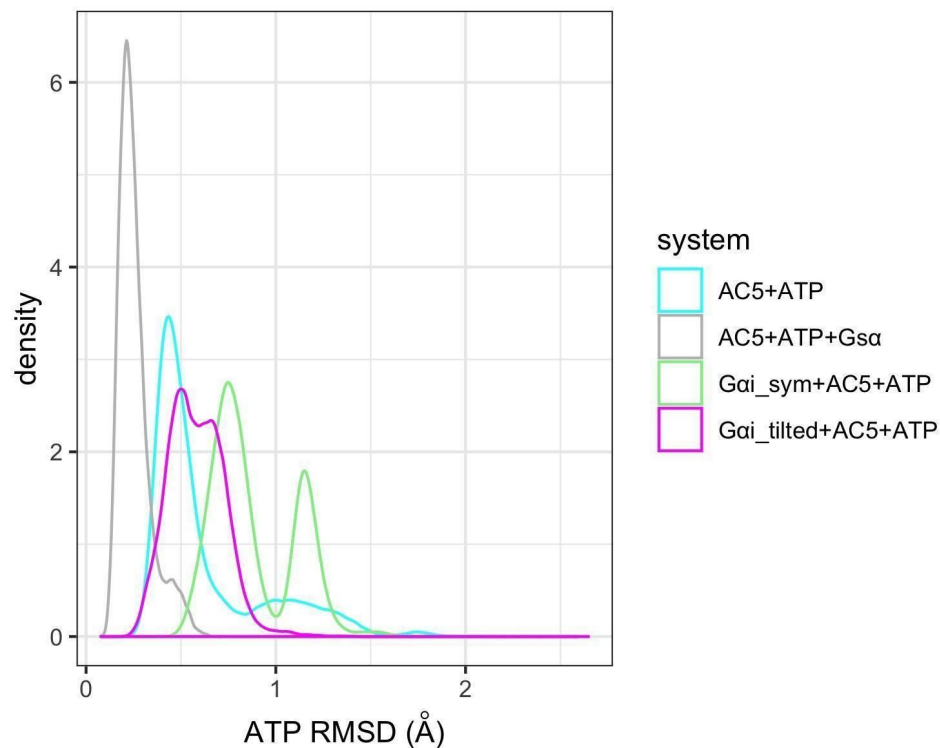


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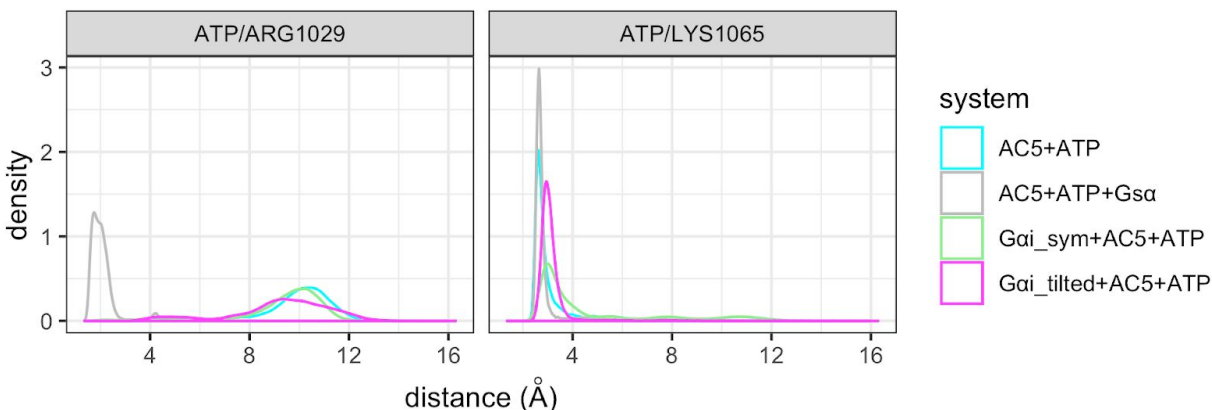


