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Article

# Allosteric Inhibition of Adenylyl Cyclase Type 5 by G-Protein: A Molecular Dynamics Study

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

**Keywords:** molecular dynamics; flexibility; protein-protein interactions; allostery; enzyme activity; docking; homology models

## 1. Introduction

One of the most studied signal transduction pathways is the intricate control of cyclic AMP (cAMP) generation, which is a universal second messenger based on G-protein coupled receptors (GPCR) in eukaryotes [1]. cAMP has a role in a vast number of biological systems, including but not limited to hormone secretion [2], smooth muscle relaxation [3], olfaction [4], learning, and memory [5–7].

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

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

ACs' function is regulated by several modulators, which are either stimulators or inhibitors of cAMP synthesis. These include the stimulatory G-protein subunit alpha ( $G_s\alpha$ ), which is released from its cognate receptor and binds to and activates the AC enzyme via the subunit interaction with the C2 domain [10,14–16] upon GPCR activation [14,16,17], the inhibitory G-protein subunits  $G_{\alpha i}$  and  $G_{\beta\gamma}$ , calcium ions, calmodulin, and a variety of kinases. AC isoforms integrate several signals and they differ from each other for their modulators and for the different tissues where they are more abundant [18–21]. Although all nine transmembrane ACs are expressed in the brain, specific ACs are particularly abundant in specific brain regions, and AC5 is highly expressed in the striatum; therefore, it seems to be involved in signal transduction networks that are crucial for synaptic plasticity in the two types of medium spiny neurons [22].

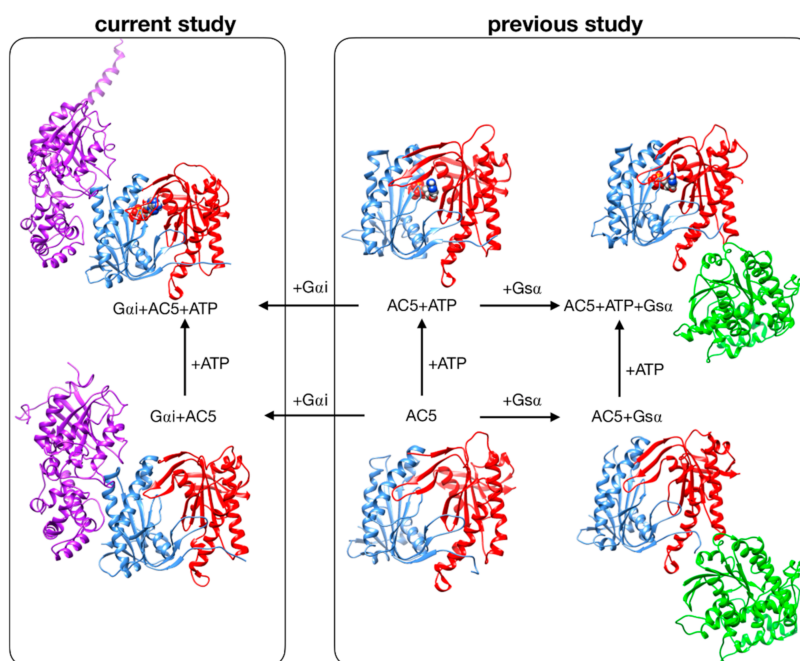
Structural information on the AC cytoplasmic catalytic core [21] and on a complex containing both AC catalytic domains bound to an active conformation of the stimulating  $G_s\alpha$ , with or without a bound ATP analog, is available [23]. However, the transmembrane regions contain six predicted membrane-spanning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

To gain insight into the functional mechanism of AC, some studies at the molecular level have been conducted using all-atom molecular dynamics (MD) simulations. In our previous work, we studied the stimulation mechanism of AC5 by performing MD simulations of AC5 alone, AC5 + ATP, AC5 +  $G_s\alpha$ , and AC5 + ATP +  $G_s\alpha$  [25]. We chose the mouse AC5 isoform among the other isoforms, since this isoform notably plays a key role in a variety of neuronal GPCRs-based signal cascades [19,26,27]. We extensively characterized the flexibility of the four states, the protein–protein interfaces, the ATP mobility, the  $G_s\alpha$  binding site, and the  $G_{\alpha i}$  putative binding site on C1 and the effect of ATP and  $G_s\alpha$  on these properties. Our study showed that both ATP and  $G_s\alpha$  binding have significant effects on the structure and flexibility of adenylyl cyclase. The comparison between the simulations of AC5 + ATP and AC5 + ATP +  $G_s\alpha$  helped to explain how  $G_s\alpha$  binding enhances enzyme activity and could therefore aid product release. Our simulations also suggested that ATP binding could influence the binding of the inhibitory G-protein subunit  $G_{\alpha i}$ , if the potential binding site within domain C1 were to be involved.

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

Despite these recent studies, the impact that  $G_{\alpha i}$  would have if ATP were already bound in its AC5 pocket and also whether  $G_s\alpha$  and  $G_{\alpha i}$  could nevertheless bind simultaneously to AC5 in the presence of ATP are yet to be clarified. In the present study, we used the same approach applied to investigate the stimulation mechanism in our previous work [25]. We have used all-atom molecular dynamics simulations to study the impact of ATP and  $G_{\alpha i}$  on the structure and flexibility of AC5. As in

our previous study, we considered only the cytoplasmic domains of AC5, since they are capable of reproducing many of the regulatory properties of the wild-type enzyme and therefore can be used as working models to investigate the regulation mechanisms of AC [31,32]. Since no structural data are available for the complex AC5 + G $\alpha$ i, we computed docking experiments using representative structures for G $\alpha$ i and AC5 + ATP obtained from our MD simulations, and we considered two distinctive poses. The all-atom microsecond-scale simulations of AC5 in complex with G $\alpha$ i with or without ATP studied here (see Figure 1) were compared with our previous simulations of AC5, AC5 + ATP, AC5 + Gs $\alpha$ , and AC5 + ATP + Gs $\alpha$  in order to help explain how binding changes the properties of AC5 and notably to understand the inhibition effect of G $\alpha$ i.



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

## 2. Materials and Methods

### 2.1. Models

Models of the cytoplasmic domains of AC5 and G $\alpha$ i protein were built by homology to known proteins using Modeller v9.12 [33]. In each case, 100 homology models were generated, and the model with the lowest DOPE score [34] was selected.

