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Molecular Details of cAMP Generation in Mammalian Cells: A Tale of Two Systems

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Abstract

The second messenger cAMP has been extensively studied for half a century, but the plethora of regulatory mechanisms controlling cAMP synthesis in mammalian cells is just beginning to be revealed. In mammalian cells, cAMP is produced by two evolutionary related families of adenylyl cyclases, soluble adenylyl cyclases (sAC) and transmembrane adenylyl cyclases (tmAC). These two enzyme families serve distinct physiological functions. They share a conserved overall architecture in their catalytic domains and a common catalytic mechanism, but they differ in their sub-cellular localizations and responses to various regulators. The major regulators of tmACs are heterotrimeric G proteins, which transduce extracellular signals *via* G protein-coupled receptors. sAC enzymes, in contrast, are regulated by the intracellular signaling molecules bicarbonate and calcium. Here, we discuss and compare the biochemical, structural and regulatory characteristics of the two mammalian AC families. This comparison reveals the mechanisms underlying their different properties but also illustrates many unifying themes for these evolutionary related signaling enzymes.

Keywords

cAMP signaling; class III nucleotidyl cyclases; enzyme regulation; soluble adenylyl cyclases; transmembrane adenylyl cyclases

Introduction

The second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) was discovered by Earl Sutherland during his studies of hormonal regulation of metabolism in mammalian heart and liver.[1,2] Subsequent studies revealed cAMP to be a prototypical second messenger, modulating physiological processes in all domains and kingdoms of life (its presence in plants remains controversial). The effects of cAMP are mediated by distinct targets in different kingdoms. In mammalian cells there are at least three known types of cAMP effector proteins: protein kinase A (PKA), exchange proteins activated by cAMP (EPACs), and cyclic nucleotide gated ion channels (CNGs and HCNs),[3] plus a potential fourth target recently identified, Phosphodiesterase type 10.[4] The second messenger is universally generated by adenylyl cyclases (AC; EC 4.6.1.1), enzymes that catalyze the cyclization of ATP to generate cAMP and inorganic pyrophosphate. Nucleotidyl cyclases (i.e. enzymes generating cAMP and the related second messenger cGMP from GTP) are a diverse family

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of enzymes that can be separated into six classes[5,6] (see below), with all known eukaryotic nucleotidyl cyclases belonging to a single class (class III). In many organisms and cell types, several different adenylyl cyclases (ACs) and/or guanylyl cyclases (GCs) are expressed simultaneously.[7] Such heterogeneity reflects the multitude of cellular processes that are regulated by cyclic nucleotide second messengers in eukaryotic cells.

In mammals, cAMP is formed by either of two types of widely expressed Class III ACs (Figure 1). A family of transmembrane adenylyl cyclases (tmACs) encoded by nine distinct genes termed type I through type IX was discovered first, and this family represents the more widely studied source of cAMP. tmACs are directly regulated by heterotrimeric G proteins and generate cAMP in response to hormones and neurotransmitters which signal through G protein-coupled receptors (GPCRs).[8] As their name implies, tmACs are obligatory membrane proteins. The individual regulatory properties of each isoform have been reviewed extensively, [7,9,10] and specific tmAC isoforms have been linked to a subset of the physiological responses mediated by tmACs; for example, learning and memory,[11–14] cardiac myocyte function,[15] and olfaction[16] are, at least in part, mediated by tmACs types I and VIII, type V, and type III, respectively.

A second, independent source of cAMP in mammalian cells is the more recently discovered, soluble adenylyl cyclase (sAC).[17] sAC is uniquely regulated by bicarbonate[18] and calcium,[19,20] and it is insensitive to heterotrimeric G protein regulation.[17] sAC is widely expressed[21] and is not strictly a soluble protein; it is present at discrete sub-cellular localizations in a wide variety of cells.[22] Isoform diversity from the single confirmed sAC gene in mammalian genomes is generated by complex alternative splicing.[23,24] Physiological processes demonstrated to be regulated by sAC include sperm activation,[25,26] pH regulation in epididymis,[27] and TNF activation of granulocytes.[28] Its unique regulation by bicarbonate suggests it also contributes to other processes responsive to carbon dioxide and/or functions as a metabolic sensor in cells.[29]

The catalytic mechanisms and regulation of both mammalian G protein-regulated tmAC catalytic domains and a bicarbonate-regulated, bacterial sAC-like cyclase has now been studied kinetically and crystallographically. Here, we discuss the biochemical, structural and regulatory characteristics of these two families of mammalian ACs. This comparison reveals the mechanisms underlying their different properties and illustrates many unifying themes for these evolutionary related signaling enzymes.

Classification of Nucleotidyl Cyclases

The known nucleotidyl cyclase sequences have been grouped into six classes based on sequence homology within their catalytic portions.[5,6] The AC enzymes from *Escherichia coli* and a number of related Gram-negative prokaryotes belong to class I. Class II is comprised of the “toxin” ACs from pathogens such as *Bordetella pertussis*[30] and *Bacillus anthracis*.[31] These ACs are secreted enzymes that translocate into host cells where they disrupt intracellular signaling by flooding host cells with supraphysiological amounts of cAMP. Eukaryotic adenylyl and guanylyl cyclases belong to class III, the broadest class that contains members from bacterial and animal kingdoms.[32] Classes IV, V and VI are relatively recently defined, and are comprised of one or a few prokaryotic members.[6,33,34]