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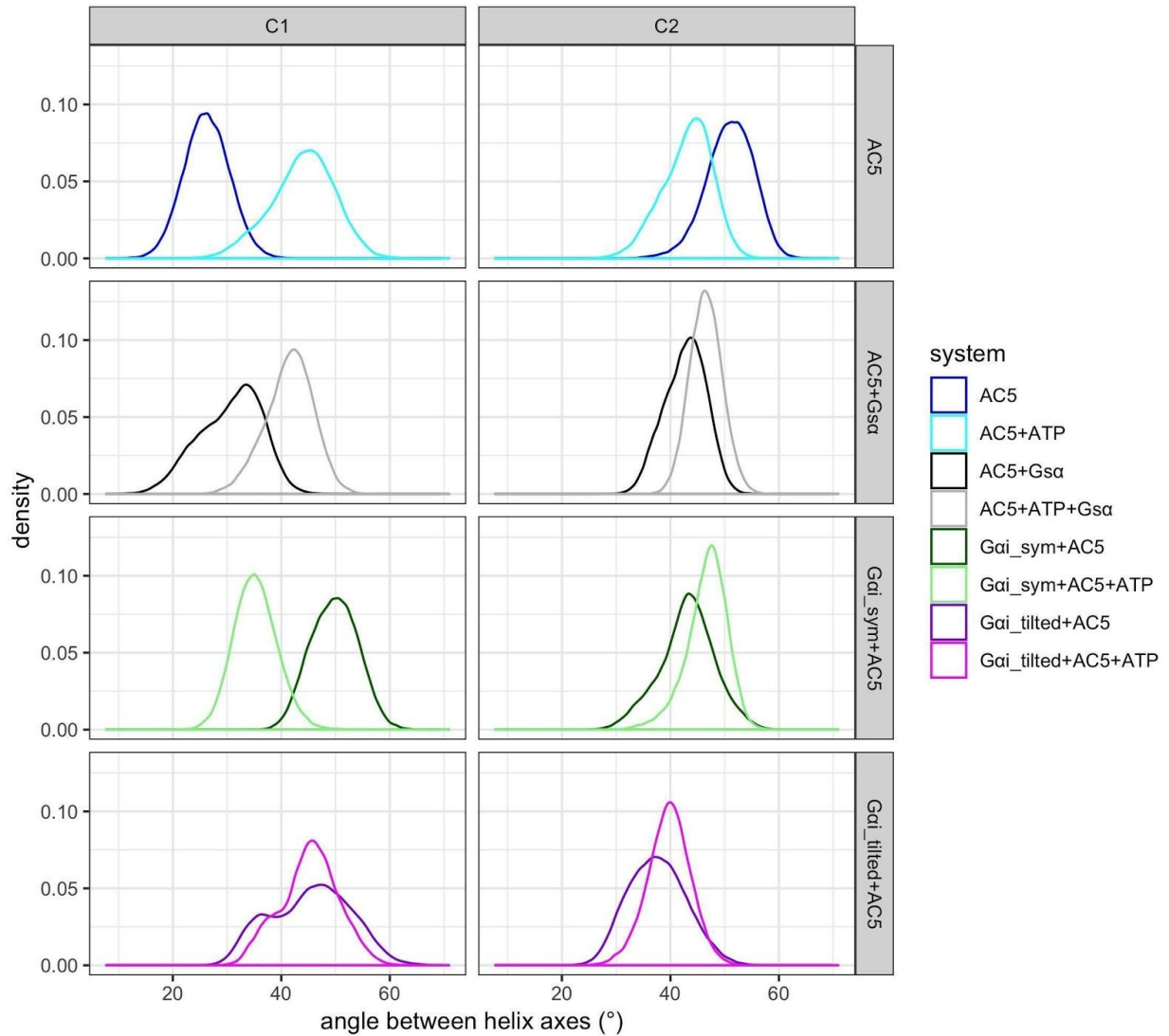