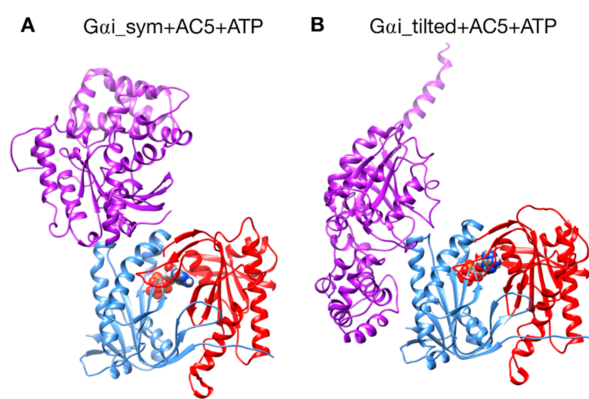
For AC5, the model was taken from the MD simulation of our previous study [25] by considering the structure closest to the center of the largest cluster of the last 500 ns simulation of AC5 + ATP. The model we studied earlier [25] was a homology model for the cytoplasmic domains of mouse AC5 (UniprotKB-P84309) with bound ATP, which was generated using the structure 1CJK [13] as template (template/model sequence identity was 98% for the C1 domain, spanning residues 455–643 of P84309, and 57% for the C2 domain, spanning residues 1063–1257 of P84309).

For G $\alpha$ i, a homology model spanning residues 9–355 of the mouse sequence (UniprotKB-P08752) bound with GSP (a GTP analogue) and Mg<sup>2+</sup> ion was generated using Modeller with three templates:



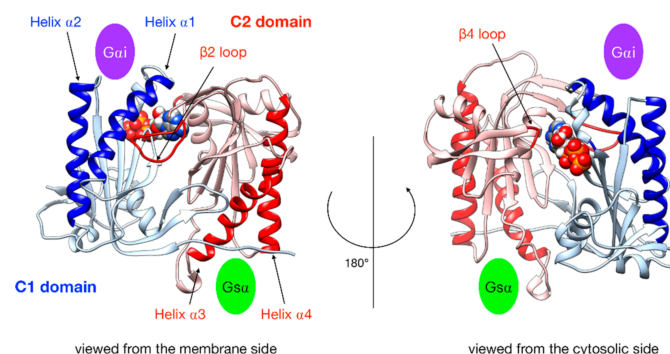
1CJK [13] (bovine Gs $\alpha$  38% sequence identity), 1AS3 [35] (rat G $\alpha$ i, 81% identity), and 1AGR [36] (rat G $\alpha$ i, 87% sequence identity). This model was used in docking (see below). In addition, we considered a model of G $\alpha$ i sampled from MD simulation: a simulation of 1  $\mu$ s was run starting from the homology model. The structure closest to the center of the largest cluster observed during the simulation was used for docking. In physiological condition, G $\alpha$ i has post-translational modifications, including a myristoylation on its N-terminus tail that is required for the inhibition of AC5, which affects the conformation and dynamics of G $\alpha$ i [37]. As the present study is focused on the impact of G $\alpha$ i binding on the dynamics and conformation of AC5, we did not include such myristoylation in our model of G $\alpha$ i.

Models of AC5 and G $\alpha$ i were docked using CLUSPRO [38–40] to generate the G $\alpha$ i + AC5 + ATP complexes shown in Figure 2, by setting as “attractive residues” (see Figure S1) some residues in the proximity of the presumed binding site from ref [24]. These residues are subject to extra attractive forces during the docking calculation [40].



**Figure 2.** The two different configurations of the G $\alpha$ i + AC5 + ATP complex simulated in this study. (A): G $\alpha$ i<sub>sym</sub> + AC5 + ATP: G $\alpha$ i has an orientation symmetrical to the Gs $\alpha$  protein in the AC5 + Gs $\alpha$  complex. (B): G $\alpha$ i<sub>tilted</sub> + AC5 + ATP: G $\alpha$ i protein is tilted with respect to AC5.

A first complex was built by docking the model of AC5 sampled from simulation with the homology model of G $\alpha$ i. The resulting complex (see Figure 2A) locates G $\alpha$ i in an orientation similar to Gs $\alpha$  with respect to AC5 in the 1CJK complex, with the G $\alpha$ i protein binding in the groove formed by the two  $\alpha$ -helices termed  $\alpha$ 1 and  $\alpha$ 2 in Figure 3. This orientation is called the symmetrical orientation. The resulting complex is denoted G $\alpha$ i<sub>sym</sub> + AC5 + ATP.



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

A second complex was built by docking the model of AC5 sampled from MD simulation with the model of G $\alpha$ i sampled from MD simulation. In the resulting complex, G $\alpha$ i is tilted compared to the G $\alpha$  orientation with respect to AC5: the G protein is in contact not only with the helix groove, but also with residues on the side of the C1 domain (see Figure 2B). The resulting complex is denoted G $\alpha$ i\_tilted + AC5 + ATP.

We would like to stress that both G $\alpha$ i\_sym + AC5 + ATP and G $\alpha$ i\_tilted + AC5 + ATP complexes are compatible with the membrane location deduced from the recent full-length structure of AC9 [41], as shown in Figure S2.

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

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

## 2.2. All-Atom Molecular Dynamics Simulations

Molecular dynamics simulations were performed with the GROMACS 5 package [42–46] using the Amber99SB-ILDN force field for proteins that has been shown to yield an accurate description of many structural and dynamical properties of proteins [47–50]. Side chain protonation states of titratable amino acids were assigned using a value of pH = 7.4 with the help of the pdb2pqr software [51]. Capping acetyl and methyl-amino groups were added to the N and C termini of both AC5 domains and G $\alpha$ i to avoid strong artifacts between non-natural termini [23,36]. The four states we study (G $\alpha$ i\_sym + AC5, G $\alpha$ i\_sym + AC5 + ATP, G $\alpha$ i\_tilted + AC5, G $\alpha$ i\_tilted + AC5 + ATP) were each placed in a truncated octahedral box and solvated with TIP3P water molecules [52] to a depth of at least 11 Å. The solute was neutralized with potassium cations, and then K<sup>+</sup> Cl<sup>−</sup> ion pairs [53] were added to reach a physiological salt concentration of 0.15 M. Parameters for ATP and GTP were taken from [54]. The parameters for Mg<sup>2+</sup> came from [55]. This new set of parameters was developed to improve the kinetic properties of Mg<sup>2+</sup> ions with water and with the phosphate ion, and it was implemented in Amber99. This new set of parameters also provided a better description of the structure of Mg<sup>2+</sup>-phosphate binding than previous sets (these interactions are naturally important in our simulations in the presence of ATP) [55]. Hence, the combination of Amber 99SB-ILDN and the new set of parameters of Mg<sup>2+</sup> ions is currently the best choice to reproduce the dynamics of AC5 and G $\alpha$ , and to properly describe the interactions of Mg<sup>2+</sup> with AC5 and ATP.