Class III adenylyl cyclases domain topologies

Class III catalytic domains are found in a number of different contexts with a variety of regulatory domains, which confer modulation by a wide array of signals. Despite sensitivity to diverse regulators, the several crystal structures of class III ACs revealed a conserved basic architecture of the catalytic centers (see below). These structures, together with a

number of biochemical studies, show that activity requires dimerization of two catalytic domains[35] (Figure 2). In the case of mammalian receptor GCs[36] and many bacterial ACs,[37,38] where each protein contains only a single catalytic domain, homodimerization creates two symmetrical active sites at opposite poles of the dimer interface. In contrast, mammalian tmACs, soluble guanylyl cyclases (sGC), and the known isoforms of mammalian sACs form an active cyclase *via* heterodimerization of structurally similar catalytic domains (C_1 and C_2).[24,36,39] These two catalytic domains can reside on separate polypeptide chains, as in sGCs (true heterodimer), or on a single polypeptide, as in tmACs and sAC (pseudo-heterodimer; from now on also designated as heterodimer). In heterodimeric cyclases, C_1 only carries a subset of the active site residues essential for catalysis; the remaining essential residues are contributed by C_2. Therefore, as described in detail below, only one of the two sites formed at a heterodimeric interface is active.[40] It is assumed that the primordial cyclase was a homodimer, and gene duplication permitted diversification as well as mutation of catalytic residues within the second “active site”. An unusual mycobacterial AC homodimer showed structural asymmetry and half-of-the-sites-reactivity which could be the evolutionary step before degeneration of the second site.[41] In tmACs, this pseudo-symmetrical “active” site apparently evolved to serve a regulatory function as part of an effector binding site (see below).

The different topologies and domain interactions of representative class III proteins are shown in Figure 2. Among mammalian adenylyl cyclases, the nine tmAC genes encode a variable N-terminal region, followed by a six transmembrane helices domain (TM1), the first catalytic domain C_1, another six transmembrane helices domain (TM2), and the second catalytic domain C_2. In contrast, the sAC gene encodes a number of spliced forms that contain no detectable TM domain, and the two N-terminal catalytic domains C_1 and C_2 are separated by a relatively short stretch of approximately 20 amino acid residues.

Evolution of Mammalian Adenylyl Cyclases

sAC appears to be the most ancient among mammalian cyclases; its sequence is more similar to ACs from cyanobacteria and myxobacteria than to other mammalian cyclases[17] (Figure 3). Additionally, mammalian sAC and the CyaC cyclase from the cyanobacteria *Spirulina platensis* are similarly regulated; both are synergistically activated by bicarbonate and calcium ions.[20,42] Bicarbonate-regulated sAC-like cyclases are also found in *Anabaena PCC 7120*, *Stigmatella aurantiaca*, *Mycobacterium tuberculosis*, [43] and the thermophilic bacterium *Chloroflexus aurantiacus*,[44] and sAC-like proteins were cloned and expressed from sea urchin[45] and *Plasmodium falciparum*, the parasite responsible for the most lethal form of malaria.[46] Database searches identify sAC-like ACs in the genomes of all mammals studied (human, chimpanzee, dog, cow, rabbit, mouse, and rat), as well as in chicken, ciona intestinalis, and insects (mosquito and honey bee). The human, chimpanzee, dog, and cow genomes predict a second locus encoding a sAC-like gene, but its activity or physiological relevance is unknown. Surprisingly, corresponding genes have yet to be found in the genomes of organisms such as *Caenorhabditis elegans* and *Drosophila melonogaster*, and it has been proposed that these organisms lost their sAC-like cyclase during evolution.[47]

G protein responsive tmACs first appear in metazoans. Structurally related cyclases have been characterized from *M. tuberculosis* and protozoans, but these are not thought to be G protein regulated.[37,48] Interestingly, the *M. tuberculosis* tmAC-like cyclase is analogous to half of a mammalian tmAC; it consists of a single six transmembrane helices domain followed by a cytoplasmic loop and has to homodimerize for activity.[37] Fungal ACs, although they occupy a separate branch of the nucleotidyl cyclase family tree (Figure 3), appear to combine features found in both tmACs and sAC. The adenylyl cyclases from the yeast *Saccharomyces cerevisiae* and the pathogenic fungi *Cryptococcus neoformans* and

Candida albicans are G protein regulated[49–51] and were recently shown to be bicarbonate responsive.[52]

Molecular Structure

As stated above, the functional catalytic unit of class III enzymes is formed by either homodimerization of a single catalytic domain or heterodimerization of structurally similar C_1 and C_2 catalytic domain monomers. The first crystal structure of a class III AC catalytic domain to be solved was a non-physiological mammalian C_2 homodimer,[53] followed by crystal structures of a mammalian C_1C_2 heterodimer,[40,54] two trypanosomal catalytic cores,[55] several mycobacterial ACs,[41,56,57] and a cyanobacterial sAC homolog.[38] These structures confirmed the conserved architecture of class III catalytic domains (Figure 4(a)) previously suggested by their sequence similarities (Figure 5).Within each monomer, a central seven-stranded β-sheet is shielded from the solvent by helices α2, α3, and α5. The two monomers interact head-to-tail and are held together in a wreath-like fashion by an extended clamp formed by β5, the C-terminal part of β4, and the loop in between. The active site of the catalytic entity is located at the dimer interface and formed by residues from both catalytic domains. Two highly conserved Asp residues (Asp396 and Asp440 in Figure 5; residue numbers refer to the tmAC heterodimer described by Tesmer *et al.*[40] if not stated otherwise) and the phosphate tails of the bound substrate, ATP, coordinate two magnesium ions, called ion A and ion B.[38,54] The substrate phosphates are further bound by positively charged residues (Arg484, Arg1029*, Lys1065*; the asterisk (*) indicates a C_2 residue) and main-chain atoms of the N terminus of helix α1. The ribose moiety of the substrate occupies a mainly hydrophobic pocket with a Ser conserved in tmACs (Ser1028*) but replaced by an Ala conserved in sAC homologs (Ala1149* in CyaC), a difference likely important for the characteristic substrate affinities of the two AC families (see below). The adenine base is bound in a hydrophobic cleft and recognized through interactions with two polar residues. A highly conserved Lys (Lys938*) binds to the ring nitrogen N1 of the adenine, and an Asp (Asp1018*) interacts with the amino group N6; the Asp in tmACs is functionally replaced by a Thr found in sAC-like enzymes at this position.[38,43] The Lys and the Asp are replaced in GC enzymes by Glu and Cys, respectively. Making the corresponding amino acid substitutions in GCs converts the substrate specificity to ATP,[58,59] indicating that these two residues dominate substrate selection. However, additional factors contribute to substrate selection and turn-over.[60] AC enzyme specificity is not switched to GTP when analogous mutations are introduced,[57,58,60] perhaps due to an impact on formation of the dimer interface caused by alteration of these residues.[57]

For both mammalian tmACs and sAC, active cyclase is formed *via* heterodimerization of two similar catalytic domains. Differences between the two catalytic domains within either cyclase result in a non-symmetrical dimer interface; i.e. the two centers formed are not identical. In fact, only one site possesses all the residues essential for catalysis.[40] The site at the other pole of the dimer center is degenerate, and in tmACs, this degenerate center is part of the binding site for the tmAC specific regulator forskolin[40,53] (see below). Thus, because C_1 and C_2 contribute distinct essential residues to the lone catalytic center, even though their overall structure is the same, the two catalytic domains serve different functions.