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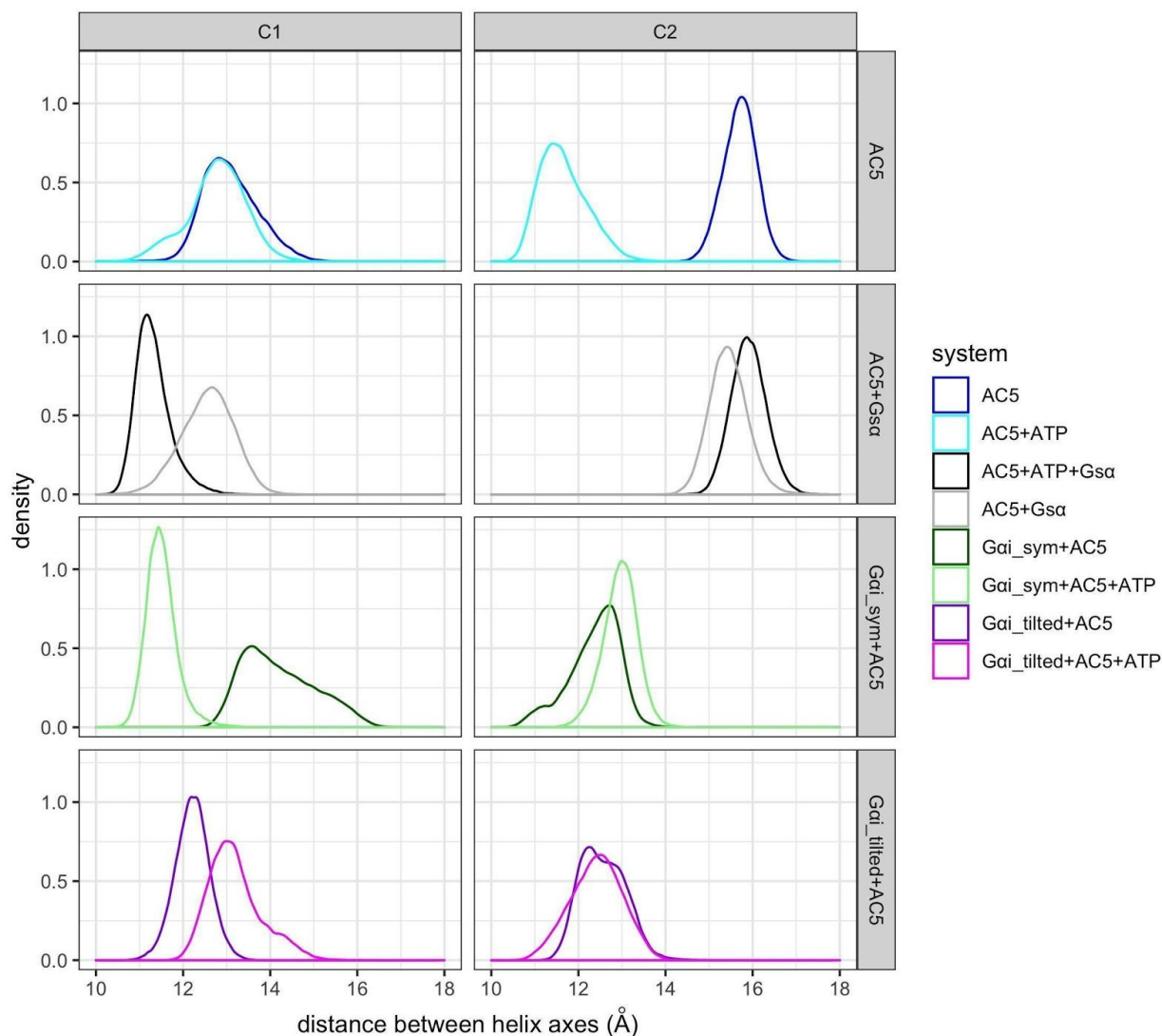