Long-range electrostatic interactions were treated using the particle mesh Ewald method [56,57] with a real-space cutoff of 10 Å. We used virtual interaction sites for the hydrogens, and bond lengths were restrained using P-LINCS [45,58], allowing a time step of 4 fs [59]. The translational movement of the solute was removed every 1000 steps to avoid any kinetic energy build-up [60]. After energy minimization of the solvent and equilibration of the solvated system for 10 ns using a Berendsen thermostat ( $\tau_T = 1$  ps) and Berendsen pressure coupling ( $\tau_P = 4$  ps) [61], the simulations were carried out in an NTP ensemble at a temperature of 310 K and a pressure of 1 bar using a Bussi velocity-rescaling thermostat [62] ( $\tau_T = 1$  ps) and a Parrinello–Rahman barostat ( $\tau_P = 1$  ps) [63]. Simulations were carried out using typically between 72 and 120 computer cores depending on the system size, which allowed a production rate of about 100 ns/day. Analysis was carried out on a 1.1  $\mu$ s production segment for each simulation, following a 400 ns equilibration period as in our previous study [25].

## 2.3. Analysis of All-Atom Molecular Dynamics Simulations

We analyzed our all-atom MD simulations using average structures, time-averaged properties such as the RMSD (root mean square deviation), angle between helices, distance between helix axes,

distance between the ATP/Mg<sup>2+</sup> ion and some key residues, and specific geometrical measurements described below, protein–protein and protein–ligand interface characteristics and, in some cases, residue-by-residue conformational and dynamic properties.

When RMSD distributions indicated the existence of distinct conformations, a cluster analysis was carried out using the gromos algorithm of GROMACS [64], using an RMSD cutoff equal to 1.5 Å on backbone atoms, on the conformations collected in the production phase. Clusters accounting for less than 100 ns were discarded.

The C1/C2 interface was characterized using three quantities: the gap volume, the change of accessible surface area upon binding ( $\Delta$ ASA), and the Gap index [65,66]. The Gap index, defined by the gap volume between two protein chains divided by the interface area, measures the shape complementarity at protein–protein interfaces [66]. The gap volume was computed by the SURFNET software [65], and the interface area was calculated using a local implementation of the Lee and Richards algorithm [67] and the same radii.

In order to characterize G protein binding sites, as in our previous work [25], we computed the angle  $\alpha_{C2}$  between the pairs of  $\alpha$ -helices within domain C2 that bind Gs $\alpha$  (termed  $\alpha_3$  and  $\alpha_4$  in Figure 3) and also the angle  $\alpha_{C1}$  between the quasi-symmetric pair of helices within domain C1 (termed  $\alpha_1$  and  $\alpha_2$  in Figure 3) that binds G $\alpha_i$  in the present study. The angles were measured using helical axes derived from the residues that remain in stable  $\alpha$ -helical conformations throughout the simulations (C1: 408–420 and 468–475, C2: 910–918 and 978–988) as defined in [25]. We also computed the distances between the center of the helices in each domain ( $d_{C1}$  and  $d_{C2}$ , respectively).

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

To characterize the ATP binding site, we computed two distances between ATP and two key residues for AC5 activity (distance between O<sub>2</sub> $\gamma$  and LYS 1065 and between O<sub>2</sub> $\alpha$  and ARG 1029) and the distances between ASP 396 and ASP 440 and the two Mg<sup>2+</sup> ions [68]. For comparison purposes, we here use the numbering scheme from reference [68]; the corresponding numbering in P84309 is LYS 1245, ARG 1209, ASP 475, and ASP 519.

Data plots were generated with the ggplot2 package in R [69,70], and structure images are generated with UCSF Chimera [71].

### 3. Results

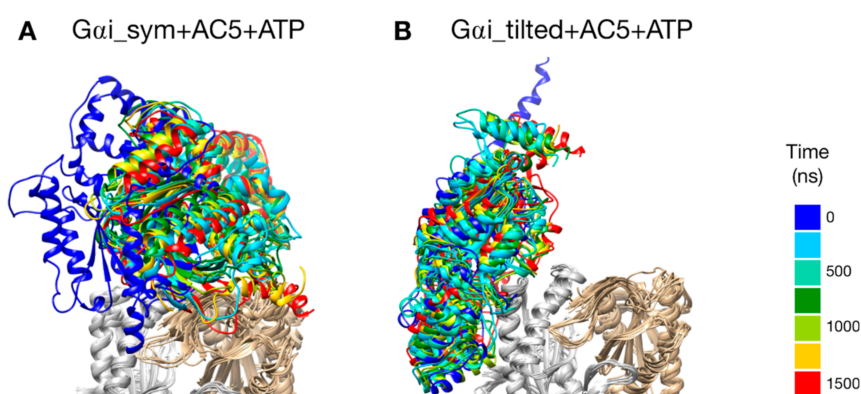
#### 3.1. Overview of Simulations

In the absence of any structural information on the catalytic domains of the enzyme with the inhibiting G-protein subunit G $\alpha_i$ , we use a combination of homology modeling, molecular dynamics, and protein–protein docking to get insight on the inhibition mechanism at the molecular level and the impact of the ligand or protein on the conformation and dynamics of AC5.

We have studied the behavior of two molecular species (see Figure 1): AC5 bound to the inhibiting G-protein subunit G $\alpha_i$  (G $\alpha_i$  + AC5) and AC5 bound to both ATP and G $\alpha_i$  (G $\alpha_i$  + AC5 + ATP). For both complexes, we considered two different relative conformations: one called G $\alpha_i$ \_sym + AC5 where the G $\alpha_i$  protein has an orientation symmetrical to the Gs $\alpha$  protein in the AC5 + Gs $\alpha$  complex, which is presented in Figure 2A, and one called G $\alpha_i$ \_tilted + AC5, where the G $\alpha_i$  protein is tilted, which is presented in Figure 2B. For each of these species, we generated 1.5  $\mu$ s MD trajectories in an aqueous environment with a physiological salt concentration (0.15 M KCl). The first 400 ns of each trajectory were treated as the equilibration of the system, and analysis was carried out only on the remaining 1.1  $\mu$ s. In order to understand the effect of G $\alpha_i$  and ATP on AC5, we used the MD simulations for isolated AC5, AC5 with ATP and two Mg<sup>2+</sup> ions in its active site (AC5 + ATP), AC5 bound to the activating G-protein subunit Gs $\alpha$  (AC5 + Gs $\alpha$  + GTP), and AC5 bound to both ATP and Gs $\alpha$  (AC5 + ATP + Gs $\alpha$  + GTP) obtained in our previous work [25,72]. Data are shown in Tables S1–S5 and in Figures S3–S14.