The Catalytic Mechanism of Class III Cyclases

The overall structure as well as most residues involved in substrate binding and catalysis are conserved among class III cyclases. The positions with variations mostly show functionally conserved substitutions (see above) which define a limited number of subfamilies within class III.[32] Therefore, it is likely that class III cyclases share a common catalytic mechanism. The small differences between individual cyclases or subfamilies, such as the

Δ-loop in trypanosomal ACs,[55] mostly confer unique mechanisms of regulation upon these enzymes.

The simplest reaction mechanism compatible with stereochemical studies on class III cyclases[61] is the now widely accepted in-line attack of the 3′-hydroxyl group of the ribose at Pα occurring simultaneously to the release of the Pβγ pyrophosphate (Figure 6); i.e. a pseudo-bimolecular nucleophilic substitution (S_N2) mechanism. The function of ion B seems to be a stabilization of the bound substrate as well as the subsequently produced pyrophosphate through interactions with Pβ and Pγ.[54] Ion A, in contrast, serves to stabilize the pentacovalent transition state at Pα and to activate the 3′-hydroxyl of the ribose as attacking group, either directly or through a magnesium-activated base such as a magnesium-bound water or hydroxyl ion.

Tesmer *et al.* proposed a substrate-induced active site closure as part of the catalytic cycle, based on a more closed form observed for tmAC after, e.g. the ATP analog ATPαS had been soaked into the active site.[40,54,62] The closure is due to movements of α1 and an “arm” sub-domain formed by β7, β8, and the loop in between them toward the dimer center (Figure 7(a)). High-resolution data for a sAC-like enzyme revealed, however, that at least in this enzyme ATPαS binds in a conformation not suitable for the reaction to occur. In contrast, α,β-Me-ATP binds in a productive conformation with the 3′-hydroxyl and the bond to be broken arranged in a straight line[42] (Figure 6). Interestingly, sAC complexed to α,β-Me-ATP has an even more open active site which is again due to movements of α1 and β7/ 8. Based on these results, we proposed a modified model for the catalytic role of the α1 and β7/8 movements: substrate binding fully opens the active site, and the subsequent closure drives catalysis by separating the products cAMP and pyrophosphate. [42] Consistent with this model, the tmAC–G_sα complexes with P-site inhibitors, which presumably resemble an enzyme/product complex, are similar to the closed enzyme conformation.[63] During active site closure, Pγ and Pβ would be shifted toward the active site exit. Pyrophosphate dissociation would finally enable the active site to open again by releasing interactions to α1 and β7/8. These interactions explain why P-site ligands (see below) require either a polyphosphate tail or pyrophosphate for efficient inhibition; the interactions of pyrophosphate are essential for trapping the enzyme in its closed conformation.

Although class II cyclases are unrelated to class III enzymes in topology and quarternary structure, it appears that they utilize a similar catalytic mechanism. The monomeric catalytic cores of two class II enzymes, edema factor from *Bacillus anthracis* and CyaA from *Bordetella pertussis*, comprise a single active site at the interface between two structurally distinct domains.[31,64] Recent studies revealed that this active site, although non-homologous to the class III catalytic center, provides two ion binding sites which seem to correspond functionally to ion A and ion B sites.[65,66] This similarity, together with the lack of structural homology might indicate that these two cyclase classes are a result of convergent evolution of a two-ion active site for this substitution reaction at Pα. This conclusion might also apply to the DNA polymerase ‘palm domains’ which catalyze an intermolecular substitution at Pα. Part of the palm domain shows a topology also found in class III cyclases[67] and a very similar arrangement of two magnesium ions catalyzing its related reaction.[68]

Regulation of Adenylyl Cyclases

In mammals, cAMP is involved in a multitude of physiological processes.[9] In fact, this single second messenger can modulate seemingly disparate functions within a single cell.[69,70] The co-existence of sAC and tmACs, along with a broad family of cAMP degrading phosphodiesterases (PDEs),[71] permitted a revision of the original models for cAMP signal transduction in order to explain this paradox. These original models relied

upon diffusion through the cytoplasm of the second messenger generated at the plasma membrane to elicit cAMP-dependent responses at intracellular organelles and structures. However, modern methods for measuring cAMP *in situ* (for example, using FRET-based and conductance-based sensors) revealed that diffusion is restricted.[72–74] This observation suggests the existence of additional sources of cAMP localized closer to the second messenger site of action. In the revised model (Figure 1), cAMP is formed, acts, and is degraded in independently regulated cAMP signaling micro-domains at the plasma membrane or at intracellular sites.[75,76]

Spatial restriction and independent modulation of the cyclases in different micro-domains would be achieved by the plethora of regulatory mechanisms of sACs and tmACs. Both families are represented by multiple isoforms and their activities are modulated through protein regulators and small-molecule ligands. We discuss here physiologically relevant regulatory mechanisms as well as the modulation of AC activity with synthetic compounds.

