Review

Structure, mechanism, and regulation of soluble adenylyl cyclases — similarities and differences to transmembrane adenylyl cyclases ☆

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ABSTRACT

The second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) regulates a wide range of physiological processes in almost all organisms. cAMP synthesis is catalyzed by adenylyl cyclases (ACs). All ten mammalian AC isoenzymes (AC1–10) belong to AC Class III, which is defined by sequence homologies in the catalytic domains. Nevertheless, the mammalian AC can be separated into two distinct types, nine transmembrane enzymes (tmAC; AC1–9) and one soluble AC (sAC; AC10). tmACs are mainly regulated by heterotrimeric G-proteins as part of the G-protein coupled receptor pathways, while sAC is directly activated by bicarbonate and Ca^{2+} and acts as a sensor for ATP, Ca^{2+}, and bicarbonate/CO_2/pH at various intracellular locations. Mammalian sAC has been implicated in processes such as sperm activation, glucose metabolism, and prostate and skin cancer, making it a potential therapeutic target, and first sAC-specific inhibitors have been developed. Mammalian sAC appears evolutionarily closer related to microbial Class III ACs than to tmACs, and sAC-like bicarbonate activated ACs are indeed found in lower organisms and can contribute, e.g., to virulence regulation in microbial pathogens. Here, we review work on the architecture, catalysis, and physiological and pharmacological regulation of sAC-like enzymes, with a main focus on the mammalian enzyme. We further compare the biochemical, regulatory, and structural characteristics of sAC-like enzymes to the evolutionarily and structurally related mammalian tmACs, pointing out common features as well as sAC-specific properties and modulators. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

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1. Introduction

Cyclic adenosine 3′,5′-monophosphate (cAMP) was discovered as a signaling molecule in the 1950s during studies on hormonal regulation of mammalian metabolism [1]. Subsequent studies described cAMP as a prototypical second messenger, which passes on signals reaching the cellular membrane to intracellular targets [2]. cAMP is found in all domains and kingdoms of life – only its occurrence in plants is still not fully clarified – and it contributes to a very wide range of physiological processes, from virulence mechanisms in bacteria and fungi to metabolic regulation and transcription in mammals [3–6]. cAMP targets distinct effector proteins in the different kingdoms. In *Escherichia coli*, the transcription factor "catabolite gene activator protein" (CAP), also known as "cAMP responsive protein" (CRP), acts as a major effector [3]. In mammalian cells, at least four types of cAMP effectors are known: protein kinase A (PKA), cyclic nucleotide gated ion channels (CNGs and HCNs), small G-protein regulating "exchange proteins activated by cAMP" (EPACs), and phosphodiesterases (PDEs) [3,7].

Cellular levels of cAMP are controlled through its synthesis by adenylyl cyclases (AC; EC 4.6.1.1) and its degradation by PDEs [4,5,8]. AC enzymes catalyze an intramolecular cyclization of ATP to cAMP under release of pyrophosphate (see Section 2.2.2) [4]. ACs, and the guanylyl cyclase (GC) enzymes generating the related second messenger cGMP from GTP, form the nucleotidyl cyclase family, which comprises six evolutionary distinct classes defined by sequence homologies within their catalytic domains [4,9]. The AC from *E. coli* and several AC enzymes from related gram-negative prokaryotes belong to Class I [4]. Class II comprises secreted "toxin" ACs from pathogens such as *Pseudomonas aeruginosa* and *Bordetella pertussis*, which translocate into host cells and disrupt intracellular signaling [6]. All known eukaryotic nucleotidyl cyclases, ACs and GCs, belong to the ubiquitous Class III, which also comprises AC from many prokaryotes and all known GC [4,9]. The Classes IV–VI were defined more recently and so far comprise only one or few prokaryotic members [4].

Several different Class III AC and/or GC isoforms are expressed simultaneously in many tissues and organisms, reflecting the variety of cellular processes regulated by cyclic nucleotides. In mammals, there are ten AC genes encoding differently regulated AC isoforms called AC1 to AC10 [5,10]. AC1–9 form an extensively studied subfamily of so-called transmembrane ACs (tmACs). Mammalian tmAC activity is mainly regulated by heterotrimeric G proteins upon activation of G protein-coupled receptors (GPCRs) by extracellular hormones and neurotransmitters [10]. The different tmAC isoforms contribute to distinct physiological activities, such as AC1 to memory formation [11] and AC5 to cardiac myocyte function [12]. A second type of AC, referred to as "soluble AC" (sAC), is encoded by the AC10 gene [4]. A mammalian sAC activity was discovered in the 1970th but more intense studies commenced only after isolation of the first sAC cDNA in 1999 [13,14]. Significant progress in recent years revealed that sAC activity is sensitive to variations in physiological ATP levels and that sAC enzymes are directly activated by Ca^{2+} and the metabolite bicarbonate, rendering them intracellular metabolic sensors and signal integrators [15–19]. Mammalian sAC is expressed in many tissues [20,21] and resides in discrete cellular locations, such as the nucleus and mid-bodies [22]. Physiological functions regulated by sAC are, e.g., sperm motility [23], pH regulation [24], apoptosis [25], and metabolic pathways such as glycolysis and mitochondrial respiration [26–28]. AC enzymes with sAC-like regulation, in particular activation by bicarbonate, have also been described in lower organisms and even in bacteria [4,29,30], suggesting an evolutionary conserved family of sAC-like, bicarbonate-sensing Class III ACs.

Mammalian sAC has been implicated in glucose-induced insulin release and in prostate and skin cancer, suggesting it as a therapeutic target for the treatment of type 2 diabetes and cancer [18,31–33]. It is also used as a target for male contraceptives and as a diagnostic marker for melanoma [34,35]. The contribution of sAC-like ACs to virulence regulation in pathogens such as *Candida albicans* and *P. aeruginosa* renders them potential targets for antifungal and antibiotic compounds [30,36]. Specific modulators for sAC-like enzymes are therefore tools for functional studies but also potential lead compounds for the development of therapeutic drugs.

Architecture, catalysis, and physiological and pharmacological regulation of Class III tmACs and sACs have been studied biochemically and structurally, revealing common features as well as properties and modulators specific for one of the two related AC types. Here, we review biochemical, regulatory, and structural characteristics of sAC-like enzymes, with a focus on the mammalian members, and compare them to tmACs.

2. sAC and sAC-like ACs — specific features and comparison to tmACs

2.1. Class III AC domain architecture

All eukaryotic ACs belong to Class III, which is defined by a conserved ~200 residue catalytic domain [4,9]. A series of crystal structures of Class III AC and GC catalytic cores revealed a conserved dimeric architecture [4,19,37–41]. There are either two Class III catalytic domains, normally referred to as C_1 and C_2, on one polypeptide chain and form a pseudo-heterodimer, or there is one catalytic domain per polypeptide that homodimerizes with a second copy (Fig. 1a). Most bacterial ACs and mammalian receptor GCs are homodimers, which feature two symmetrical active sites at the dimer interface. Mammalian tmACs and sACs have the tandem arrangement of two homologous and structurally similar catalytic domains C_1 and C_2 forming a pseudo-heterodimer (from now on referred to as heterodimer). C_1 carries only a subset of the catalytic residues and C_2 a complementary set of essential residues, so that only one of the pseudo-symmetric interface sites is active (Fig. 1a). It appears that C_1 and C_2 evolved through gene duplication and subsequent degeneration of the second site into a regulator binding site (see Refs. [4,19,42] and Section 2.4.1).

Class III AC catalytic domains appear to constitute a versatile signaling module that is fused to a variety of regulatory domains in different AC enzymes, enabling them to serve as sensors and signaling enzymes for many different stimuli [43]. This variety is particularly obvious in
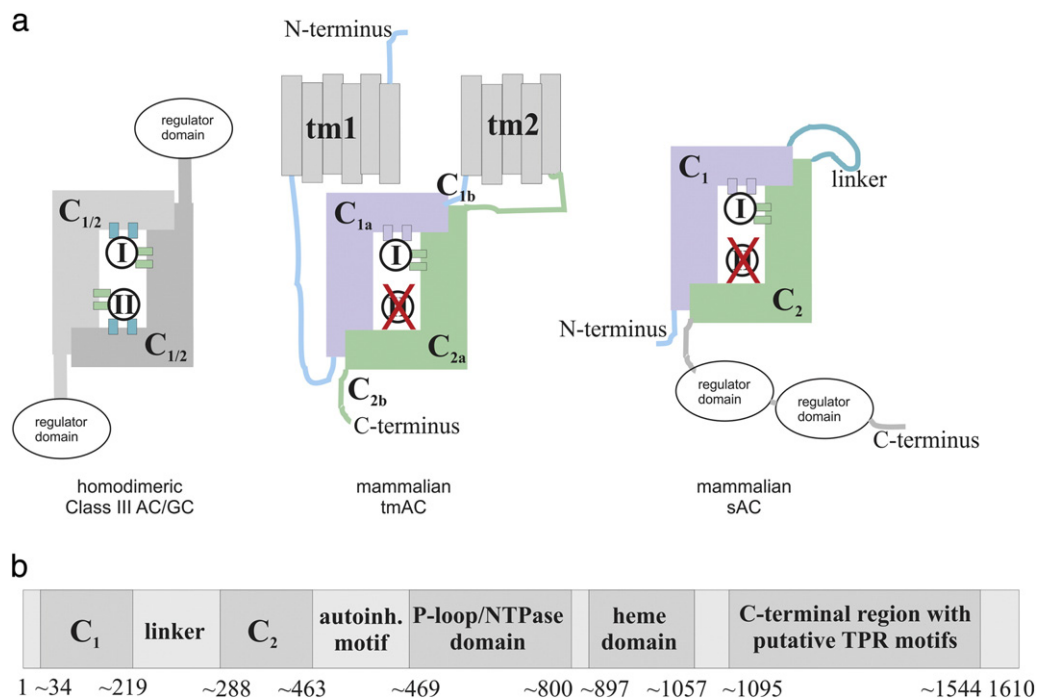
Fig. 1. Domain architecture of mammalian tmAC and sAC. (a) Some of the general architectures of Class III nucleotidyl cyclases. Homodimeric enzymes feature two symmetric, fully active centers because both catalytic domains can contribute conserved catalytic residues typical for C_1 and C_2 (they are therefore labeled as $C_{1/2}$ domains). Mammalian tmAC and sAC carry two structurally similar catalytic domains on one polypeptide. C_1 and C_2 contribute only a specific subset of the catalytic residues, resulting in one active site and a pseudo-symmetric, inactive pocket. (b) Scheme of the domains and sequence regions in mammalian sAC. The domains/regions are not drawn to scale, residue numbers given at the bottom refer to human sAC.