### 3.2. Stability of $G\alpha i$ + AC5 Complexes in the Presence and in the Absence of ATP

The two types of complexes behave differently in the presence of ATP. In the  $G\alpha i_{sym}$  + AC5 + ATP simulation, the  $G\alpha i$  protein reallocates significantly with respect to AC5 toward the C2 domain, ending in a configuration where it is in contact with the C2 domain; see Figure 4A. The peculiarity of this system is also apparent in the rest of the study and will be commented on later. On the contrary, in the  $G\alpha i_{tilted}$  + AC5 + ATP system, the  $G\alpha i$  protein fluctuates around its initial position without significant reallocation, indicating that this complex is very stable; see Figure 4B. We quantified the fraction of interface contacts between  $G\alpha i$  and AC5 that are conserved throughout the simulation time as well as the total number of interface contacts as a proxy of the interface size. For  $G\alpha i_{sym}$  + AC5 + ATP, the fraction of conserved contacts at the  $G\alpha i$ /AC5 interface drops rapidly below 25%, while the total number of contacts increases significantly (see Figure S3). In  $G\alpha i_{tilted}$  + AC5 + ATP, 50 to 75% of the initial contacts are conserved during the simulation time, and the total number of contacts also tends to increase, although less dramatically (Figure S3). In the absence of ATP, both systems maintain between 50 and 75% of their initial contacts, with more moderate reallocation and variation in terms of contact number (see Figures S3 and S4).

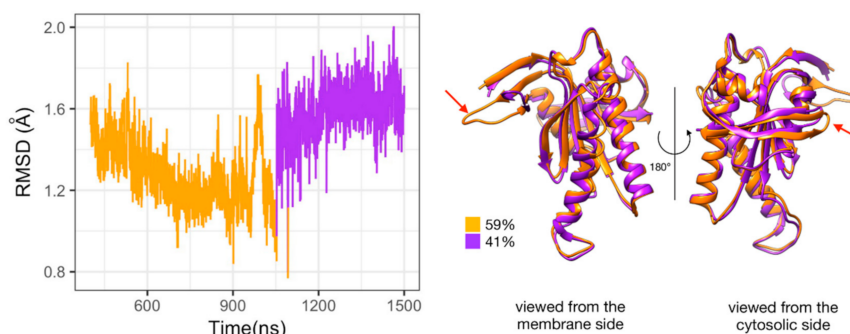


**Figure 4.** Snapshots of the  $G\alpha i$  + AC5 + ATP complexes observed during the simulations, viewed from the membrane side.  $G\alpha i$  structures extracted every 250 ns are colored on a rainbow scale from blue to red. The C1 domain of AC5 is colored in gray and the C2 domain in beige.

### 3.3. Impact of $G\alpha i$ on AC5 + ATP

We begin by considering the global impact of  $G\alpha i$  on AC5 + ATP by computing the RMSD on backbone atoms separately on each AC5 domain. RMSD calculations with respect to the average MD structure of each AC5 domain show that  $G\alpha i$  binding has a significant effect on both the structure and the dynamics of the enzyme (see Table S1 and Figure S5 where in order to allow comparison with the results obtained in our previous study [25], values for AC5, AC5 + ATP, AC5 +  $Gs\alpha$ , and AC5 + ATP +  $Gs\alpha$  were also included). On the one hand, the domain C1 is slightly rigidified by the binding of  $G\alpha i$ . On the other hand, the C2 domain visits several conformational substates involving the ATP binding pocket ( $\beta 2$  loop and  $\beta 4$  loop) in the  $G\alpha i_{sym}$  + AC5 complex (see Figure 5). These two substates also lead to two different substates for ATP (see Figure S6) which is more mobile, increasing the average RMSD from 0.6 Å for AC5 + ATP to 0.9 Å for  $G\alpha i_{sym}$  + AC5 + ATP (see Table S1 and Figure S7). In the case of  $G\alpha i_{tilted}$  + AC5 + ATP, the C2 domain visits a specific substate close to the one sampled in AC5 + ATP where the mobility of ATP is unchanged due to the presence of  $G\alpha i$ .



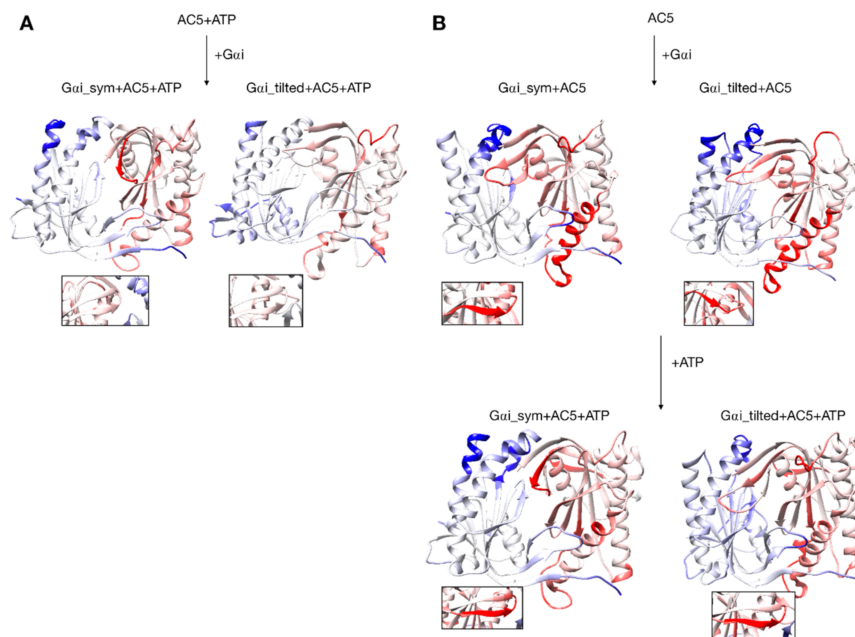


**Figure 5.** Substates of domain C2 observed during the simulation of  $G\alpha_i$ \_sym + AC5 + ATP complex. Left side: Root mean square deviation (RMSD) time series for the C2 domain, colored according to cluster membership. Right: structures closest to the center of each cluster, and relative size of each cluster as percentages. Prominent structural changes are indicated by red arrows.