Isoform Diversity

In mammals, nine genes encode tmACs. These are numbered AC I through IX, according to their time of discovery, and each displays unique regulatory properties.[7,9,10,77] Besides the different tissue distribution of these isoforms, there are also reports of alternative splicing of some of the tmAC genes, increasing their isoform complexity. Three tmAC VIII variants were identified in rat brain.[78] In addition to a “full-length” isoform, one isoform is missing potential glycosylation sites from an extracellular loop and is postulated to be targeted to distinct membrane compartments. A third isoform is missing a region, which seems to be important for calcium/calmodulin regulation. Alternatively spliced forms of tmAC III in mammalian germ cells,[79] tmAC IV in uterine myometrium,[80] and of tmAC V and VI have been identified,[81–83] but their biochemical properties or physiological significance have not yet been elucidated.

sAC is present in all tissues thus far examined.[18,24] Although encoded by a single confirmed gene (a second potential sAC gene in humans has not yet been confirmed to be functional), multiple sAC isoforms with distinct regulatory properties are generated by alternative splicing. The longest known cDNAs encode the full-length sAC protein of approximately 187 kDa (sAC_{fl}). The activity, originally purified from rat testis resided in a protein of approximately 50 kDa,[17,18] which was later found to be generated by alternatively splicing[23] (sAC_t). This truncated protein comprises little more than the two N-terminal catalytic domains,[23] and much of what we know about the biochemical activity of sAC has been determined from this more easily purified sAC_t isoform. Both protein products, sAC_{fl} and sAC_t, are expressed in mouse testis,[26] and while the two isoforms are similarly regulated by small molecules (see below), they display distinct intrinsic specific activities.[17,19] The truncated isoform has a tenfold higher specific activity due to the presence of an auto-inhibitory domain within sAC_{fl},[84] suggesting sAC_{fl} will be subject to additional modes of regulation.

Additional sAC isoforms generated by alternative splicing have been identified in somatic tissues.[24] At least two distinct human sAC cDNAs predicted proteins with an altered or removed C_1 catalytic domain. One splice variant contained a 37 base-pair insertion between exons 3 and 4 leading the authors to postulate a new start codon in exon 4. Such a sAC isoform would possess only one catalytic domain (C_2) and would be predicted to require homodimerization or heterodimerization with an as yet unknown isoform (possessing C_1) for activity. Another variant skipped exon 4, splicing from exon 3 in-frame to exon 5; this protein would be missing one of the essential magnesium binding aspartates provided by C_1, suggesting it too would have to dimerize to produce an active adenylyl cyclase. Preliminary

studies in our laboratory confirm the widespread distribution of these spliced forms in human tissues and predict the existence of a complex pattern of alternative splicing of sAC in a number of human and rodent somatic tissues (J. Farrell & Y. Chen, unpublished results).

Protein Regulators

The major regulators of tmACs are heterotrimeric G-proteins. The α-subunit of G_s ($G_s\alpha$) stimulates most if not all the tmAC isoforms. In contrast, $G_i\alpha$ selectively inhibits tmACs I, V, and VI, and individual tmAC isoforms also display unique regulation by G protein βγ subunits.[7,10] Despite their structural similarities (described above), the three-dimensional structures reveal differences that might explain sAC's insensitivity to heterotrimeric G-protein regulation. A region implicated in Gβγ binding, specifically the extended loop between β3 and α3 in tmAC II C_2,[85] is missing in sAC (Figure 5). A second and possibly more important region for Gβγ responsiveness maps to the C_{1b} domain next to but outside of the structurally conserved catalytic core domain.[86] The binding surface for $G_s\alpha$ is again located on tmAC C_2 and is formed by the α1/α2 loop, the N terminus of α2, and the C terminus of α3 (Figures 4(a) and 7(a)).[40] sAC C_2 contains a 21 residue insertion between α1 and α2 which likely blocks $G_s\alpha$ access sterically, and a shortened C terminus in α3 (Figure 5). It remains to be seen whether the large insertion between α1 and α2 of sAC is responsible for an unknown sAC specific regulatory mechanism. A similar situation is encountered with the potential $G_i\alpha$ binding site which was mapped to the α1/α2 and the α3/β4 region of C_1 by mutagenesis:[87] in addition to sequence differences, sAC contains a larger loop between α1 and α2 than tmACs (Figure 5) which should prevent $G_i\alpha$ binding.

A second major protein regulator of tmACs is the calcium receptor protein calmodulin. Calcium-loaded calmodulin directly stimulates tmAC isoforms type I and type VIII,[78,88–90] and possibly also tmAC III.[91] Activation in type I proceeds *via* interaction with the C_{1b} regulatory domain located between the first catalytic domain and the second set of transmembrane domains[92,93] while calcium/ calmodulin regulation of tmAC VIII occurs *via* interaction with the C-terminal C_{2b} domain.[94] Calcium/calmodulin can also modulate tmAC I and III activity indirectly *via* calcium/calmodulin regulation of CaM kinases.[95,96]

tmACs can also be regulated by other protein kinases. PKA regulates types V, and VI[97,98] and PKC regulates types II, V, and VI.[99–101] tmACs are also subject to regulation *via* other post-translational modifications, including the NO-dependent inhibition of types V and VI[102] likely mediated by *S*-nitrosylation; and the N-linked glycosylation of tmAC VIII.[78,103] Surprisingly, an RGS protein, RGS2, which would have been predicted to modulate tmAC activity indirectly by acting as GTPase activator on heterotrimeric G proteins, directly regulates tmAC III, V and VI.[104] More regulation mechanisms for tmACs await to be identified; the transmembrane regions, for example, also seem to have a regulatory role.[105]