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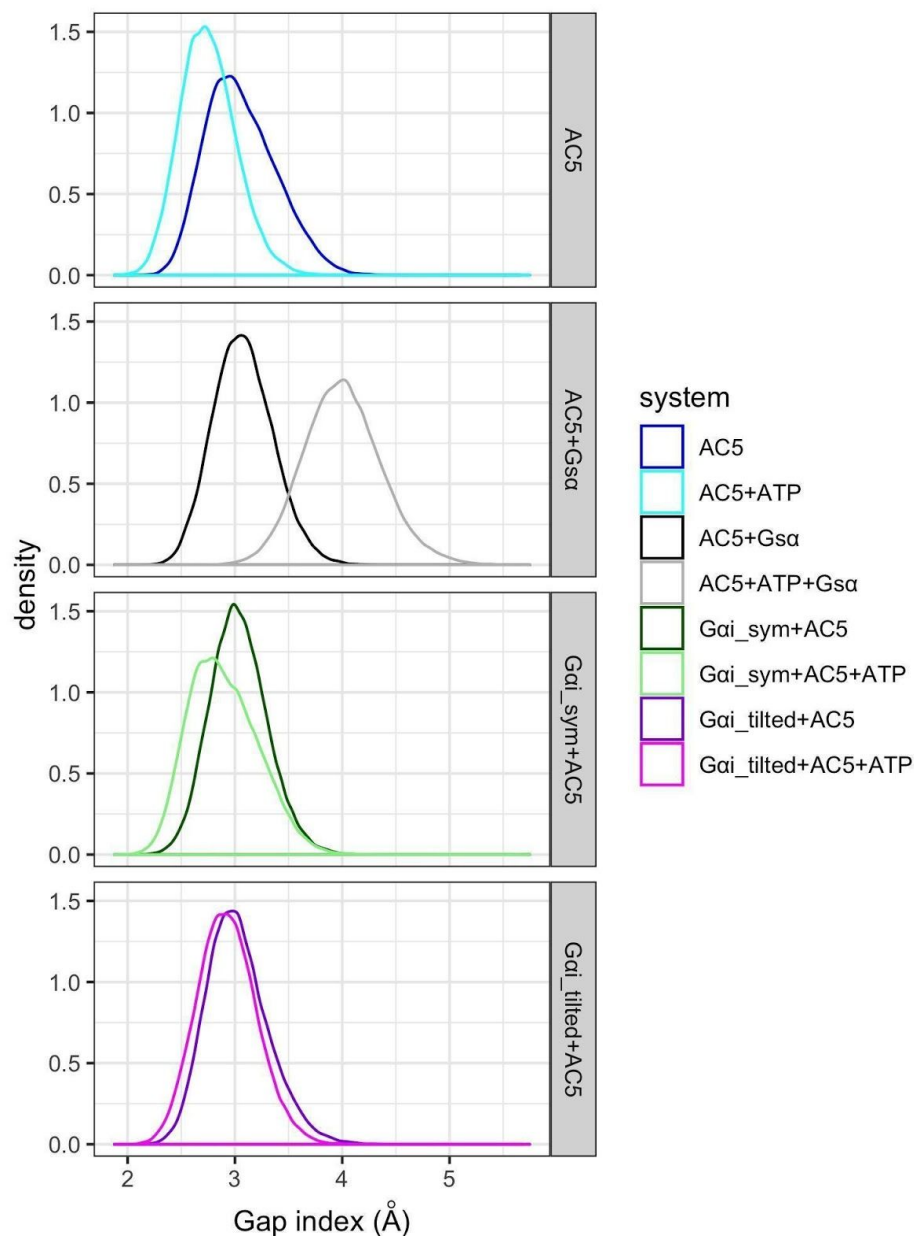