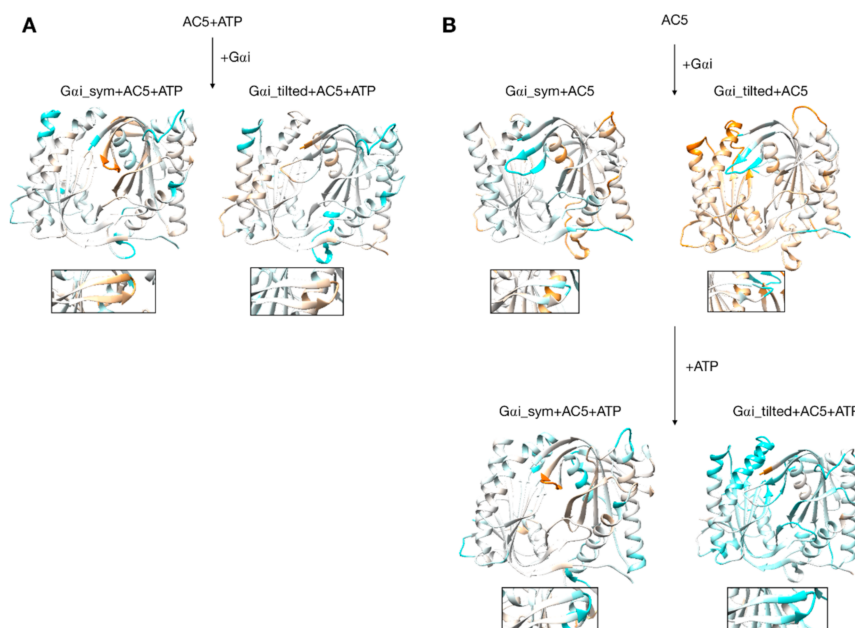
The presence of several substates upon binding of  $G\alpha_i$  is in contrast with the stabilization of a specific substate upon the binding of ATP and/or  $Gs\alpha$ . Indeed, in our previous work, we observed that the C2 domain can visit several substates when AC5 is isolated, and the presence of either ATP alone or ATP and  $Gs\alpha$  stabilizes two distinct substates. In the former case, a single substate for the  $\beta_2$  loop is selected (the longest-lived substate in isolated AC5) in a closed conformation. In the latter, an opening of loop  $\beta_2$  away from the active site is observed. The selection of a specific substate is correlated to the mobility of ATP and its reactivity [25].

Despite the decrease in flexibility of the C2 domain, which is also observed when  $Gs\alpha$  is bound to AC5 + ATP, in the presence of  $G\alpha_i$ , ATP is still rather mobile (see Table S1), and for  $G\alpha_i$ \_sym + AC5 + ATP, an increase in mobility is observed: this impact is opposite to the one observed in AC5 + ATP +  $Gs\alpha$ , where a higher stability of ATP is observed (average RMSD equal to 0.3 Å). In both simulations in the presence of ATP, the interactions between the terminal phosphate group of ATP and LYS 1065 (belonging to loop  $\beta_2$ ) and the interactions between the penultimate phosphate and ARG 1029, a key functional residue, are absent (see Figure S8): the arginine side chain is separated from its target oxygen atom by roughly 9 Å. Moreover, it is known that ATP has stronger interactions with the C1 domain via its associated two  $Mg^{2+}$  ions, notably with residues ASP 396 and ASP 440. For  $G\alpha_i$ \_tilted + AC5 + ATP, these interactions are stable and are not affected by the presence of  $G\alpha_i$ . On the contrary, for  $G\alpha_i$ \_sym + AC5 + ATP, these interactions are absent, justifying the increase of ATP mobility (see Table S3).

$G\alpha_i$  binding also turns out to have more global effects on AC5 + ATP. First, the angles and the distance between the pairs of  $\alpha$ -helices in both AC5 domains are modified. In  $G\alpha_i$ \_sym + AC5 + ATP, the angle between the helices  $\alpha_1$  and  $\alpha_2$  in domain C1 is significantly reduced (by 9°, see Table S1) and the angle between the helices  $\alpha_3$  and  $\alpha_4$  in domain C2 is slightly increased (by 4°). In  $G\alpha_i$ \_tilted + AC5 + ATP, an opposite effect is observed: the angle between the helices  $\alpha_1$  and  $\alpha_2$  in domain C1 is hardly affected (increased by 1°, see Table S1 and Figure S9), and the angle between the helices  $\alpha_3$  and  $\alpha_4$  in domain C2 is slightly decreased (by 3°). In addition, the distance between the C2 helices in  $G\alpha_i$ \_tilted + AC5 + ATP complexes is maintained around 13 Å, whereas our earlier results indicate that it is around 16 Å in the AC5 + ATP +  $Gs\alpha$  complex (see Table S1 and Figure S10). In both simulations, the C1/C2 interface remains mostly as tight as in isolated AC5 + ATP (gap index from 2.8 Å for AC5 + ATP to 2.9 Å once  $G\alpha_i$  is bound, Table S1 and Figure S11) involving a movement of helix  $\alpha_3$  (see Figure 6). In terms of flexibility,  $G\alpha_i$  binding mainly flexibilizes the binding site region of ATP in AC5, although it also decreases the flexibility of the C-terminals of helices  $\alpha_1$  and  $\alpha_3$  (see Figure 7A).



**Figure 6.** Changes in conformation induced by  $G\alpha_i$  protein. **(A)** scenario where ATP is already bound to AC5 when  $G\alpha_i$  interacts, **(B)** scenario where ATP is not yet bound to AC5 when  $G\alpha_i$  interacts. More intense colors (blue for domain C1 and red for domain C2) correspond to larger movements compared to the preceding structure (i.e., AC5 + ATP for A and AC5 and AC5 +  $G\alpha_i$  for B) on a scale of 0 to 4 Å. The insets display the  $\beta_4$  loop.



**Figure 7.** Changes in flexibility induced by G proteins. **(A)** Scenario where ATP is already bound to AC5 when  $G\alpha_i$  interacts, **(B)** scenario where ATP is not yet bound to AC5 when  $G\alpha_i$  interacts. More intense colors (orange for increased flexibility and cyan for decreased flexibility) correspond to differences with respect to the preceding structure on a scale of  $-1.2$  to  $+1.2$  Å. The insets display the  $\beta_4$  loop.

### 3.4. Impact of $G\alpha_i$ on Apo AC5 and Further Impact of ATP on AC5 + $G\alpha_i$

Although it seems probable that ATP is already bound to AC5 based on our previous study [25], we also consider the scenario where ATP is not already present when  $G\alpha_i$  binds on AC5. We begin by considering the global impact of  $G\alpha_i$  on AC5.  $G\alpha_i$ \_sym and  $G\alpha_i$ \_tilted have different effects on the



C1 domain of AC5:  $G\alpha_i$ \_sym slightly rigidifies it as attested by the RMSD calculation (Table S1 and Figure S5), whereas  $G\alpha_i$ \_tilted flexibilizes it, and this flexibility concerns the binding helices  $\alpha_1$  and  $\alpha_2$  (Figure 7B). For the C2 domain, in both  $G\alpha_i$ \_sym + AC5 and  $G\alpha_i$ \_tilted + AC5 complexes, the  $\beta_2$  loop and the  $\beta_4$  loop are rigidified upon the addition of  $G\alpha_i$  (see Figure 7B). Their conformations slightly differ in both complexes: the  $\beta_2$  loop is more closed with  $G\alpha_i$ \_tilted than with  $G\alpha_i$ \_sym, whereas the  $\beta_4$  loop, at the back of the structure, is half open with  $G\alpha_i$ \_tilted compared to with  $G\alpha_i$ \_sym (see Figure S12). In  $G\alpha_i$ \_sym + AC5, the C2 domain visits several conformational substates involving the helices  $\alpha_3$  and  $\alpha_4$  (see Figure S13).