In contrast to tmACs, no regulatory proteins or post-translational modifications have yet been identified for sAC, but there is evidence for intramolecular protein modulation in particular sAC isoforms. As mentioned above, sAC_{fl} displays diminished specific activity relative to the sAC_t isoform; the difference has been mapped to a putative auto-inhibitory domain located just downstream from the second catalytic domain.[84] Neither the structural consequences of this domain nor any mechanism of disinhibition of cyclase activity are yet known. Generally, the molecular mechanisms of mammalian AC regulation through their regulatory domains as well as through other regulatory proteins, including $G_s\alpha$, $G_i\alpha$, Gβγ and calmodulin, are poorly understood. $G_s\alpha$ increases the affinity of C_1 for C_2,[39,106] but in addition to stabilization of the heterodimer, more subtle rearrangements are necessary to explain the observed levels of stimulation and the negligible influence of the activator on

substrate affinity.[40,107] $G_s\alpha$ stimulation of tmACs was therefore proposed to proceed *via* an induced rotation of the two catalytic domains into a proper arrangement,[40] but due to the lack of a mammalian tmAC structure in absence of $G_s\alpha$, this idea was based on a comparison with the non-physiological IIC_2 homodimer.[53] A similar mechanism was proposed for regulation of a mycobacterial class III AC through its pH sensing domain. In the inactive state, the pH-sensing domain prevents proper orientation of the two catalytic domains. Activation is mediated by a helix-to-loop transition in the linker between regulatory and catalytic domains which enables a large repositioning of the two catalytic domains relative to each other as well as major conformational changes of active site regions.[56] The only other structurally characterized regulatory domain of a class III AC is the isolated GAF-region of a the cyanobacterial CyaB2 AC,[108] which could not reveal how it translates ligand binding into a change of activity of the catalytic domain. Taken together, repositioning of the two domains of the class III catalytic core appears to constitute a mechanism for regulating its activity, but a firm and detailed understanding of the regulation of mammalian AC enzymes through regulatory domains and proteins will require further structural and mechanistic studies.

Regulation by Small Molecules

Forskolin

Forskolin is a diterpene compound isolated from plants that activates all mammalian tmACs[10,109] with the exception of tmAC IX.[110,111] It occupies part of the second, degenerated “active site” in tmACs (Figure 4(a)),[40] and it has been speculated to exploit the binding site of an as yet unidentified endogenous regulatory ligand. Forskolin has been suggested to activate tmACs by inducing dimerization and/or active site rearrangements,[40] but this idea remains speculative because the structure of a tmAC in the absence of forskolin is not yet known. Mammalian sAC is insensitive to forskolin,[17,112] and two major changes in sAC, an insertion of four residues in the loop between β2 and β3 and an Ala to Arg exchange at the ribose binding site, seem to render its second, degenerated active site too small to accommodate forskolin (Figure 5).

Substrate ATP, ATP-analogs and P-site inhibitors

Comparison of the tmAC and sAC/CyaC active sites reveals striking similarity;[38,40] all 18 residues important for substrate binding and catalysis are either conserved or show small and mostly functionally conservative variations (Figure 5). Two of these small differences appear to distinguish tmACs and sAC-like enzymes. An Asp conserved in tmACs (position 1018*) and involved in recognition of the adenine base of the substrate ATP is functionally replaced by a threonine (CyaC Thr1139*) in sAC-like enzymes (Figure 4(b)). Secondly, a Ser in tmACs (Ser1028* in tmAC IIC2) is replaced by a conserved Ala(Ala1149*inCyaC)insAC-likeenzymes.Ser1028* appears to be a polar interaction partner for the ribose oxygen atoms in an otherwise hydrophobic environment; this difference likely explains the higher substrate affinity of tmACs (10^{-4} – 10^{-5} M)[113] compared to sAC-like enzymes (10^{-3} M).[20,38]

The relatively low affinity for ATP displayed by mammalian sAC may represent a physiological adaptation. Its K_m value in the millimolar range implies sAC would be sensitive to physiologically relevant fluctuations in intracellular ATP levels (found to be 1–3 mM in most cell types) and function as a cellular ATP sensor. Interestingly, longer isoforms of mammalian sAC contain a Walker A (“P-loop”) motif often found in nucleotide binding pockets. This motif is dispensable for catalytic activity,[17,84] but it might be involved in an as yet unknown nucleotide-dependent mechanism of sAC regulation. The mechanism of

substrate inhibition observed for the catalytic cores of sAC enzymes at high ATP levels[20] is also unknown.

P-site inhibitors are adenosine analogs that potently inhibit tmACs.[114] Crystal structures of a tmAC in complex with P-site inhibitors revealed they bind to the active site, along with the reaction product pyrophosphate.[40,63] Binding to the active site was initially surprising; kinetic studies had suggested that P-site ligands behave as uncompetitive inhibitors.[115] However, Dessauer *et al.* elegantly explained this behavior by demonstrating that in the presence of pyrophosphate, the enzyme can be trapped in the inactive, closed conformation, the form with greatest binding affinity for the inhibitor.[63,114,116] In contrast, the substrate binds preferably to the active, open form of the enzyme. The same differences between the adenine and sugar binding pockets of sAC and tmACs, which decreases AC's affinity for ATP, should also explain its diminished sensitivity to inhibition by P-site ligands.[117] Interestingly, a new series of P-site inhibitors, such as the compoundPMC-6 (1*R*,4*R*-3-(6-aminopurin-9-yl)-cyclopentanecarboxylic acid hydroxyamide), contain a metal chelating moiety[118,119] and can discriminate between some tmAC isoforms;[119] PMC-6 inhibits type II and III modestly, but tmAC V with high potency.

A class of synthetic nucleotide analogs identified as competitive AC inhibitors are 2′(3′)-*O*-(*N*-methylanthraniloyl)-substituted nucleotides (MANT-nucleotides). [120] The crystal structure of a tmAC catalytic domain in complex with MANT–GTP showed the nucleotide bound to the ATP binding site but with guanine in a reversed orientation relative to the adenine ring (Figure 7(b)).[121] The MANT fluorophore binds to a hydrophobic pocket at the interface between C^1 and C_2 and prevents the “open” to “closed” domain rearrangement, thereby identifying a novel target site for inhibitor development. However, similar to most P-site ligands, MANT compounds are more potent against tmACs than against sAC but display limited tmAC isoform selectivity.[117]

Catalysis by class III ACs proceeds *via* an in-line attack of the 3′-hydroxyl group in concert with the release of pyrophosphate. Therefore, it should have been surprising that ACs are inhibited only by R_p–ATPαS, whereas the S_p form serves as substrate.[38,61] Comparing the conformation of CyaC-bound R_p–ATPαS and α,β-Me–ATP reveals that replacement of the pro-R oxygen will cause ATPαS to bind in a nonproductive conformation and, therefore, be an inhibitor.[38] Pα is turned around so that the pro-S oxygen, which is more favorable for magnesium coordination than sulfur, coordinates ion A (Figure 4(b)). This rotation moves Pα away from the position suitable for in-line attack by the 3′-hydroxyl. In contrast, S_p-ATPαS can bind in a productive conformation by coordinating ion A with its unmodified pro-R oxygen.