microbial Class III AC [43], which thereby regulate diverse functions from metabolic adaptation to virulence [6]. The mycobacterial Class III AC Rv1625c is an example for a more tmAC-like microbial enzyme [44]. However, microbial ACs appear generally more closely related to sAC than to tmACs (see Refs. [4,45] and Section 2.3), and even several sAC-like bicarbonate-activated ACs have been identified in microorganisms. The sAC-like AC in *Cryptococcus neoformans*, e.g., features Leu-rich repeats and a protein phosphatase-like domain and integrates signals such as bacterial peptidoglycans and CO_2 levels to regulate environmental adaptations and virulence [30,46]. CyaB, a sAC-like AC from *P. aeruginosa*, features an N-terminal "membrane-associated sensor 2" (MASE2) domain and integrates CO_2 sensing with regulation through a Chp chemosensing system [36,47]. Many other regulatory modules can be found fused to Class AC catalytic domains [43]. The mycobacterial AC Rv1264 is regulated by a pH-sensing domain [48], and small-molecule binding modules such as GAF (first identified in cGMP-binding PDE, AC, and the *E. coli* transcription factor FhlA) domains make AC responsive to regulators such as cGMP [43]. However, the ligand is not known for many of these AC regulating domains.

Mammalian AC isoforms also comprise significant protein regions outside the catalytic domains. They likely serve regulatory functions but might also contribute to intracellular localization. Among the mammalian AC, the nine tmACs feature variable N-terminal regions, followed by a six transmembrane (tm) helices domain (tm1), the first catalytic domain C_{1a}, a second six transmembrane helices domain (tm2), and the second catalytic domain C_{2a} (Fig. 1a) [4,49]. Linker and tm regions likely provide additional functions other than membrane anchoring and fusing different protein parts. However, little is known beyond a function of the C_{1b} linker fusing C_{1a} to tm2 in regulation by free Ca^{2+} and by Ca^{2+}/calmodulin and a likely contribution of N-terminus and the C_{2b} region C-terminal from C_{2a} to Ca^{2+}/calmodulin recognition. In sAC, the catalytic domains C_1 and C_2 are positioned at the N-terminus and connected by a ~68 residue linker that forms a death domain like subdomain with the ~33 residue N-terminal tail of the protein (Fig. 1b) [19]. C-terminal from this C_1–C_2 tandem, full-length mammalian sAC comprises a ~1100 residue C-terminal region (CTR), mostly with little understood function and apparently without a tm region (Fig. 1b) [4]. The sAC-CTR starts with a small motive mediating an autoinhibitory effect (see Ref. [50] and Section 2.4.1). It likely acts together with other sAC-CTR domains, such as the neighboring putative NTPase domain. p NTPase-like domain and weak sequence similarities further C-terminal, sAC was suggested to belong to the "signal transduction ATPases with numerous domains" (STAND) family [51,52]. In STAND proteins, such as the apoptosis regulator Apaf-1 and the transcriptional activator MalT, intrinsic NTPase activity regulates the protein's signaling status through conformational changes in linker and/or effector domains. In MalT, e.g., a NTPase domain and a tetratrico peptide repeat (TPR)-related domain enable ATP and maltotriose to induce activation through the modulation of interactions with an inhibitory protein and of MalT oligomerization [52–54]. Its intrinsic ATPase activity switches MalT off again [52]. Besides the NTPase-like domain, the sAC-CTR contains three regions with putative TPR modules [20], but a MalT- and STAND-related regulation of sAC remains to be confirmed. Physicochemical sequence analysis and expression of sAC fragments in *E. coli* in fact revealed a domain that can bind a heme ligand and that covers part of the first predicted TPR region, making a TPR-like structure less likely [20]. This sAC heme-binding domain (sAC-HD) shows no sequence similarity to the heme domain of the related Class III enzyme soluble guanylyl cyclase (sGC), which acts as physiological receptor for the activator nitric oxide (NO). The sAC-HD instead might be remotely related to PAS domains, which are also found in bacterial Class III AC and sGC [43,55], or it might represent a novel heme domain type [20]. Nevertheless, NO and another potential gaseous signaling molecule, carbon monoxide (CO), can bind to sAC-HD. A variety of sAC isoforms comprising different CTR parts appears to be generated through alternative splicing (see Refs. [56,57] and Section 2.4.1),

and sAC species comprising the HD are found at least in testis and skeletal muscle [20]. sAC-HD thus makes sAC a candidate for the missing link between NO and cAMP signaling, e.g., in sperm cells [58,59]. However, the physiological interaction partners and/or functions of sAC-HD remain to be clarified. For the remaining sAC-CTR, where no well defined sequence motifs and homologies have been identified besides the features discussed above, we also have to await further studies to understand how they contribute to the regulation of sAC activity and localization.

2.2. Catalytic domain structure and catalytic mechanism - general and sAC-specific features

2.2.1. Catalytic domain structure

The first crystal structure for an active Class III AC catalytic core, a heterodimer of a mammalian tmAC5-C_1 and tmAC2-C_2 domain [42], revealed a head-to-tail arrangement of these structurally similar domains resulting in a compact core. This arrangement was subsequently confirmed as general Class III setup through crystal structures of a variety of AC and GC catalytic cores [4,37–40], including the bacterial sAC-like AC CyaC [17]. Only recently, crystallization and structure solution were reported for the mammalian sAC catalytic core (sAC-cat) and several sAC-cat ligand complexes [19,45,60,61]. The structure of this human sAC-cat will now be described in order to provide information on the architecture of a typical Class III cyclase catalytic core, and sAC specific features will be explicitly pointed out and compared to tmACs.

The overall sAC-cat structure shows the typical Class III pseudo-heterodimer arrangement of structurally similar C_1 (residues 34–219; numbering refers to human sAC if not stated differently) and C_2 (288–463) domains (Fig. 2a, top left; C_2 residues and secondary structure elements are labeled by an asterisk) [19]. Both domains feature the generic Class III monomer fold with a central seven-stranded β-sheet shielded from solvent by helices α2, 3, and 5 [4,37]. The dimer is stabilized through a clamp-like extension formed by the β4 C-terminus and β5 strand of both pseudo-monomers. Interestingly, molecular replacement phasing with the available Class III AC structures was not successful and the sAC-cat structures had to be solved using experimental phasing strategies [19,61]. However, only small differences in overall structure to other Class III AC were identified. sAC-specific features are its short N-terminal extension and the C_1–C_2 linker. The ~33 residue region N-terminal from C_1 forms an extended tail (residues 1–12) and two additional α-helices (αN1, αN2; Fig. 2a, right). With two of the three α-helices in the ~68 residue C_1–C_2 linker (αL1-3), they form a death domain-like four helix bundle (NLH-bundle) [19]. This linker starts with three consecutive Pro (220–222), located at a corner of the core particle and next to a partially solvent accessible hydrophobic patch. Part of the hydrophobic surface interacts with the N-terminus of a symmetry related monomer, and these sAC regions have been speculated to serve as interaction sites for other proteins or sAC domains in solution [19].