The conformation of the binding helices in both AC5 domains is significantly altered by  $G\alpha_i$ .  $G\alpha_i$  notably displaces helix  $\alpha_3$  (Figure 6B). In both  $G\alpha_i$ \_sym + AC5 and  $G\alpha_i$ \_tilted + AC5, the angle between the helices  $\alpha_1$  and  $\alpha_2$  in domain C1 is significantly increased (by  $24^\circ$  and  $19^\circ$ ; see Table S1 and Figure S9). On the contrary, the angle between the helices  $\alpha_3$  and  $\alpha_4$  in domain C2 is significantly decreased (by  $7^\circ$  and  $12^\circ$ ), and the helices are also closer to each other (Table S1 and Figure S10). The C1/C2 interface remains as tight as in isolated AC5, in contrast with what we observed previously with the binding of  $G\alpha$ , which resulted in a looser C1/C2 interface (gap index equal to  $3.8 \text{ \AA}$ , see Table S2 and Figure S11).

In the scenario where ATP is not already bound to AC5 when  $G\alpha_i$  interacts, we can also analyze the effect of ATP addition on the pre-formed  $G\alpha_i$  + AC5 complex. As shown in Figure 7B, the addition of ATP notably rigidifies AC5. The interface at the  $G\alpha_i$ /AC5 interface is quite loose in the  $G\alpha_i$ \_tilted + AC5 complex (gap index equal to  $4.6 \text{ \AA}$ , see Table S2), and the addition of ATP tends to tighten this interface (gap index equal to  $4.2 \text{ \AA}$ ). On the contrary, in the  $G\alpha_i$ \_sym + AC5 complex, the initial  $G\alpha_i$ /AC5 interface is made more loose by the addition of ATP (gap index from  $3.2 \text{ \AA}$  in  $G\alpha_i$  + AC5 to  $5.4 \text{ \AA}$  when ATP is bound), which is probably due to the change of interface as observed by the losing of 75% of the native contacts by adding ATP (see Figure S3). By comparison, in the AC5 +  $G\alpha$  complex, the gap index decreased from  $3.2$  to  $2.7 \text{ \AA}$  upon ATP addition (Table S2). Despite the variation of the values of gap index for the  $G\alpha_i$ /AC5 interface upon the binding of ATP, all the values are typical of obligate protein–protein interfaces [73].

#### 4. Discussion

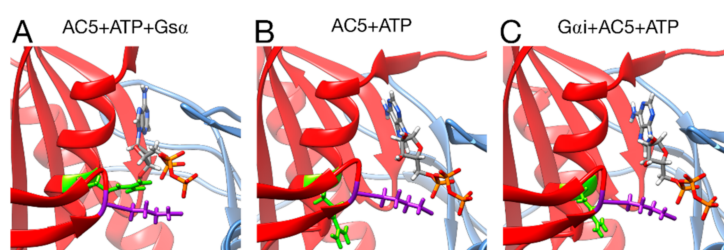
As already observed in our previous work [25], microsecond-scale simulations are necessary to investigate the allosteric coupling existing within AC5 and the effect of the binding of  $G\alpha_i$ . Similarly to van Keulen and Rothlisberger [28], we studied the scenario where  $G\alpha_i$  binds to AC5 in the absence of ATP, but we could not exclude the possibility that ATP is bound on AC5 when  $G\alpha_i$  binds, based on our previous work [25]. Due to the lack of structural information on the complex between  $G\alpha_i$  and AC5, we generated docked models using CLUSPRO, which is a server that achieves good results in the blind community-wide assessment CAPRI [74]. In order to reduce possible structural bias, we used as input isolated protein models sampled from MD simulations and integrated putative interface residues [24] in the form of a soft constraint by specifying attractive residues for the docking calculation. We considered two different docking poses in our study:  $G\alpha_i$ \_sym and  $G\alpha_i$ \_tilted. In the former, the  $G\alpha_i$  protein is bound to AC5 in a symmetrical fashion compared to what is known for the AC5 + ATP +  $G\alpha$  complexes (Figure 2A) and similar to the model postulated by Dessauer et al. [24]. In the latter, the  $G\alpha_i$  protein is rotated and tilted onto the C1 domain (Figure 2B). Both complexes stay bound during the simulations, but a greater stability is obtained for the  $G\alpha_i$ \_tilted configuration (see Figure 4), suggesting more biological relevance. In the case where  $G\alpha_i$  binds on AC5 + ATP in a symmetrical fashion, an allosteric effect is observed: a closure on the  $G\alpha_i$  site is coupled with an opening on the  $G\alpha$  site and an opening of the  $\beta_2$  loop of C2, as observed for the binding of  $G\alpha$ . Despite that, this conformation seems to be less likely because the complementarity is very low and the interface is unstable, as already mentioned above. On the contrary, the  $G\alpha_i$ \_tilted configuration is also very similar to the one reported by van Keulen and Rothlisberger in their recent work where they studied the complex between myristoylated  $G\alpha_i$  and AC5 in the absence of ATP [28], although

the starting conformations of AC5 and  $G\alpha_i$  are quite different, as well as the docking procedures. Given the inherent complexity of protein–protein docking, such convergence toward similar models despite different approaches is an additional support to the biological relevance of this model.

On the experimental side, several mutations on AC5 are known to impact its regulation by  $G\alpha_i$ : ASP411, MET414, THR415, and ASP418 in helix  $\alpha_1$ , LEU472 and VAL476 in helix  $\alpha_2$ , and VAL479 in the loop connecting helix  $\alpha_2$  to a  $\beta$ -strand [24] (see Table S4 for the numbering scheme). In our simulations of the  $G\alpha_i$ \_tilted + AC5 complex, these residues are remarkably persistent, i.e., present more than 80% of the time at the interface between AC5 and  $G\alpha_i$  with and without ATP; see Table S4. Not only are these residues persistent at the interface, but they also form stable interactions with particular side chains on  $G\alpha_i$  belonging to the switch I, switch II and switch III regions [75] (see Table S5 and Figure S14). The high stability of such interactions observed in our MD simulations suggest a functional role for these residues, which is consistent with the fact that the mutations affecting them have an impact on the regulation of AC5 by  $G\alpha_i$ , as observed experimentally [24].

The binding of  $G\alpha_i$  slightly rigidifies the C2 domain in all simulations, and an opening of the  $G\alpha_i$  binding site is coupled with a closure of the  $G\alpha$  binding site, in particular in the  $G\alpha_i$ \_tilted + AC5 complexes with and without ATP, as observed in van Keulen and Rothlisberger's simulation [28]. As in the case of the AC5 +  $G\alpha$  complex, ATP stabilizes the  $G\alpha_i$ /AC5 interface when  $G\alpha_i$  is tilted, whereas the latter is less complementary. All these changes involve coupling through AC5 over distances of tens of angstroms.