Bicarbonate

Bicarbonate directly and specifically activates sAC enzymes; tmACs are insensitive.[18] Indeed, sAC-like ACs are the only known signaling proteins directly regulated by bicarbonate. Crystallographic studies on CyaC did not expose the bicarbonate-binding site, but flash soaking experiments revealed an induced active site closure through a ~4–5 Å tilt of β-strands 7 and 8 and the intervening loop, accompanied by a ~3 Å shift of α1 (Figure 7(c)).[38] These shifts push the β/γ-phosphates of the bound ATP analog and could facilitate separation of the ultimate products, cAMP and pyrophosphate, during catalysis (see above). The bicarbonate-induced closed conformation resembles the conformation of tmAC in complex with product analogs.[63] Thus, bicarbonate appears to increase sAC activity by facilitating the open/ closed transition assumed to occur during catalysis, which is consistent with kinetic studies revealing an effect on k_{cat}.[20]

The precise bicarbonate binding site in sAC-like ACs remains unclear. A substrate binding residue, Thr1139* in CyaC, which is conserved in bicarbonate-regulated sAC-like enzymes and is replaced by an Asp in tmACs (Asp1018* in tmAC IIC_2), was postulated to be important for bicarbonate recognition.[43] However, mutagenesis at this position severely reduced the enzyme's activity, preventing a clear interpretation of the experiments. Crystallographic identification of the bicarbonate binding site is severely hampered by the high EC_{50} value of 10–25 mM.[18,20] Such a low affinity, which is necessary for sensing physiologically relevant bicarbonate levels (bicarbonate concentrations in cells and body fluids vary between 5 mM and 25 mM), reflects a weak and possibly transient interaction that is difficult to study.

Calcium

The activities of the different mammalian tmACs display both positive and negative regulation by calcium.[10,122] The isoform specific activation is mediated by calcium-loaded calmodulin and was discussed above. All tmAC isoforms are directly inhibited by high micromolar concentrations of calcium.[123] Presumably, at these concentrations, calcium non-productively competes with magnesium for the tmAC ion A site.[123,124] Specific tmAC isoforms (types V and VI) are also inhibited by sub-micromolar concentrations of calcium.[123-] Although the mechanism is not understood in detail, calcium and magnesium appear to bind to two distinct enzyme conformations and compete either by binding to the same ion site or by binding to allosterically coupled sites.[123,124]

In contrast to tmACs, calcium supports sAC activity even when it is the only divalent cation available.[20] Therefore, although the ion binding residues are conserved between sAC and tmACs, sAC is uniquely catalytically active with calcium in the ion A site. In the presence of magnesium, high micromolar concentrations of calcium stimulate the activity of sAC-like ACs by increasing the enzyme's affinity for the substrate ATP from a K_M of about 10 mM to K_M=1 mM.[20] X-ray crystallography revealed that calcium replaces magnesium at the ion B pocket of the active site, coordinating the β and γ-phosphates of the bound substrate analog.[38] Therefore, while sAC is active with either magnesium or calcium occupying both ion sites, its activity is highest with magnesium in the ion A site and calcium in the ion B site. All groups in contact with ion B are conserved between sAC and tmACs, yet this type of calcium effect has not been described in tmACs.

Calcium may play a second modulatory role on sAC. Sub-micromolar concentrations of calcium were observed to stimulate native sAC protein immunoprecipitated from testis.[19] Stimulation was independent of calmodulin and appeared to be due to an increase of v_{max}, not substrate affinity. These results suggest that post-translational modifications or interaction with an unknown regulatory protein may alter the calcium affinity of the enzyme or confer some additional modulatory mechanism.

Atypical AC inhibitors

In addition to the nucleotide analog inhibitors described above, a number of other, unrelated compounds have been described which inhibit mammalian AC activity. Catechol estrogens (CE) and tyrphostins with two vicinal hydroxyl groups were independently identified as non-competitive cyclase inhibitors,[125–127] yet they seem to share a common binding site and inhibitory mechanism.[42] For CE, the mechanism was identified from a crystal structure of the trimeric complex of the sAC-homolog CyaC with a substrate analog and the inhibitor.[42] CE is bound to a hydrophobic patch near the active site, and its vicinal hydroxyl groups chelate the catalytic magnesium ion, distorting the active site and trapping the enzyme–substrate complex in a non-productive conformation (Figure 7(d)). CEs inhibit both sAC and tmACs with comparable affinities, but sequence variations in the binding pocket indicate

that more specific ligands for this site might exist.[42] Although catechol estrogens are known to be enriched in certain human tissues,[128,129] it remains unclear whether they represent physiological AC regulators or whether more specific physiological regulators exist for this binding site. In either event, CEs and tyrphostins represent promising starting compounds for the development of more specific cyclase inhibitors.

Another non-physiological compound acting as non-competitive inhibitor of tmACs is calmidazolium, [130] which is a known chelator for divalent ions. It is tempting to speculate that calmidazolium exploits the CE interaction site and inhibitory mechanism.

Because of similarities in catalytic cleft topology and the nature of the reactions catalyzed,[67] inhibitors of DNA polymerase and HIV reverse transcriptase were tested for effects on adenylyl cyclases. Foscarnet[131] and antiviral acyclic nucleoside derivatives of 9-(2-phosphonylmethoxyethyl)adenine[132] proved to be potent inhibitors of nucleotidyl cyclases, and these compounds may provide novel ideas for the development of specific inhibitors.

Finally, novel sAC inhibitors were recently identified in a high-throughput chemical library screen.[26,75] One particular compound, KH7,[26,28] is non-competitive with ATP, but its structure bears little similarity to CEs or P-site inhibitors, suggesting it may define yet another inhibitory principle. It shows a pronounced selectivity for sAC relative to tmACs or guanylyl cyclases, yet it appears to potently inhibit all thus far identified sAC-like enzymes in a wide range of organisms throughout evolution (K. Hess, E.M.B., L.R.L. & J.B., unpublished results).