The active site of the Class III cyclase catalytic core, with seven highly conserved catalytic residues, is located at one pole of the pseudo-dimer interface and formed by residues from both, C_1 and C_2 (Fig. 2a,b). However, C_1 and C_2 provide distinct catalytic residues, and since C_1 lacks essential C_2 residues and the other way round, the pseudo-symmetric site at the other pole of the dimer interface is inactive and instead serves as a regulatory site (see Section 2.4.1). In the active site, two conserved Asp residues (Asp47 and Asp99) form binding sites for two divalent ions, ion A and ion B (Fig. 2b). The ion sites are normally occupied by Mg^{2+} and contribute to substrate binding (ion B) and turnover (ion A), respectively (see Section 2.2.2). Ion A and Arg416* interact transiently with the α-phosphate (see Section 2.2.2), while ion B is coordinated by the β- and γ-phosphates, which are further bound by main chain atoms at the α1 N-terminus and by polar side chains (Ser49, Thr52, Asn412). A mainly hydrophobic cleft formed by
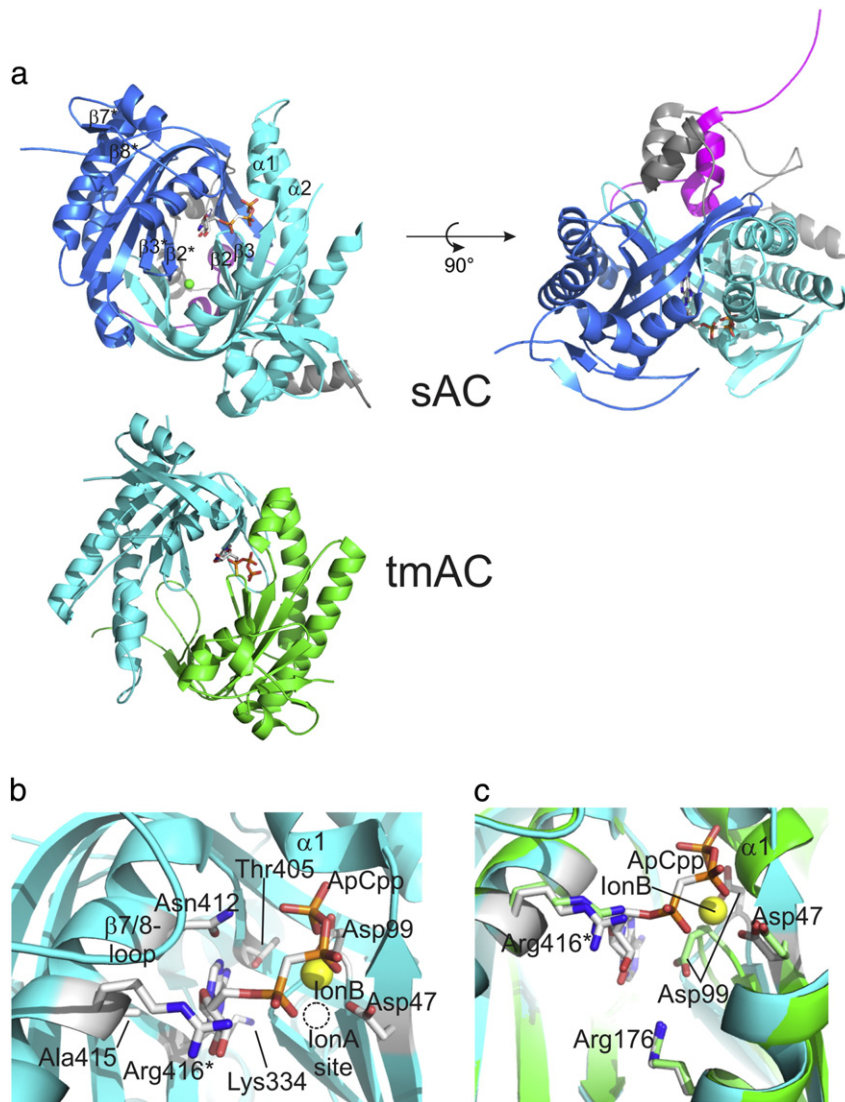
Fig. 2. Structure of the sAC catalytic core and comparison to tmAC. (a) Crystal structure of the human sAC-cat complex with the substrate analog ApCpp (PDB ID: 4CLK) in two orientations. The front view (left) shows the entrance to active site (containing the substrate analog in stick presentation; upper right) and regulatory pocket (containing chloride as green sphere; lower left) formed at the interface of the C_1 (cyan) and C_2 (blue) domains. The side view (right) shows the death domain like helix bundle on the back of the pseudo-heterodimeric core, formed by helices from N-terminus (magenta) and C_1–C_2 linker (gray), respectively. Only selected secondary structure elements are labeled. For comparison, a front view of a tmAC/ATPαS/forskolin complex (1CJK) is shown below sAC. The bound Gsα protein (positioned at the lower left tmAC corner) is omitted for clarity. (b) Active site of a sAC/ApCpp complex (4CLK). The ATP analog and catalytically important residues are shown in stick presentation. The Ca^{2+} in the ion B site is shown as a yellow sphere, and the missing ion A is indicated by a dotted circle. (c) Overlay of the sAC/ApCpp complex (cyan; 4CLK) with apo sAC (green; 4CLF), showing that Asp99 moves away from Arg176 to allow binding of nucleotide and ion B. Selected residues are shown in stick presentation, ion B as a sphere.

Ala97, Phe296, Leu345, Phe336, Val406, and Val441 accommodates the substrate base, and adenine is supposed to be recognized through two polar interactions, between Lys334 and the ring nitrogen N1 and between Thr405 and the 6-amino group, which further forms two main chain contacts (Fig. 2b). These interactions are considered the major determinants for the ATP or GTP specificity of Class III AC and GC, respectively [4,62]. The Lys334 interaction is not seen in the available sAC-cat/nucleotide complexes and might be transient (see Section 2.2.2). The Thr appears typical for sAC-like AC and is functionally replaced by Asp in most other Class III AC [4,62]. The Thr (can act as H-bond donor and acceptor) has been speculated to cause the significant GC side activity of sAC, since it appears to enable a GTP binding mode excluded by Asp (obligate H-bond acceptor) [45]. Also other residue combinations have been observed in microbial AC [62]. In mammalian GC, the Lys and Thr/Asp are replaced by Glu and Cys, respectively, adapting the residues to the H-bond donor/acceptor pattern of guanosine. Mutating these positions converted Class III cyclase specificity in some cases but not in others

[4,62,63]. Additional factors thus appear to contribute to substrate selection, such as steric complementarity between base and active site and the dimer arrangement, which is influenced by these residues due to the dimer interface location of the active site [38,64].

The substrate ribose also occupies a mainly hydrophobic pocket and only forms a polar contact with its ring oxygen to Asn412. In tmACs, the ring oxygen further appears to interact with a conserved Ser (Ser1028* in tmAC2-C_2) that is replaced by a conserved Ala in sAC-like AC (Ala415* in hsAC). This difference is speculated to be relevant for the lower ATP affinity characteristic for sAC-like AC [17].

Interestingly, in the sAC-cat apo structure Asp99, one of the two conserved Asp, is shifted from its ion coordinating position (Fig. 2c). An Asp side chain rotation opening the ion sites had previously been observed in the bacterial GC Cya2 [38] but in sAC, the whole active site loop β2/3 comprising Asp99 is significantly shifted toward the sAC-specific residue Arg176, resulting in a Arg176/Asp99 salt bridge and an overlap of the β2/3-loop with the ion sites [19]. Comparison to a sAC-cat/ATP analog complex structure shows no major differences

in overall structure but dramatic rearrangements of the regions comprising the two conserved Asp (Fig. 2c). A partial unwinding of the small, catalytic helix α1 [17,42] releases the interaction between the backbone of Ser49 and Asp47, allowing the Asp to tilt into position for ion binding. The necessary space is opened up through a β2/3-loop rearrangement, which further reorients Asp99 away from Arg176 and in position for substrate binding. The thereby formed ion B site is occupied by a Ca²⁺ ion (see Sections 2.2.2 and 2.4.1) in the sAC-cat/ApCpp complex [19]. This substrate-induced active site rearrangement has not been observed for tmACs and might contribute to the lower ATP affinity of sAC-like AC (see Sections 2.2.2 and 2.4.1).

2.2.2. General catalytic mechanism of Class III AC and sAC-specific features

Overall structure, active site architecture, and residues involved in substrate binding and catalysis are conserved or show only functionally conserved substitutions in Class III cyclases (see Refs. [4,38] and the structure-based comparison and alignment in Ref. [45]), suggesting that only small variations from a generic catalytic mechanism occur in this enzyme family. The widely accepted Class III mechanism, initially suggested based on stereochemical studies and now supported by many biochemical and structural data, involves an intramolecular attack of the substrate's ribose 3′-hydroxyl group at Pα and simultaneous release of Pβγ (Fig. 3a) [4,65]. This pseudo-binuclear nucleophilic substitution (S_N2) mechanism with an in-line arrangement of the attacking 3′-OH and the Pα-O bond being broken implies a pentavalent transition state with an additional negative charge at Pα, which is stabilized by ion A and Arg416* (Fig. 3a). Ion A is therefore called the catalytic ion. Due to this temporary completion of the ion A site, this ion appears to bind transiently during catalysis and is rarely observed in AC/substrate analog complexes, most likely only when a non-productive nucleotide conformation assists in an artificial ion A coordination [17,66]. In a CyaC complex with Rp-ATPαS, the thio-modification causes an non-productive α-phosphate orientation resulting in a stable complex with ion A, whereas in CyaC and sAC complexes with Ca²⁺/ApCpp, the triphosphate assumes a conformation suitable for the in-line reaction and no ion A is bound [17,19]. Ion A

might also contribute to deprotonation of the attacking 3′-hydroxyl, either directly or through a Mg²⁺-activated water molecule [4,17,66]. Ion B contributes to binding, and possibly proper orientation, of the ATP substrate through interactions with Pβ and Pγ [17,19,66], and Ca²⁺ appears to enable a stronger interaction than Mg²⁺ in this site (see Section 2.4.1). The γ-phosphate is further bound by Asn412, Thr52, and several backbone atoms in a pocket at the α1 N-terminus, and the β-phosphate also interacts with the α1 N-terminus, via Ser49 and the Ile48 carbonyl oxygen. This phosphate binding mode is generally seen in Class III cyclase complexes with NTPs [4,37], only the substrate-induced formation of this phosphate-binding pocket, along with ion sites, through partial α1 unwinding (see also Section 2.2.1) has so far only been observed for sAC-cat [19]. The requirement of an active site rearrangement in sAC might contribute to its lower ATP affinity compared to tmACs (see Section 2.4.1). In the sAC/ApCpp complex, the α-phosphate already interacts with Arg416*, the conserved Class III residue that will stabilize the transition state (see above), while no such interaction with the substrate ground state is assumed for tmACs/nucleotide complex, but it remains to be seen whether this contact is also formed with ATP rather than an analog [19,42]. In sAC, Arg176 is positioned opposite the ribose hydroxyls and might facilitate the deprotonation of the 3′-OH.