Turning now to the enzymatic function of AC5, it is known that specific hydrogen bonds between AC5 and ATP play an important role in the production of cAMP from ATP, as already shown in hybrid QM/MM free energy calculations, notably the hydrogen bond between the highly conserved ARG 1029 and the  $\alpha$  phosphate group of ATP [68]. The present simulations show that this interaction is not formed upon the binding of  $G\alpha_i$  and other ones are lost (for example between LYS 1065 and ATP, see Figure S8), when on the contrary, it is formed upon the binding of  $G\alpha$  (see Figure 8). This structural effect of  $G\alpha_i$ , which stabilizes the active site of AC5 in a state that prevents ATP to form the required interactions for catalysis, provides a structural basis for the enzymatic inhibition of AC5 by  $G\alpha_i$  observed experimentally [24].



**Figure 8.** Interactions between ATP and key residues in different complexes. (A) active AC5 + ATP +  $G\alpha$ , (B) inactive AC5 + ATP complex, (C) inactive  $G\alpha_i$ \_tilted + AC5 + ATP complex. Key residues of C2 are shown as sticks and colored in purple (LYS 1065) and green (ARG 1029). The C1 and C2 subunits of AC5 are colored blue and red, respectively. For clarity, the region 394–428 of C1 is omitted from the representation.

The binding of  $G\alpha_i$  inhibits AC5 by increasing the flexibility of the active site allowing a high mobility of ATP without changing the complementarity of the C1/C2 interface. It was proposed previously [24] that the inhibitory effect of  $G\alpha_i$  might be due to the disruption of the active site of AC5 or a decrease of the affinity between C1 and C2 domains. Our simulation study is in line with this first hypothesis. The further inactivation of AC5 + ATP by  $G\alpha_i$  does not exclude the possibility that ATP is bound to AC5 during the inhibition process, and it may have a role in the tight regulation of the enzyme.

Based on our previous study [25] and the current one, we can speculate on the regulation mechanism of AC5 by considering only the  $G\alpha_i$ \_tilted configuration. In the absence of ATP, based on

our simulations, only  $G_{s\alpha}$  could interact with AC5, due to the close conformation of the binding site on the C1 domain. However, we cannot exclude the existence of  $G_{\alpha i}$  + AC5 based on our study. The binding of ATP induces an opening of the angle between the pair of  $\alpha$ -helices of domain C1 ( $\alpha_{C1}$ ) and a closure of the pair of  $\alpha$ -helices of domain C2 ( $\alpha_{C2}$ ), which have a similar value close to the one observed upon the binding of  $G_{s\alpha}$ . If  $G_{\alpha i}$  binds first AC5 + ATP, the closure of the angle  $\alpha_{C2}$  does not allow the binding of  $G_{s\alpha}$ , and the ATP pocket remains close. When  $G_{\alpha i}$  dissociates from AC5 + ATP, AC5 undergoes a conformational change that allows the binding of  $G_{s\alpha}$ . On the contrary, if  $G_{s\alpha}$  binds AC5 first, the enzyme is active thanks to the stabilization of ATP in its pocket and the formation of specific hydrogen bonds and cycles between AC5 + ATP +  $G_{s\alpha}$  (favorable to  $G_{\alpha i}$  binding) and AC5 +  $G_{s\alpha}$  (unfavorable to  $G_{\alpha i}$  binding). If  $G_{s\alpha}$  dissociates, after cAMP release, AC5 is in an apo conformation, and there is no need for further inhibition. If  $G_{s\alpha}$  dissociates from AC5 + ATP, then the conformation of AC5 becomes accessible to  $G_{\alpha i}$  for the inhibition. Another possibility is that due to the open conformation of the binding site on the C1 domain,  $G_{\alpha i}$  can bind to the AC5 + ATP +  $G_{s\alpha}$  complex to form a ternary complex, whose existence is still unknown.

## 5. Conclusions

We perform all-atom molecular dynamics simulations in an attempt to better understand the regulation of adenylyl cyclase, which is a key enzymatic player in cellular signaling cascades. Microsecond-scale simulations of the G-protein subunit  $G_{\alpha i}$  bound to the cytoplasmic domains of adenylyl cyclase in the presence and in the absence of ATP in two different conformations help to better understand some features of this important signal transmission protein, since no structural information on this complex is available. They notably provide information on a single, non-chimeric adenylyl cyclase isoform, AC5, which is bound to the inhibitory G-protein in the presence and in the absence of ATP.

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

Our results also show that  $G_{\alpha i}$  binding to the C1 domain does not impact C1/C2 interface complementarity, flexibilizes the C1 domain, and significantly closes the angle between the C2  $\alpha$ -helices that cannot bind  $G_{s\alpha}$  when  $G_{\alpha i}$  is in tilted conformation. The simultaneous binding of ATP and  $G_{\alpha i}$  in a tilted conformation at the AC5 interface results in a rigidification of the C2 domain, without affecting the C1/C2 interface complementarity, and a slight increase of the angle between the C2  $\alpha$ -helices. Hence,  $G_{\alpha i}$  has an important impact on AC5 dynamics, and its effects are enhanced when ATP binds by increasing the conformational freedom of the bound ligand, thus putting it in an unfavorable configuration within its binding site and not allowing the establishment of key interactions between ATP and AC5, which therefore leaves AC5 completely inactive.

Our simulations also show that ATP has a crucial role in the regulation of AC5, and we cannot exclude the presence of ATP during the inhibition. Our previous simulations already showed that ATP binding could influence the binding of the inhibitory G-protein subunit  $\alpha i$  at the C1 domain. Here, we propose that the presence of ATP is needed to induce the competition between  $G_{s\alpha}$  and  $G_{\alpha i}$  to tightly regulate AC5. However, based on our results, we cannot exclude the existence of the  $G_{\alpha i}$  + AC5 + ATP +  $G_{s\alpha}$  complex and the  $G_{\alpha i}$  + AC5 +  $G_{s\alpha}$  complex. For the latter, other molecular dynamic studies of this hypothetical ternary complex concluded that if it exists, it would be inactive [29,30]. Further studies have to be conducted to shed light on this point.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2218-273X/10/9/1330/s1>, Figure S1:  $G_{\alpha i}$  + AC5 + ATP complexes, viewed from the membrane side.  $G_{\alpha i}$  is colored in purple, AC5 is colored in blue (C1 domain) and red (C2 domain). In green, residues labeled as "attractive" for the docking: residues 484–486 and 554–558 in AC5 (numbered according to P84309), which are at the entry of the binding site suggested