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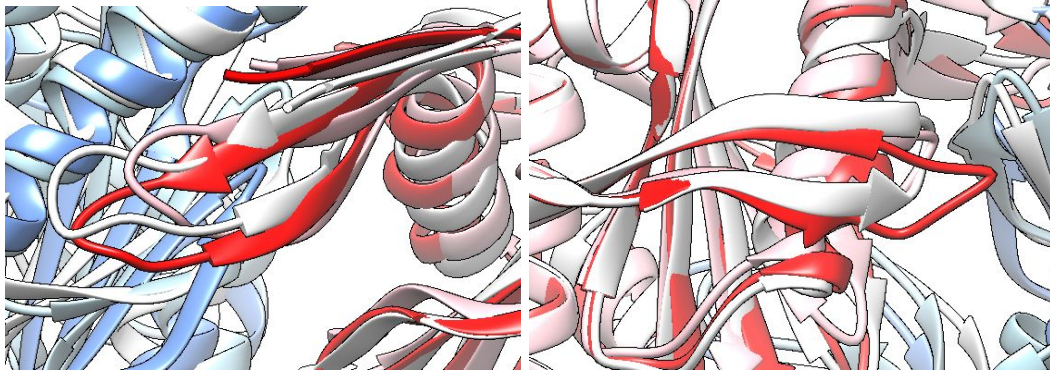


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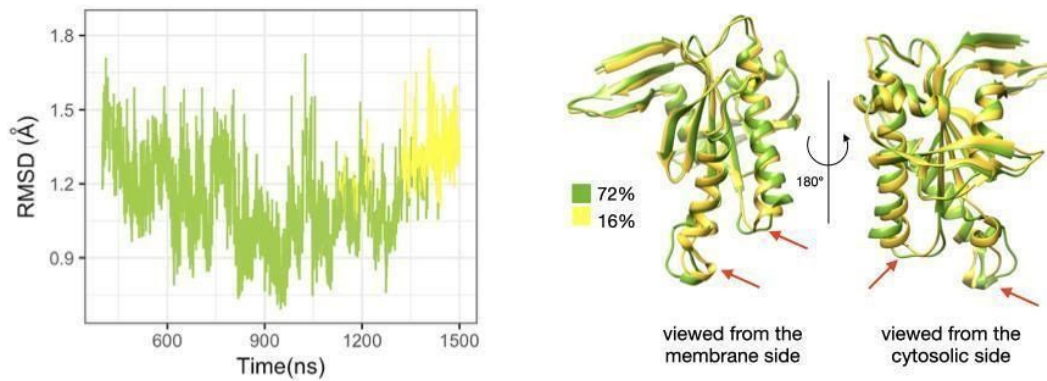


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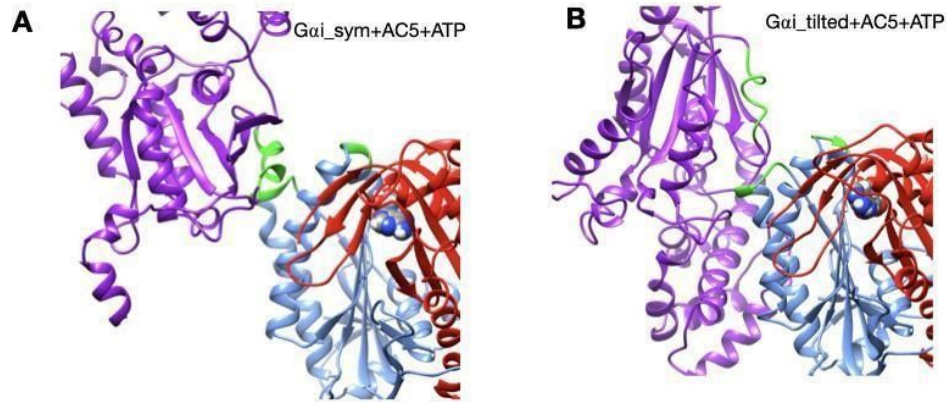


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S2. Tables

Table S1. Average and standard deviation of backbone RMSD for the C1 and C2 domains of AC5, angle between helices (α_{C1} and α_{C2}), distance between helices axes (d_{C1} and d_{C2}), Gap index for the interface C1/C2. ^a: data in italic are from our previous study (Frezza E, Martin J, Lavery R. A molecular dynamics study of adenylyl cyclase: The impact of ATP and G-protein binding. PLOS ONE. 2018;13: e0196207. doi:10.1371/journal.pone.0196207; Frezza E, Martin J, Lavery R. A molecular dynamics study of adenylyl cyclase: the impact of ATP and G-protein binding. Zenodo; 2018. doi:10.5281/zenodo.1213125).