Structures of sAC-cat in complex with the products cAMP and PPᵢ, alone and in combination, show binding modes for the nucleoside and the β/γ-phosphates that are very similar to those with substrate analog [19]. It thus appears that they do not move significantly during catalysis and suggest that product formation proceeds mainly via an α-phosphate movement toward the 3′-OH (Fig. 3a). However, the conserved adenine-recognizing residue Lys334 interacts with the base of substrate and product, respectively, only indirectly via a water molecule. It indeed appears that the full interactions required for base recognition are formed only after initial binding of the nucleotide, since binding affinities of sAC and the bacterial GC Cya2 for their substrate and the respective other nucleotide are similar, yet turnover rates differ [19,38]. Soaking of sAC-cat crystals with ATP shows that the sAC retains its activity within the crystal lattice, so that a transient Lys334/adenine interaction has to be assumed [17,19]. Similar to the sAC/nucleotide
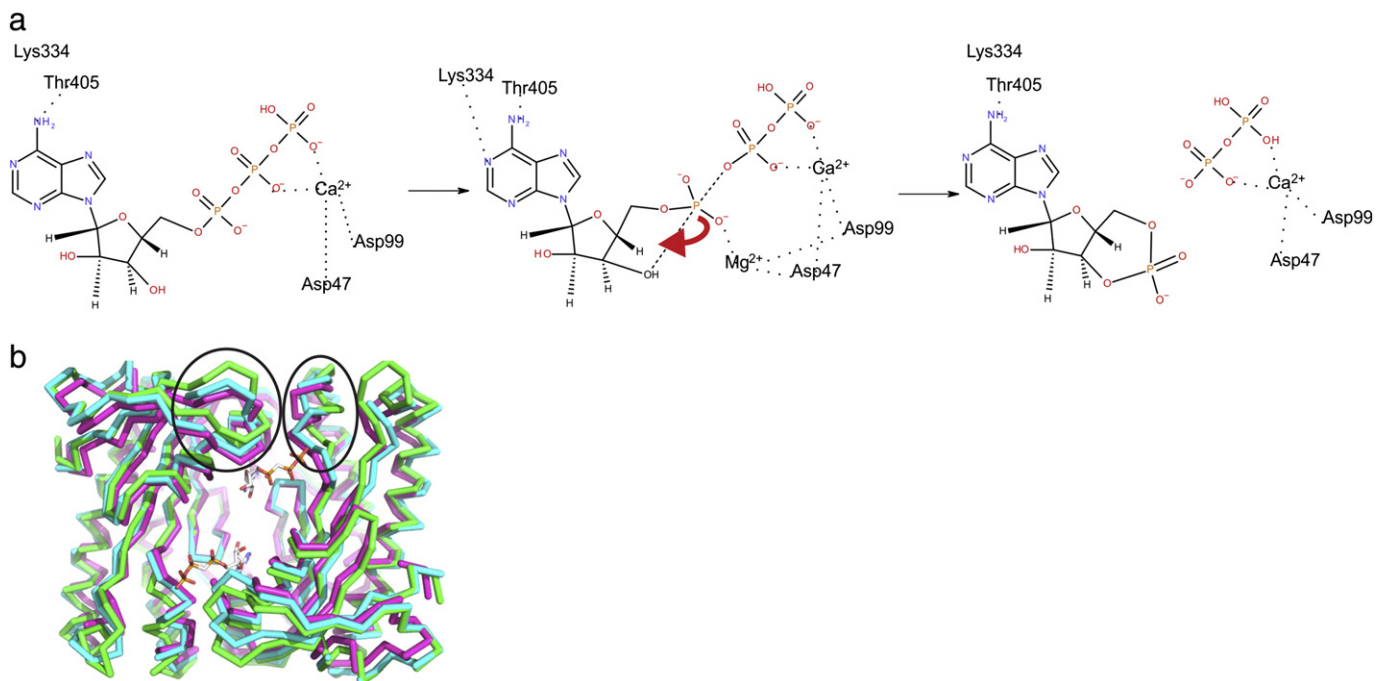
Fig. 3. Catalytic mechanism of Class III AC. (a) Catalytic mechanism of Class III AC, with residue numbers referring to human sAC. A major interaction for substrate binding is mediated by the divalent cation in the ion B site (here shown as Ca²⁺). The substrate base and the α-phosphate appear to form transient interactions with Lys334 and ion A (here shown as Mg²⁺), respectively. Arrow: Movement of α-phosphate. (b) Active site closure movement of Class III AC. An overlay of CyaC in complex with ApCpp (green; PDB ID: 1WC0), ATPαS (cyan; 1WC1), and ATPαS in presence of bicarbonate (magenta; 1WC6) shows that the β7/8-loop and α1 (circled) move from open to closed to further closed positions.

complexes, this base contact is not observed in a tmAC/ATP complex [67], suggesting that such a transient nucleotide discrimination during turnover is a general Class III cyclase feature. In contrast, the Lys/adenine interaction is observed in several AC complexes with ATP-analogs [4,37], but it might be visible because of a slightly shifted position of the artificial, reaction-incompetent substrate analog. It is tempting to speculate that a temporary shift of the substrate AMP moiety toward the conserved base-recognizing Lys supports elongation and ultimate breaking of the covalent bond between α- and β-phosphate (Fig. 3a). Lys144, located at the other end of the active site cleft, appears to act as a "PP$_i$ receptor", helping to keep the terminal phosphates in position or even slightly tearing Pβ away from Pα.

In the sAC/cAMP complex, the product phosphate position appears to clash with the ionA site, likely contributing to the transient binding of this ion [19]. The cAMP product then appears to be released first. PP$_i$ binds with higher affinity [19] and its release has in fact been observed to be rate-limiting for tmACs under certain conditions [68]. A reorientation of Asp399* and the surrounding β2/3* active site loop was observed upon formation of the trimeric complex of sAC-cat with the two products cAMP and PPi and is speculated to constitute an enzyme state involved in catalysis or product release [19]. A steric clash with Asn412 is another mechanism suggested to contribute to product release [37], but its relevance also remains to be confirmed.

An active site closure movement of two structural elements, α1 and a β7/β8 module, appears to contribute to Class III AC catalysis [4,17,42]. It was first proposed as a substrate-induced active site closure [42]. An alternative model was later based on CyaC structures and the substrate analog ApCpp, which might be a better substrate analog for AC than other nucleotides because it allows proper alignment of the 3′-hydroxyl and the bond to be broken (see above) [17]. In this model, substrate binding actually opens the active site further before the closure movement then contributes to substrate turnover by supporting separation of the α- and βγ-phosphates [17] (Fig. 3b). Product dissociation would finally release the interactions to α1 and β7/8 and allow active site opening. Consistent with such an opening upon PP$_i$ release, tmAC complexes with P-site inhibitors (adenosine derivatives requiring a polyphosphate tail or pyrophosphate for efficient inhibition; see Section 2.4.2), which are assumed to resemble enzyme/product complexes, show a closed enzyme conformation [42,66,69]. However, the series of sAC-cat complex structures with various ligands, including substrate analog and products from substrate turnover, are all obtained with the same crystal form and in a rather closed conformation [19]. Crystal packing might restrict the conformations accessible to the protein, but it retains full activity in the crystals and thus appears to be able to access all states necessary during catalysis [19,45]. The substrate-induced active site opening thus might be more local in sAC, making it compatible with the packing, and it might occur more transient before a closure as proposed in the initial model. However, the exact role of the open/closed transition of α1 and the β7/8 module and identification of the dynamic active site processes during substrate binding and turnover clearly require further studies.

Interestingly, Class II ACs are structurally unrelated to Class III enzymes but appear to employ a related catalytic mechanism. The monomeric Class II catalytic domains have a single active site with two ion binding sites, which appear to functionally resemble the Class III ion A and ion B sites [70]. Similarly, a Class III-like mechanism with an in-line S$_N$2 reaction catalyzed by using two divalent cations is assumed for Class IV AC [71]. Since Class II, III, and IV show no significant similarity in their overall structure, it is assumed that their common mechanistic features are the result of a convergent evolution process [71].