by mutations in [24], and residues 209–216 in G $\alpha$ i (numbered according to P08752), which are in the switch II region (201–221) involved in the binding [24]. The switch I and switch III regions [75] are indicated by arrows. Figure S2. Superimposition of the G $\alpha$ i + AC5 + ATP complexes simulated in this study superimposed with the full-length structure of AC9 bound to Gs $\alpha$  (PDB structure 6R3Q [41]). Blue: C1 domain of AC5, red: C2 domain of AC5, purple: G $\alpha$ i, gray: full-length AC9 bound with Gs $\alpha$ . Membrane location from [41]. Figure S3. Number of contacts at the AC5/G $\alpha$ i interface. Contacts are defined using a 5 Å cut-off between heavy atoms. Top row: Fraction of initial contacts (t = 0) that are maintained as a function of time. Bottom row: total number of contacts between AC5 and G $\alpha$ i; dashed horizontal lines indicate the number of contacts at t = 0. Figure S4: Snapshots of the G $\alpha$ i + AC5 complexes observed during the simulations without ATP, viewed from the membrane side. Structures extracted every 250 ns are colored on a rainbow scale from blue to red. The C1 domain of AC5 is colored in gray, and the C2 domain is colored in beige. Figure S5: RMSD distribution for C1 and C2 domains, with respect to average structures, in the simulations with and without ATP. Data for AC5 and AC5 + Gs $\alpha$ , with and without ATP, taken from [25,72]. All the densities are computed with ggplot2 in R [66,67], using default parameters (Gaussian kernel density estimator and 512 equally spaced points). Figure S6: Substates of ATP and C2 observed in the G $\alpha$ i\_sym + AC5 + ATP simulation. A: Structural clusters obtained using gromos (cutoff = 1.5 Å on backbone atoms for C2 and cutoff = 1 Å for ATP), B: average structures viewed from the membrane side, C: close-up view on the  $\beta$ 4 loop, from the cytoplasmic side. The average structures are colored in gray (400 ns < T < 1100 ns) and yellow (1200 ns < T < 1500 ns). Figure S7: RMSD distribution for ATP. Data for AC5 + ATP and AC5 + ATP + Gs $\alpha$  taken from [25,39]. Figure S8: Distance between ATP and key residues of C2. Data for AC5 + ATP and AC5 + ATP + Gs $\alpha$  are from our previous study [25,72]. ATP/ARG1029: distance between the O2 $\alpha$  of ATP and the center of mass of terminal hydrogen atoms, which are covalently bound to N $\epsilon$  of ARG 1029. ATP/LYS1065: distance between the O2 $\gamma$  of ATP and the center of mass of the terminal hydrogen atoms, which are covalently bound to N $\zeta$  of LYS 1065. Figure S9: Distribution of the angles between helix axes (helix  $\alpha$ 1 and helix  $\alpha$ 2 in C1, helix  $\alpha$ 3 and helix  $\alpha$ 4 in C2, see Figure 3). Data for AC5 and AC5 + Gs $\alpha$ , with and without ATP, taken from [25,72]. Figure S10: Distribution of the distance between helix axes (helix  $\alpha$ 1 and helix  $\alpha$ 2 in C1, helix  $\alpha$ 3 and helix  $\alpha$ 4 in C2, see Figure 3). Data for AC5 and AC5 + Gs $\alpha$ , with and without ATP, taken from [25,39]. Figure S11: Distribution of the C1/C2 interface gap index. Data for AC5 and AC5 + Gs $\alpha$ , with and without ATP, taken from [25,72]. Figure S12. Local comparison of C2 loops in average structures without ATP. White: AC5, red/blue: G $\alpha$ i\_tilted + AC5, pink/cyan: G $\alpha$ i\_sym + AC5. Left panel:  $\beta$ 2 loop, right panel:  $\beta$ 4 loop. Figure S13. Substates of domain C2 observed during the simulation of G $\alpha$ i\_sym + AC5 complex, without ATP. Left: RMSD time series for the C2 domain, colored according to cluster membership. Right: Structures closest to the center of each cluster, and the relative size of each cluster as percentages. Prominent structural changes are indicated by red arrows. Figure S14. The persistent interface residues in G $\alpha$ i\_tilted + AC5 + ATP simulation. The C1 domain is colored in blue, the C2 domain is colored in red, G $\alpha$ i is colored in purple. Important residues on C1 from reference [24] that are persistent at the interface with G $\alpha$ i are shown as sticks, which are colored in orange and labeled with a residue numbering from [24]. Residues on G $\alpha$ i that form stable interactions from Table S5 are shown in sticks and colored in pink (switch I), yellow (switch II), and cyan (switch III). Table S1. Average and standard deviation of backbone RMSD for the C1 and C2 domains of AC5, angle between helices ( $\alpha$ C1 and  $\alpha$ C2), distance between helices axes (dC1 and dC2), Gap index for the interface C1/C2, RMSD for ATP. a: data in italics are from our previous study [25,39]. Table S2: Mean values and standard deviation of Gap index for the G $\alpha$ i/AC5 interface. a: data in italics are from our previous study for the Gap index for the Gs $\alpha$ /AC5 interface [25,72]. Table S3. Distance between Mg<sup>2+</sup> ions and the aspartate residues (ASP 396 and ASP 440 in C1 domain). Table S4. Persistence of residues of AC5 mutated in reference [24] at AC5/G $\alpha$ i interface in our simulations. The fraction of time each residue is part of the AC5/G $\alpha$ i interface is shown as a percentage, and the number of contacting residues on G $\alpha$ i is between parentheses. Interface residues are defined using a 5 Å cut-off between heavy atoms. Residues in italics have limited or no impact on the binding. <sup>1</sup>: For the sake of comparison, residue numbering refers to reference [24]; the corresponding numbers in Uniprot sequence P84309 are between parentheses. <sup>2</sup>: In Ref. [24], the double mutant M414/T415 was studied; hence, we monitor the presence of M414 or T415. Table S5. Stable interactions between key residues of AC5 from reference [24] and residues of G $\alpha$ i. Interactions that appear more than 50% of the time in each simulation are listed with the corresponding fraction of time between parentheses. Interactions are defined using a 5 Å cut-off between heavy atoms. Interactions that are common to simulations with and without ATP for a given system are highlighted in bold. For the sake of comparison, AC5 residue numbering refers to reference [24]; the corresponding numbers in Uniprot sequence P84309 are between parentheses. G $\alpha$ i numbering refers to the Uniprot sequence. <sup>1</sup>: G $\alpha$ i residues belonging to the switch I region, <sup>2</sup>: G $\alpha$ i residues belonging to the switch II region, <sup>3</sup>: G $\alpha$ i residues belonging to the switch III region [75].

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