System	RMSD C1 (Å)	RMSD C2 (Å)	α_{C1} (°)	d_{C1} (Å)	α_{C2} (°)	d_{C2} (Å)	Gap index C1/C2 (Å)	RMSD ATP (Å)
AC5+ATP ^a	<i>0.9 ± 0.2</i>	<i>1.2 ± 0.2</i>	44 ± 6	12.8 ± 0.7	43 ± 5	11.7 ± 0.6	2.8 ± 0.3	0.6 ± 0.3
AC5+ATP+Gsa ^a	<i>0.8 ± 0.1</i>	<i>1.1 ± 0.2</i>	42 ± 5	11.3 ± 0.4	47 ± 3	15.9 ± 0.4	3.8 ± 0.5	0.3 ± 0.1
Gai_sym+AC5+ATP	0.8 ± 0.1	1.4 ± 0.2	35 ± 4	11.5 ± 0.5	47 ± 4	13.0 ± 0.4	2.9 ± 0.3	0.9 ± 0.2
Gai_tilted+AC5+ATP	0.9 ± 0.1	0.9 ± 0.2	45 ± 5	13.2 ± 0.6	40 ± 4	12.4 ± 0.6	2.9 ± 0.3	0.6 ± 0.1
AC5 ^a	<i>1.0 ± 0.2</i>	<i>1.2 ± 0.4</i>	26 ± 4	13.1 ± 0.6	50 ± 4	15.7 ± 0.4	3.1 ± 0.3	
AC5+Gsa ^a	<i>1.0 ± 0.2</i>	<i>0.9 ± 0.2</i>	31 ± 6	12.6 ± 0.6	43 ± 4	15.4 ± 0.4	3.1 ± 0.3	
Gai_sym+AC5	0.9 ± 0.1	1.2 ± 0.2	50 ± 4	14.2 ± 0.8	43 ± 5	12.4 ± 0.6	3.0 ± 0.3	
Gai_tilted+AC5	1.2 ± 0.2	1.1 ± 0.3	45 ± 7	12.2 ± 0.4	38 ± 5	12.6 ± 0.5	3.0 ± 0.3	

Table S2: Mean values and standard deviation of Gap index for the Gai/AC5 interface. ^a: data in italic are from our previous study for the Gap index for the Gsa/AC5 interface.

System	Gap Volume (Å ³)	Δ ASA (Å ²)	Gap index (Å)
Gai_sym+AC5+ATP	7569 ± 504	1413 ± 157	5.4 ± 0.7
Gai_tilted+AC5+ATP	5640 ± 469	1345 ± 152	4.2 ± 0.6
Gai_sym+AC5	2850 ± 449	881 ± 101	3.2 ± 0.4
Gai_tilted+AC5	5561 ± 562	1225 ± 138	4.6 ± 0.7
AC5+ATP+Gsa ^a	3286 ± 333	1244 ± 123	2.7 ± 0.5

<i>AC5+Gsa^a</i>	3424 ± 361	1067 ± 63	3.2 ± 0.4
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Table S3. Distance between Mg ions and the arginine residues (Asp 396 and Asp 460 in C1 domain).

System	Mg1/ASP396 (Å)	MG1/ASP440 (Å)	MG2/ASP396 (Å)	MG2/ASP396
<i>AC5+ATP^a</i>	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
<i>AC5+ATP+Gsa^a</i>	2.4 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
Gai_sym+AC5+ATP	2.9 ± 0.1	7.2 ± 0.9	2.6 ± 0.1	5.2 ± 0.3
Gai_tilted+AC5+ATP	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.1