2.3. Evolution of mammalian AC and Class III subfamilies

Class III AC catalytic cores are assumed to have evolved divergently [9]. They are fused to a large variety of regulatory domains (see Section 2.1)

and show recognizable homology but low overall sequence identity [9,43], suggesting that they might be evolutionary old. Only few positions are strictly conserved, because they are critical for the general structure of Class III AC or because they directly contribute to catalysis [4,43]. Even among the catalytic residues, such as the specificity-determining residues, functionally conservative substitutions are seen (see Section 2.2.1). Based on such positions, four Class III subclasses have been defined [43].

sAC was proposed to be most ancient among the mammalian cyclases based on a higher catalytic core sequence similarity to ACs from cyanobacteria and myxobacteria than to other mammalian cyclases [4,14]. To form C$_1$–C$_2$ cyclases, such as mammalian sAC and tmAC (see Section 2.1), a homodimeric primordial cyclase apparently underwent gene duplication and diversification, and evolutionary closeness to bacterial or mammalian cyclases appear even distinct for sAC's C$_1$ and C$_2$ domain [4,72]. Including the growing number of available crystal structures in such comparisons has revealed previous alignment errors but supports a closer relationship of bacterial AC to sAC than to tmACs, although no larger structural features specific for subfamilies and thus defining such relationships are evident (see Ref. [45] and Section 2.2). Further evolutionary details will likely require additional Class III AC crystal structures and careful correction of sequence alignments. However, mammalian sAC forms a cAMP signaling system with PDE2 and PKA in the matrix of mitochondria [28,73,74], which are assumed to have evolved from proteobacteria, and this metabolic regulation function is speculated to constitute an evolutionary early acquired role of sAC [75]. Also, mammalian sAC and several bacterial Class III ACs are similarly regulated by bicarbonate and Ca^{2+} (see Refs. [4,16,17,29] and Section 2.4.1), suggesting an evolutionary closeness. Another indication for such a closeness are bacterial ACs with similarities to the sAC-CTR, such as a mycobacterial AC and three *Chloroflexus* proteins that feature a P-loop containing putative NTPase domain C-terminal from C$_2$ (see Section 2.1) [72,76].

sAC-like ACs were identified in the genomes of many eukaryotic organisms, but only few of them have been cloned and further studied. sAC gene has been reported for chicken, ciona intestinalis, corals, mosquito, honey bee, dogfish, and all mammals analyzed (human, chimpanzee, dog, cow, rabbit, mouse, and rat) [4,77,78]. Surprisingly, a sAC-like gene has not yet been identified in other organisms, including the widely used model organisms *Caenorhabditis elegans* and *Drosophila melonogaster*, and it has been speculated that these lineages lost their sAC-like cyclase during evolution [79]. The physiological relevance of a second sAC-like locus in the genomes of human, chimpanzee, dog, and cow remains to be clarified [4].

Appearance of G-protein responsive tmACs starts only in metazoans, but related cyclases not regulated by G-proteins have been characterized from *Mycobacterium tuberculosis* and protozoans [80,81]. However, most microbial ACs appear either more closely related to sAC or not closely related to either sAC or tmACs. Conversely, fungal ACs appear to feature properties from both sAC and tmAC, although they seem to form a separate branch in the Class III cyclase phylogenic tree [4]. The AC enzymes from *Saccharomyces cerevisiae* and from the pathogenic fungi *C. albicans* and *C. neoformans* are regulated by G-proteins [82,83], but they lack obvious tm regions and are also regulated by bicarbonate [84].

2.4. Regulation of sAC-like AC

Like other signaling enzymes, Class III ACs are highly regulated, most often through various signals and mechanisms such as small-molecule ligands, posttranslational modifications (PTMs) and regulatory proteins. The regulation of sAC-like AC clearly distinguishes them from tmAC and other Class III AC. sAC-like ACs show an increase in apparent ATP affinity in presence of Ca^{2+}, and their turnover rates are increased by bicarbonate [16,17,29]. Mammalian tmACs, in contrast, are not responsive to bicarbonate but are mainly regulated by heterotrimeric G-proteins [10]. The regulation of tmACs has been reviewed extensively (see, e.g., Refs. [5,10,85]) and will be discussed here only in comparison to sAC

regulation rather than in detail. Furthermore, we will focus here on general regulation features of sAC-like enzymes and on mechanisms applying to mammalian sAC enzymes, but not on mechanisms specific for sAC enzymes from lower organisms.

2.4.1. Physiological regulation

2.4.1.1. Isoform expression and localization. The single confirmed mammalian sAC gene and the nine mammalian tmAC genes encode isoforms with distinct regulation. They show different tissue expression patterns, which can also depend on the developmental state [85,86]. sAC was initially identified in testis, where its functional relevance is long known [14], but it was subsequently also identified in many additional tissues [15,20,21,56,87]. Alternative splicing has been reported for sAC and for several tmAC isoforms, but the physiological significance of the tmAC variants is largely unexplored [4]. For sAC, in addition to a 187 kDa full-length enzyme (sAC$_{fl}$) smaller splice variants appear to be formed. A ~50 kDa sAC form was initially obtained during isolation of sAC activity from rat testis and termed sAC$_t$ (sAC truncated) [14]. It is restricted to the N-terminal part of the full-length protein covering C$_1$ and C$_2$ and was later found to be formed through alternative splicing [57]. sAC$_{fl}$ and sAC$_t$ are both regulated by Ca^{2+} and bicarbonate, which directly act on the catalytic core (see below) [14,57]. However, sAC$_{fl}$ appears to display lower basal activity due to the presence of a small auto-inhibitory module C-terminal from C$_1$ (see Ref. [50] and below), and its large CTR (see Section 2.1) should mediate additional sAC$_{fl}$-specific regulation and localization mechanisms that remain to be established. A physiological role also remains to be identified for two sAC splice variants of ~80–85 kDa, which were identified in several human tissues and cell lines but would require a partner protein for activity due to a missing or incomplete C$_1$ domain [4,56].

Subcellular localization contributes to mammalian sAC regulation. While all tmAC isoforms appear restricted to the cell membrane, sAC can dynamically localize to different areas of cAMP regulation, such as the nucleus or mitochondria [22,27,28,74,88,89]. This spatio-temporal distribution of sAC contributes to the dynamic formation of intracellular, locally restricted cAMP signaling domains through interplay with a large variety of cAMP-degrading PDEs [8,22,89–91]. The different regulation of PDEs, tmACs and sAC isoforms, and their tissue-specific and dynamic expression and localization provides differently regulated and localized cAMP signaling systems, explaining how cAMP can regulate diverse, and sometimes seemingly contradicting processes even within a single cell type [22,88]. However, the exact signals and mechanisms mediating intracellular sAC relocation remain to be identified.

2.4.1.2. Regulatory domains and proteins. The main regulators of tmACs are heterotrimeric G-proteins, whereas no regulatory proteins have yet been identified for mammalian sAC. Gα subunits bind directly to tmAC catalytic domains, leading to stimulation of all tmAC isoforms by the Gs α-subunit (Gsα), and inhibition of tmACs 1, 5, and 6 in case of Goα or Giα [10]. All available heterodimeric tmAC structures comprise bound Gsα [37], making it difficult to deduce the effects of its binding. However, comparisons to other AC structures and biochemical data suggest that G-protein binding influences stability and relative orientation of the two catalytic domains and thereby the proper orientation of the interface residues forming the active site [42,49]. Such a subunit rotation is assumed to constitute a major mechanism in Class III AC regulation [4,42] and was also proposed, e.g., for regulation of a bacterial AC by a pH-sensing domain [48] (see below). Despite the structural similarity of sAC to tmACs (see Section 2.2), smaller sAC-specific features likely explain its G-protein insensitivity. The C$_2$-α1/α2* loop as a major Gsα binding region in tmACs is shortened in sAC (and α1 in fact not present) [19]. Likewise, the extended tmAC C$_2$-α3/β3* loop implicated in G$\beta\gamma$ binding, which can have an activating or inhibiting effect on several tmACs [10,92], is missing in sAC [19,45]. The other

way round a ~8 residue insertion in the sAC α1/α2 loop, which was mapped as Giα binding site through mutagenesis, should hinder such an interaction with sAC. Other tmAC regulator proteins are RGS2 and Ca^{2+}/calmodulin [10], and while tmAC regions contributing to the interaction with Ca^{2+}/calmodulin have been mapped (see Section 2.1), no details are known about these interactions.

tmACs can be affected by S-nitrosylation and N-glycosylation, and in particular, several isoforms are phosphorylated and regulated by CaM kinase, PKA, PKC, or receptor tyrosine kinases [4,10,85]. For sAC, the only PTMs reported so far are phosphorylations. Several sAC residues were indicated as potential phosphorylation sites in mass spectrometry-based phosphoproteomics studies, but only three of them were identified in at least two such large scale experiments [93]. Tyr268 in the C$_1$–C$_2$ linker is oriented into the core of the NLH-bundle, and its phosphorylation might indicate dynamic rearrangements for this region. Thr1132 and Tyr1134 are located in the functionally uncharacterized putative TPR region [93]. The relevance of these phosphorylations for regulation of sAC activity or localization remains to be analyzed.

A variety of domains can be fused to Class III catalytic domains (see Section 2.1) and regulate AC activity or localization directly or through interaction with regulatory proteins. Examples comprise a regulator domain mediating pH sensing, and possibly also fatty acid-dependent regulation, in mycobacterial ACs and a MASE2 containing N-terminus in the bicarbonate activated CyaB from *P. aeruginosa* (see also Section 2.1) [36,48,94]. The pH sensing domain is the only case of structurally characterized fusion of catalytic and regulatory domains. The pH sensor appears to prevent proper orientation of the two catalytic domains in the inactive state and to promote a relative rotation of the catalytic domains in an active state through a helix-to-loop transition of the linker to the regulatory module [48]. Such an effect on the relative orientation of the catalytic domains is also assumed to contribute to tmAC regulation by G proteins (see above). The MASE2 domain in CyaB obviously targets the protein to the cell membrane, but the very N-terminus, C$_1$–C$_2$ linker, and a C-terminal extension also influence CyaB activity and its regulation by the protein PilG, yet the mechanisms remain to be established [36]. Similarly, the tm modules dominate the regions outside the catalytic domain in mammalian tmACs and only small stretches contribute, through unknown mechanisms, to regulation, e.g., by Ca^{2+}/calmodulin (see Section 2.1 and Ref. [85]). In mammalian sAC, a large ~1100 residue CTR appears to comprise several domains (see Section 2.1), but only two regions have been studied in more detail. A nine residue region immediate C-terminal to C$_2$ was proposed as an auto-inhibitor module based on deletion constructs and was suggested to cause the lower basal activity of sAC$_{fl}$ as compared to sAC$_t$ that ends after C$_2$ [50]. A mutagenesis screen revealed three substitutions relieving autoinhibition [50], and the sAC structure shows that they locate to the end of β8, which participates in the putative catalytic closure movement (see Section 2.2.2), and contribute to packing against β7 and the bulk catalytic core [19]. Interestingly, a similar screen also identified an activating mutation in the corresponding region of the bacterial sAC-like CyaB [36], and it will be interesting to see how this region regulates the catalytic domains, alone or in combination with other domains (such as STAND domains in mammalian sAC — see Section 2.1) or proteins (such as the CyaB regulator PilG).