Concluding Remarks

Even though cAMP has been extensively studied for over 50 years, the plethora of regulatory mechanisms controlling cAMP synthesis is just beginning to be uncovered. In mammalian cells this important second messenger can be produced by two related families of adenylyl cyclases, sAC and tmACs. These enzymes share a conserved catalytic mechanism but differ in their specific physiological functions due to altered regulation and distinct intracellular localization. Additional modes of regulation await discovery. Many questions remain about tmAC isoform divergence and individual cellular functions, and thus far, studies exploring sAC regulation focused on the catalytic domains, leaving the remainder of the protein largely unexplored.

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Abbreviations used

AC	adenylyl cyclase
α, β-Me-ATP	α,β-methylene-adenosine-5′-triphosphate
ATPαS	adenosine-5′-α-thio-triphosphate
FRET	fluorescence resonance energy transfer
GC	guanylyl cyclase

Gβγ	G protein, β and γ subunits
G_sα	stimulatory G protein, α subunit
G_iα	inhibitory G protein, α subunit
K_m	Michaelis constant
MANT	2′(3′)-O-(*N*-methylanthraniloyl)
PDE	phosphodiesterase
PKA	protein kinase A
PMC-6	1*R*,4*R*-3-(6-aminopurin-9-yl)-cyclopentanecarboxylic acid hydroxyamide
sAC	soluble adenylyl cyclase
sAC_{fl}	full-length soluble adenylyl cyclase
sAC_t	truncated form of soluble adenylyl cyclase
tmAC	transmembrane adenylyl cyclase
TM	transmembrane
v_{max}	maximum reaction velocity

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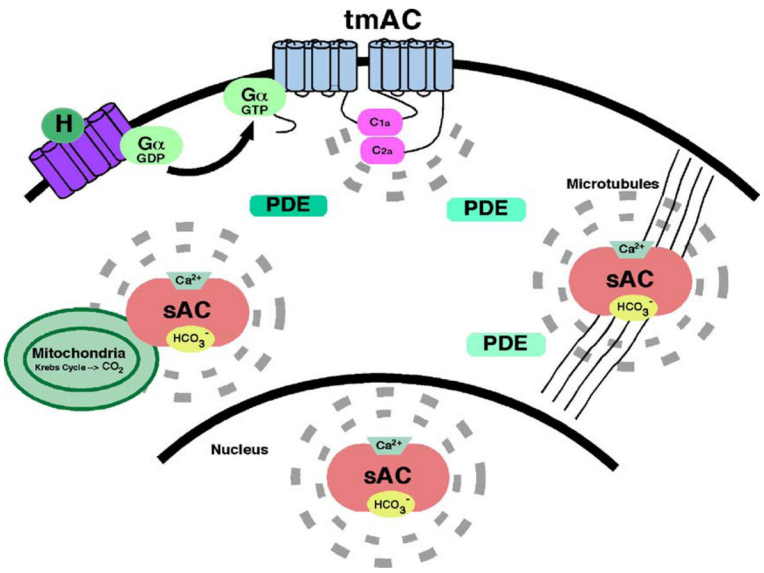

Figure 1.

Revised model for cAMP signaling. The second messenger is formed, acts, and is degraded close to its target, leading to the formation of cAMP micro-domains within the cell. H denotes a receptor-activating hormone and broken lines indicate the limited diffusion sphere of cAMP formed by the respective adenylyl cyclase.

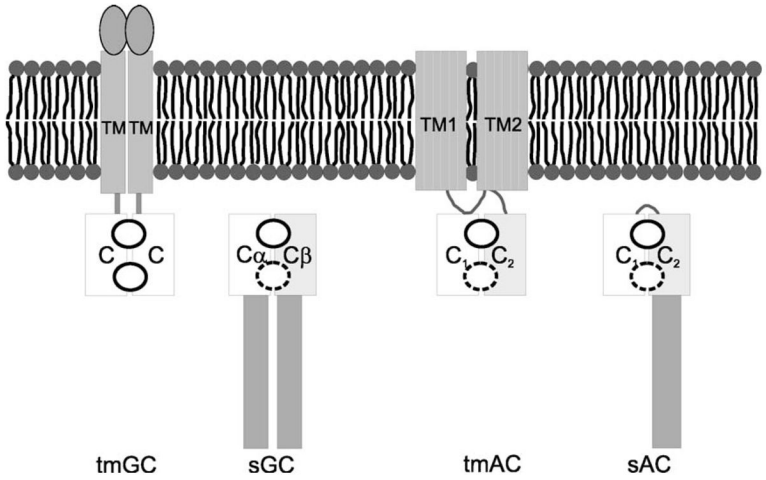

Figure 2.
Scheme of domain arrangements in various class III cyclases. The conserved, dimeric catalytic core is formed by association of two domains, which belong either to a single polypeptide or to two protein chains. Intermolecular dimers can be homodimers or heterodimers. The dimers contain either two active sites (homodimers; indicated by circles) or one active site and a degenerate, inactive site (broken circle in heterodimers and pseudoheterodimers). C, catalytic domain; TM, transmembrane domain.