2.4.1.3. ATP and GTP. Mammalian sAC and other sAC-like AC show a low apparent ATP affinity, with a K_M around 10 mM in presence of Mg^{2+}, which is two orders of magnitude lower than typically observed for other Class III AC [4]. The low substrate affinity of sAC appears relevant for two regulation mechanisms. It enables activation by Ca^{2+}, which increases the apparent affinity (see below), and it allows sAC to serve as an intracellular ATP sensor by rendering its activity sensitive to physiological changes in ATP concentrations (typically 1–3 mM) [18]. The loss of a polar interaction to the

substrate ribose due to replacement of a conserved Ser by Ala in sAC-like AC has been proposed to be mainly responsible for this typical sAC feature (see Refs. [19,45] and Section 2.2). The lower apparent ATP affinity of sAC might also contribute to an inhibitory binding mode that is specifically observed for sAC enzymes and causes substrate inhibition at ATP concentration above 5 mM [16,17]. However, a physiological relevance of substrate inhibition and the molecular details involved in this effect remain to be established.

GTP appears to bind with similar affinity as ATP to the active site of sAC, and possibly other ACs, followed by slow turnover (see Refs. [19,45] and Section 2.2). Crystallization of a sAC/GTP complex has not been successful so far, but the absence of adenine-specific interactions in sAC complexes with ATP or ATP analog likely explains the lacking discrimination during binding (see Refs. [19,45] and Section 2.2). GTP thus might cause competitive inhibition under physiological conditions, since cellular GTP concentrations appear to be slightly lower but in the same order of magnitude as ATP concentrations [95]. Interestingly, ATP can bind to one of the two active sites of homodimeric mammalian receptor GCs, which appears to contribute to their physiological activation [96]. The sites are directly neighboring to each other and possibly further coupled through dimer rotation (see above). The half-of-the-site activity and spontaneous functional diversification for two identical sites in homodimeric bacterial Class III cyclases (see below, bicarbonate) also support a regulatory function and coupling of the second active site. However, a GTP binding to the regulatory site of sAC can be excluded due to steric restrictions, and a physiological relevance of sAC inhibition through GTP binding to its active site remains to be shown.

The mammalian sAC CTR with its putative NTPase domain might mediate a second, so far uncharacterized nucleotide-dependent regulation mechanism (see Section 2.1). Again, a functional analogy might be observed for mammalian receptor GCs, where a kinase homology domain appears to contribute to ATP-dependent modulation of receptor activation [97].

2.4.1.4. Ca^{2+}. tmAC and sAC-like AC can be either activated or inhibited by Ca^{2+}. Activation of tmAC 1 and 8 is mediated by Ca^{2+}/calmodulin, which appears to bind to regions in the tmAC N- and C-terminus [85]. Low micromolar concentrations of free Ca^{2+} selectively inhibit tmAC 5 and 6, whereas high concentrations (mM) inhibit all tmACs [85]. Both effects appear to be caused by Ca^{2+} binding in the ionA/B region [67], suggesting that differences in the surrounding of the conserved, ion coordinating Asp pair (see Section 2.2) influence the binding of ions and/or substrate. Indeed, sAC-like ACs also feature the Asp pair but show a unique regulation by Ca^{2+}. In contrast to tmACs, Ca^{2+} alone, in absence of Mg^{2+} and Mn^{2+}, enables low sAC activity [45]. More importantly, Ca^{2+} stimulates the Mg^{2+}-dependent activity of sAC-like ACs, an effect that appears to modulate cAMP formation, e.g., in mitochondria and in sperm and insulinoma cells [31,98,99]. Although *in vitro* Ca^{2+} activation saturates only at high micromolar concentrations, significant stimulation of sAC activity is already observed with submicromolar Ca^{2+} [16,56]. Biochemical and structural studies show that Ca^{2+} activates sAC and its homologues through binding to the ion B site, where it functions as a stronger interaction partner than Mg^{2+} for the $\beta\gamma$-phosphates of ATP [17,19]. Thereby, it increases the apparent substrate affinity, reflected by a lowering of the K_M^{ATP} in presence of Mg^{2+} from ~10 mM to ~1 mM through Ca^{2+} addition [16,17]. Physiological sAC activity thus appears optimally supported by Mg^{2+} in the ion A site and Ca^{2+} in the ion B site. Interestingly, studies on sAC protein immunoprecipitated from testis or overexpressed in HEK293 cells indicated a biphasic activation in response to increasing Ca^{2+} concentrations [99]. An initial phase of Ca^{2+}-induced doubling in sAC activity was observed in the range of 10 nM to 10 μM, due to an increase in v_{max} rather than a lowering in K_M^{ATP}. This potent Ca^{2+} effect was also independent of calmodulin and required only the catalytic domains [99], suggesting that it might also be mediated via the ionA/B

sites. It remains to be seen, however, which mechanisms and molecular features, such as PTMs, might be responsible for this additional sAC activation effect.

2.4.1.5. Bicarbonate and forskolin. A defining property of sAC-like AC is their activation through bicarbonate binding [15,17], which makes them the only signaling proteins known to be directly regulated by bicarbonate. The equilibrium between bicarbonate and CO_2 and its role in pH homeostasis also enables them to sense changes in pH and CO_2 [100]. The EC_{50} for stimulation is in the range 10–25 mM, appropriate for sensing the physiological bicarbonate levels of 2 to 25 mM [15,16,100]. tmACs are bicarbonate-insensitive and instead can be activated by forskolin [10], a plant diterpene. Forskolin binds in the second, degenerated "active site" (Figs. 2a and 4a), which is speculated to constitute the binding site for a yet to be identified endogenous tmAC regulator [42,49]. Forskolin-dependent activation appears to involve dimer stabilization and smaller active site rearrangements [42], but details remain speculative since forskolin-free tmAC structures are not available [37]. Mammalian sAC is insensitive to forskolin [14], since Arg176 (replaced by Ala in tmACs) and a four residue insertion in the β2/3* loop restrict the space available in its second, degenerated "active site" (Figs. 2a and 4b) [19]. This sAC site instead accommodates the smaller sAC-specific activator bicarbonate [19,61]. The activator binds between Lys95 and Arg176, which is thereby released from an inhibitory interaction with the conserved active site residue Asp99 and acts as a trigger arm connecting catalytic center and regulatory bicarbonate binding sites (BBS; Fig. 4b). Bicarbonate thereby enables rearrangements of Asp99 and additional active site residues that allow ion site formation and that closely resemble changes also observed upon substrate binding [19]. The kinetic effect of bicarbonate appears to be an increase in turnover [16,17], however, indicating that substrate binding and turnover might indeed be tightly coupled (see Section 2.2.2) or that some features of bicarbonate-dependent activation could not be detected with this sAC crystal form. In fact, soaking experiments with bicarbonate and crystals of sAC/ATP analog complexes led to crystal dissolution, indicating conformational changes not compatible with the packing of these crystals [19]. Previous bicarbonate soaking experiments with crystals of the sAC-like AC CyaC in complex with ATP analogs showed an induced active site closure of β7/8 and α1, and this open-closed transition was proposed to be part of the general mechanism of substrate turnover (see Section 2.2.2) [17]. Based on mammalian sAC binding data, it has been speculated that bicarbonate might affect release of the product PP_i, possibly by influencing this open-closed transition (see Section 2.2.2 and Ref. [19]). The packing of human sAC crystals might hinder movements of α1 and β7/8, but the sAC crystals retained activity and it remains to be clarified whether this or other movements are indeed essential for sAC catalysis and bicarbonate-dependent stimulation.

Interestingly, CyaC and other homodimeric sAC-like AC have two *a priori* identical sites yet are bicarbonate regulated similar to human sAC [17]. Apparently, the two sites can act as regulatory or catalytic site, depending on the order of binding events. Such an asymmetry and coupling of sites during catalysis are supported by half-of-the-sites reactivity reported for two bacterial homodimeric Class III cyclases [38,101] and might be an evolutionary state before gene duplication and optimization of the second site for regulator binding. A consequence of combined functions as active and regulatory site appears to be similarities between such sites but also less optimal arrangements in homodimeric enzymes. Of the two positively charged, bicarbonate recognizing residues in human sAC Lys95 is conserved in all sites because the corresponding residue in active sites contributes to catalysis. Human sAC Arg176, in contrast, is not exactly conserved in bacterial sAC-like AC but might be functionally replaced by the neighboring catalytic CyaC residue Arg1150* [17,19,45]. It is tempting to speculate that this residue
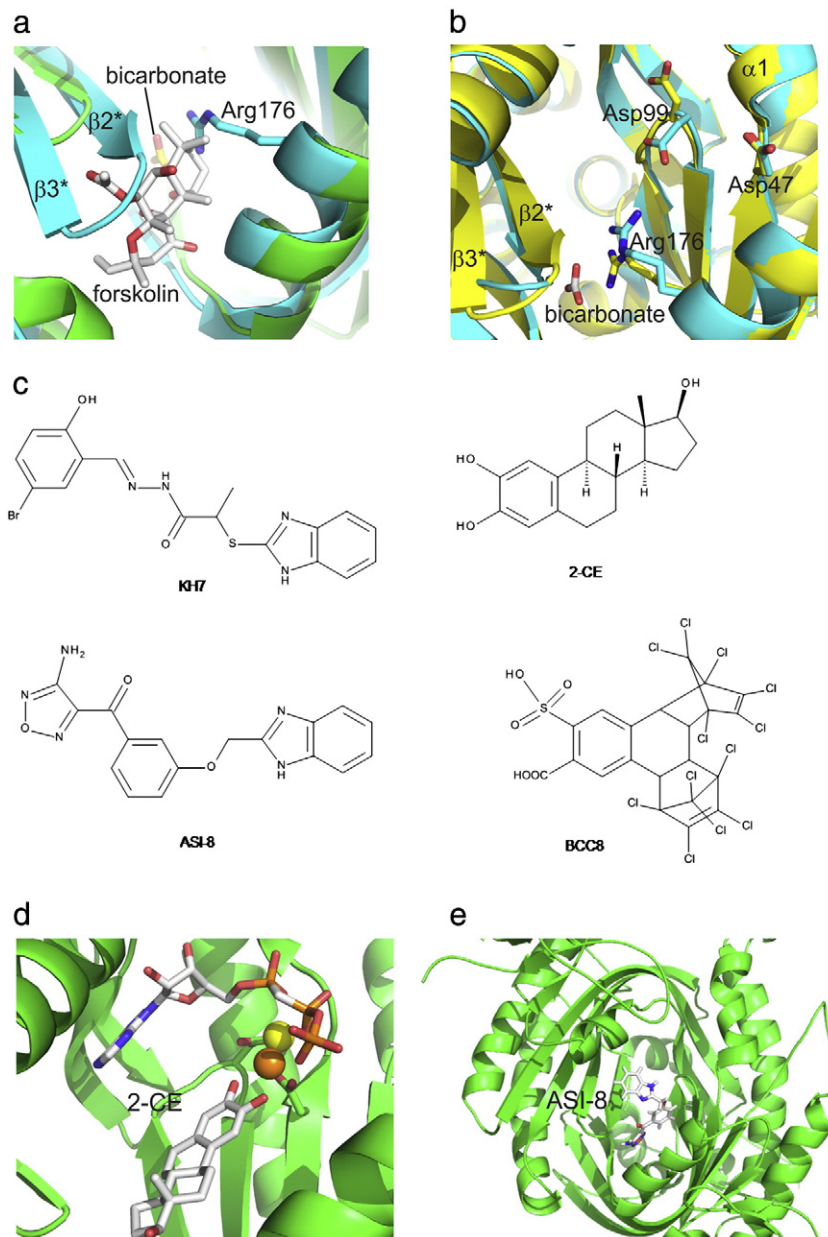
Fig. 4. Physiological and pharmacological sAC regulation. (a) Overlay of the regulator binding sites of activator complexes of sAC (cyan; PDB ID: 4CLL) and tmAC (green; 1CJT). The tmAC activator forskolin (sticks) would clash with sAC-Arg176 (sticks; labeled) and the extended sAC β2/3* loop (labeled). The smaller sAC activator bicarbonate (sticks; yellow carbon, labeled) is bound at the edge of the tighter sAC regulator pocket. (b) The overlay of apo sAC (cyan; 4CLF) and a sAC/bicarbonate complex (yellow; 4CLL) shows the rearrangements of Arg176 and Asp99 upon bicarbonate (sticks) binding. Selected secondary structure elements are labeled. (c) Chemical structures of the pharmacological sAC inhibitors 2-CE, BCC8, ASI-8, and KH7. ASI-8 appears most potent, and KH7 is best characterized and shows a potent and specific effect. (d) Crystal structure of the sAC-like AC CyaC in complex with 2-CE (2BW7). The compound (sticks, bottom) interacts with the Mg^{2+} ion A (orange), resulting in a non-productive complex with Ca^{2+} (yellow) and ATP analog (sticks, top). The ligands from the symmetric active site have been omitted for clarity. (e) Crystal structure human sAC-cat in complex with ASI-8 (4OYA). The inhibitor (sticks) occupies the center of the catalytic core (orientation as in Fig. 2a) and extends in the BBS at the lower left.

shifted during evolution to optimize bicarbonate binding after it was not required anymore for transition state stabilization.

More recently, bicarbonate-dependent stimulation was also reported for Class III GCs, in particular the mammalian receptor GC isoforms D and G [102–104]. Like in sAC, the catalytic domains of these GCs appear directly targeted by bicarbonate, but the Arg/Lys pair recognizing bicarbonate in sAC-like ACs is not conserved and the mechanism for their bicarbonate-dependent stimulation remains to be established. Furthermore, a recombinant tmAC catalytic domain heterodimer was reported to be activated by CO$_2$ rather than bicarbonate, but further studies will be required to test the physiological relevance of this effect [105].

2.4.2. Pharmacological regulation of sAC-like enzymes

cAMP is a ubiquitous signaling molecule contributing to many physiological functions. The established clinical application of inhibitors for the AC opponents, PDEs, verifies the suitability of modulating cyclic nucleotide signaling for therapy [28,106]. Since distinct AC isoforms contribute to specific processes they are also explored as therapeutic targets [34]. Pharmacological modulation of tmACs is considered, e.g., for the treatment of asthma and congestive heart failure [34], but these efforts are still in early research phases and suitable drug-like compounds largely remain to be identified (see Refs. [34,107] and below). More extensive research efforts on mammalian sAC started

only ~15 years ago [14,15], and we are just beginning to recognize sAC as an essential component of many mammalian cAMP signaling systems [4,100,108]. Thus, the potential of sAC as therapeutic target is only emerging. sAC promotes keratinocyte hyperproliferative skin diseases and is used as a diagnostic marker and drug development target for psoriasis and squamous cell carcinoma [35,109]. sAC mutations have been linked to hypercalciuria, which causes kidney stone formation and bone loss [110], and sAC was suggested as a potential target for a male contraceptive, type 2 diabetes, glaucoma, and prostate cancer [31,32, 34,111]. Furthermore, sAC-like ACs were found to regulate virulence mechanisms of human pathogens such as *P. aeruginosa*, *C. albicans*, and *Plasmodium falciparum* [30,36,84,112]. First compounds show that specific inhibition of the microbial sAC enzymes versus host sAC can be achieved [36,112], and that inhibition of the pathogens' sAC enzymes, and the bicarbonate/CO_2-sensing systems they form, are promising approaches for the development of novel anti-infectives [84,112, 113].

For using AC as drug targets, but also for functional studies on cAMP signaling, it is important to pharmacologically distinguish related Class III enzymes, such as tmAC and sAC or host and pathogen AC. A variety of Class III AC inhibitors has been described, and in particular characterized for tmACs, but most of them are either not suitable as drugs and were used for mechanistic or proof-of-principle studies, or they lack sufficient isoform selectivity [107]. However, the first encouraging results on selectively modulating Class III ACs and on using them as targets should now stimulate and support such development efforts [34,114,115]. Here, an overview of available compound classes will be given, with an emphasis on their effects on sAC.

2.4.2.1. ATP, ATP-analogs, GTP, and P-site inhibitors.
sAC-like ACs show a lower apparent ATP affinity than other Class III AC, which enables ATP sensing and Ca^{2+} activation (see Section 2.4.1). Small differences between sAC and tmAC appear to cause this sAC-specific property, while the general binding site geometry and interactions are very similar (see Section 2.2). Consistently, complexes of sAC and tmAC with close ATP analogs such as ApCpp and Rp-ATPαS are highly comparable and revealed general insights in substrate binding and catalysis (see Section 2.2) [17,19,66]. Some smaller differences between complexes appear to be due to the different modifications of the nucleotides, rather than the different AC enzymes, and revealed further mechanistic insights [4,17]. CyaC complexes with Rp-ATPαS and α,β-Me-ATP, respectively, revealed that replacing the αP-pro-R oxygen causes ATPαS to bind with the pro-S oxygen to ion A, resulting in a nucleotide conformation that prevents the in-line attack of the 3′-hydroxyl group (see Section 2.2.2) [17]. In Sp-ATPαS, in contrast, the unmodified pro-R oxygen can bind to ion A, which results in an arrangement suitable for the in-line reaction and explains why Class III ACs are inhibited by the Rp isomer of ATPαS, whereas the Sp form is a substrate [17,65]. α,β-Me-ATP, which features both oxygens as in the real substrate ATP, indeed binds with the pro-R oxygen to ion A and in the conformation required for the in-line reaction [17,19].

ApCpp and ATPαS are closely related to ATP, a substrate for hundreds of cellular proteins, and they are thus not useful as pharmacological compounds. A more selective class of adenosine derived compounds belongs to the so-called P-site inhibitors and comprises compounds such as 2′,5′-dideoxyadenosine-3′-tetraphosphate and 3′-AMP [68,69,107]. They potently inhibit tmACs non-competitively, apparently through tight binding to the active site conformation of the PP_i product complex, resulting in a dead-end complex [68]. The sAC features causing its lower apparent ATP affinity (see Section 2.2) might be responsible for sAC's lower sensitivity to inhibition by some P-site ligands [115]. Non-nucleoside P-site inhibitors, in particular PMC-6 (1R,4R-3-(6-aminopurin-9-yl)-cyclopentanecarboxylic acid hydroxyamide), can combine significant potency and tmAC isoform selectivity with membrane permeability, demonstrating the potential of P-site inhibitors for drug development [107,116].

Another class of inhibitory nucleotides are 2′(3′)-O-(N-methylanthraniloyl) (MANT)-substituted derivatives. They are more potent against tmACs than against sAC, but with limited tmAC isoform selectivity, and they were mainly used for mechanistic studies [107]. However, MANT nucleotides block the ATP binding site but position the MANT fluorophore in a less conserved hydrophobic pocket at the C_1/C_2 interface, and further developing ligands with moieties in this site might yield pharmacologically useful compounds with improved isoform specificity [117].

2.4.2.2. Forskolin derivatives, bicarbonate, and DIDS.
The plant diterpene forskolin activates mammalian tmACs 1–8, but not sAC, through not fully understood mechanisms induced by binding to the degenerated "active site" (see Refs. [10,14,42,49] and Section 2.4.1). Some forskolin derivatives indicated potential of this compound class for the development of isoform specific activators [114], but the incomplete mechanistic understanding and the availability of only one crystallizable mammalian tmAC catalytic core system have hampered such efforts.

The physiological activator of sAC-like AC, bicarbonate and Ca^{2+}, is not suitable as pharmacological sAC activator due to instability and non-specific pH effects (bicarbonate) and the multitude of effectors (Ca^{2+}). While unlikely for the Ca^{2+} site, the development of specific pharmacological ligands for the BBS site might be conceivable, in particular if neighboring pockets are also exploited, and should yield sAC specific activators, inhibitors, or blockers of bicarbonate stimulation. Two types of sAC inhibitor complexes identify such binding opportunities close to the BBS. Fragment-based screens identified several inhibitory ligands for a channel toward the BBS, including a potently inhibiting benzoimidazole (see Ref. [61] and below). The second sAC complex structure comprises 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), a compound known to block bicarbonate transporters [19]. The compound was tested based on the idea that its sulfonic acid moieties might occupy the BBS, and it turned out to be a moderately potent sAC inhibitor. It binds to an active site entrance funnel with positive surface potential and blocks entrance and channel to the BBS, but it does not occupy the BBS itself [19]. The sAC/DIDS interactions appear far from optimal, suggesting that much more potent inhibitors can be developed based on this compound and inhibition mode. Furthermore, the neighborhood to the BBS might allow the development of specific activators by exploiting both binding sites.

2.4.2.3. Catechol estrogens, tyrphostins, and other ion chelators.
Catechol estrogens (CEs) are physiologically occurring steroid derivatives that can inhibit mammalian AC enzymes [118]. 2-Hydroxy estradiol (2-CE; Fig. 4c) and 4-hydroxy estradiol (4-CE) inhibit purified mammalian sAC and CyaC potently (IC_{50} values 2–8 μM), but they inhibit some purified tmAC isoforms with comparable potency [118]. Interestingly, CEs are more selective for sAC versus tmACs when membrane preparations or cellular systems are used, with sAC still being significantly inhibited but the potency against tmACs dropping dramatically [115, 118]. The reason for this behavior is unclear, but it appears to render CEs suitable for distinguishing between sAC and tmAC in cellular systems.

CEs are present in several tissues and can inhibit cAMP accumulation in the hypothalamus [119,120], but a role as physiological sAC regulators appears less likely based on their levels and potency. Nevertheless, CEs appear interesting starting points for sAC-targeted drug development. Biochemical studies and crystal structure analysis of a CyaC/substrate analog/2-CE complex showed that CEs inhibit sAC-like AC non-competitively. They bind to a rather hydrophobic patch within the dimer center and next to the active site, where they chelate and shift the catalytic ion A and thereby trap an enzyme-substrate complex in a non-productive conformation (Fig. 4d) [118]. Variations between AC isoforms in the CE binding pocket suggest that more specific ligands can be developed, and a structure-based virtual screen with CyaC indeed yielded two inhibitors for CyaC and human

sAC (IC$_{50}$ values 1 and 11 μM, respectively, against human sAC) with negligible effects on several tmAC isoforms [121]. These compounds, 3,20-dioxopregn-4-en-21-yl 4-bromobenzenesulfonate (BCC2) and 1,2,3,4,5,6,7,8,13,13,14,14-dodecachloro-1,4,4a,4b,5,8,8a,12b-octahydro-11-sulfo-1,4:5,8-dimethanotriphenylene-10-carboxylic acid (BCC8), are not suitable for *in vivo* studies but show that there are additional scaffolds that can exploit the CE site in an isoforms specific manner. In principle, this binding site region should also allow specific inhibition of tmAC isoforms, and a CE-like binding mode was indeed predicted for the tmAC1/6-specific compound CB-7921220 [122]. Several other AC inhibiting compounds might also exploit the CE binding site and inhibition mode. A variety of catechols, such as dopamine and apomorphine, likely exert their sAC inhibiting effects *via* the CE mechanism [118,123]. The same likely applies to the catechol moiety-containing members of the tyrphostin family of kinase inhibitors, which inhibit mammalian Class III cyclases non-competitively [124]. The most potent representative, tyrphostin A25 (a.k.a. AGK82), inhibits mammalian GC-C with an IC$_{50}$ of 5.8 ± 2 μM and shows weaker effects on tmAC and sGC activity. A CE-like mechanism appears more speculative for calmidazolium, a chelating compound without catechol moiety and with a non-specific modulatory effect on several tmAC isoforms [107,125].

2.4.2.4. Other compound classes, in particular KH7 and other benzoimidazoles. A small number of compounds from chemical classes not covered above have been described as tmAC inhibitors, but they show limited potency and/or specificity [107]. Potent sAC inhibitors, in contrast, have been identified in two screening efforts. (E)-2-(1H-Benzo[d]imidazol-2-ylthio)-N′-(5-bromo-2-hydroxybenzylidene) propanehydrazide (KH7; Fig. 4c) was identified as a potent sAC inhibitor (IC$_{50}$ ~3 μM) through a high-throughput chemical library screen [59,115]. KH7 shows good membrane permeability and has no significant effect on tmACs [59,115], yet weakly activates sGC at higher concentrations (Steegborn and Buck, unpublished). KH7 has been used as pharmacological tool in many sAC studies and seems promising as a lead compound for drug development.

KH7 and closely related derivatives also inhibit sAC-like enzymes from microbial organisms and might allow the development of anti-infectives. KH7 potently inhibits the sAC-like ACβ from *P. falciparum* (PfACβ) and kills this malaria-causing pathogen [112]. A KH7 derivative, KH7.15, inhibits PfACβ, and kills *P. falciparum*, with lower potency but shows no effect on mammalian sAC. These findings suggest PfACβ as a novel anti-malaria target and KH7 derivatives as promising compounds for drug development since they can potently inhibit the pathogen's AC but also distinguish it from host ACs [112]. The parent compound, KH7, inhibits sAC-like AC from many organisms but showed no significant effect on the bicarbonate-activated AC CyaB from the bacterial pathogen *P. aeruginosa* [36]. However, screening a library of KH7 derivatives identified KH7.148 as a CyaB inhibitor (IC$_{50}$ ~10 μM) with a much weaker effect on mammalian sAC (IC$_{50}$ >100 μM) [36]. These results show again that the KH7 scaffold allows potent inhibition of sAC-like AC and furthermore bears the potential to discriminate between such ACs from different organisms, in particular pathogen and host.

The KH7 binding site and mechanism for sAC inhibition have not yet been identified, but they likely resemble those of another, recently identified group of sAC inhibitors. A fragment-based screening approach by Astex Pharmaceuticals initially yielded smaller compounds binding in the BBS or next to it and inhibiting human sAC weakly or moderately (IC$_{50}$ values 19 to >300 μM) [61]. An amino-furazan from these initial hits was further developed into potent inhibitors for human sAC through a "growing" approach, with the most potent compound (4-Aminofurazan-3-yl)-[3-(1H-benzoimidazol-2-ylmethoxy)phenyl] methanone (Astex sAC inhibitor 8, ASI-8; Fig. 4c) inhibiting with an IC$_{50}$ of 0.36 μM. A crystal structure of a sAC-cat/ASI-8 complex shows that the compound occupies the BBS and the channel connecting it to the active site (Fig. 4e) [61]. This binding mode

suggests that the compound will be selective for sAC and sAC-like AC, but activity data on AC selectivity, in particular on effects against mammalian tmACs, are not yet available. Also, the inhibition mechanism of these compounds remains to be fully understood. The larger compounds partially overlap with the ATP binding site, but this does not apply to smaller precursors already showing significant inhibitory effects, which suggests more subtle mechanisms coupling regulatory and active site. The sAC-specificity of KH7 [4,59] might indicate that it also occupies the BBS, and the benzoimidazole moiety and overall length it shares with ASI-8 might also hint at an ASI-8-like binding mode, but the exact binding and inhibition modes of KH7 remain to be established.

Acknowledgements

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