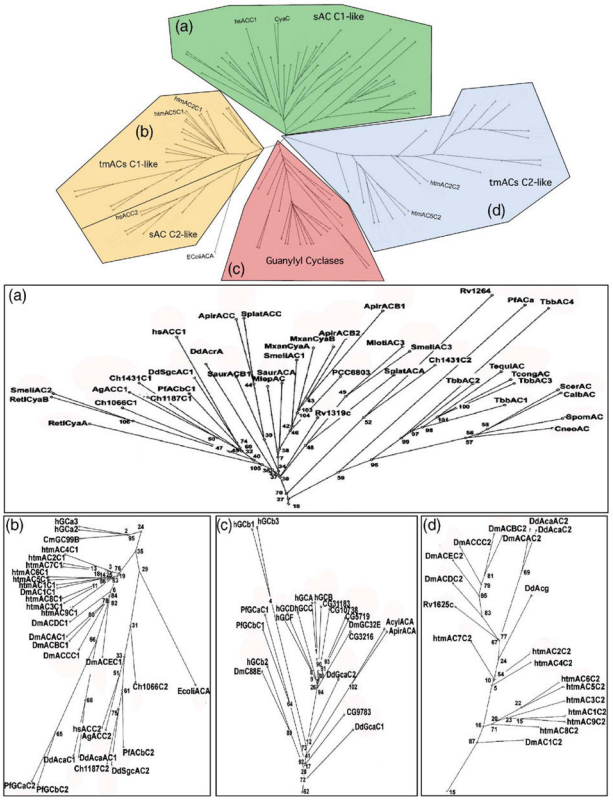

Figure 3.

Phylogenetic relationship between various class III ACs and GCs. Amino acid sequences of the catalytic regions were aligned using ClustalW[133] and represented as an unrooted tree using Fitch (Phylip 3.5; Felsenstein, J., Department of Genetics, University of Washington, Seattle) using *E. coli* CyaA as an outgroup. Numbers represent bootstrap confidence values of 1000 replicates. The individual catalytic domains from cyclases with two catalytic domains, i.e. C_1 and C_2, on a single polypeptide chain were analyzed separately. Accession numbers for the aligned amino acid sequences are as follows: human ANP-A (hGCA), P16066; human ANP-B (hGCB), P26594; human sGCα2 (hGCa2), P33402; human sGCα3 (hGCa3), Q02108; human sGCβ1 (hGCb1), Q02153; human sGCβ2 (hGCb2), O75343; human sGCβ3 (hGCb3), NP_000848; human GC-2C (hGCCC), P25092; human GC-2D (hGCD), Q02846; human GC-2F (hGCF), P51841; *Plasmodium falciparum* GCα (PfGCaC1/C2), NP_705488; *P. falciparum* GCβ (PfGCbC1/C2), AAN35978; *Dictyostelium discoideum* gca (DdGcaC1/C2), XP_643824; *D. discoideum* SgcA (DdSgcAC1/C2), XP_643219; *Drosophila melanogaster* Gyc32E (DmGC32E), Q07553; *D. melanogaster* Gyc88E (DmGC88E), NP_731974; *D. melanogaster* CG31183 (CG31183), NP_650505; *D. melanogaster* CG10738 (CG10738), NP_729905; *D. melanogaster* CG9783 (CG9783), NP_649477; *D. melanogaster* CG3216 (CG3216), NP_611532; *D. melanogaster* 99B (DmGC99B), Q07093; human tmAC-1 (htmAC1C1/ C2), Q08828; human tmAC-2 (htmAC2C1/C2), Q08462; human tmAC-3 (htmAC3C1/C2), O60266; human tmAC-4 (htmAC4C1/C2), Q8NFM4; human tmAC-5 (htmAC5C1/C2), O95622; human tmAC-6 (htmAC6C1/C2), O43306; human tmAC-7 (htmAC7C1/C2), P51828; human tmAC-8 (htmAC8C1/C2), P40145; human tmAC-9 (htmAC9C1/C2), O60503; *D. melanogaster* AC-A (DmACAC1/C2), NP_620475; *D. melanogaster* AC-B (DmACBC1/C2), NP_620474; *D. melanogaster* AC-C (DmACCC1/C2), NP_609593; *D. melanogaster* AC-D (DmACDC1/ C2), NP_620478; *D. melanogaster* AC-E (DmACEC1/C2), NP_620479; *D. melanogaster*

AC-1 (DmAC1C1/C2); *Anopheles gambiae* AgaC (AgACC1/ C2), EAA10271; human sAC (hsACC1/C2), NP_060887; *P. falciparum* ACα (PfAca), NP_701931; *P. falciparum* ACβ (PfACbC1/C2), NP_704518; *D. discoideum* Aca (DdAcaC1/C2), AAA33163; *D. discoideum* AcaA (DdAcaAC1/C2), XP_640636; *D. discoideum* AcrA (DdAcrA), AAD50121; *D. discoideum* AcgA (DdAcg), Q03101; *Trypanosoma brucei* AC-1 (TbbAC1), Q99279; *T. brucei* AC-2 (TbbAC2), Q99396; *T. brucei* AC-3 (TbbAC3), Q99280; *T. brucei* AC-4 (TbbAC4), Q26721; *T. congolense* AC (TcongAC), Q26896; *T. equiperdum* AC (TequiAC), P26338; *Mycobacterium tuberculosis* Rv1264 (Rv1624), CAB00890; *M. tuberculosis* Rv1319c (Rv1319c), Q10632; *M. tuberculosis* Rv1625c (Rv1625c), P0A4Y0; *Candida albicans* Cyr1 (CalbAC), AAG18428; *Cryptococcus neoformans* AC (CneoACC1/C2), AAG60619; *Schizosaccharomyces pombe* AC (SpomACC1/C2), P14605; *Saccharomyces cerevisiae* AC (ScerACC1/C2), P08678; *Chloroflexus aurantiacus* Chlo1066 (Ch1066C1/C2), ZP_00018085; *C. aurantiacus* Chlo1187 (Ch1187C1/C2), ZP_00018205; *C. aurantiacus* Chlo1431 (Ch1431C1/C2), ZP_00018442; *Spirulina platensis* CyaA (SplatACA), BAA22996; *S. platensis* CyaC (SplatACC), T17197; *Stigmatella aurantiaca* CyaA (SaurACA), CAA11549; *S. aurantiaca* CyaB1 (SaurACB1), T10905; *Synecocystis sp.* CyaA2 (PCC6803), BAA16969; *Anabaena pirulensis* CyaA (ApirACA), P43524; *A. pirulensis* CyaB1 (ApirACB1), NP_486306; *A. pirulensis* CyaB2 (ApirACB2), BAA13999; *A. pirulensis* CyaC (ApirACC), BAA14000; *Mycobacterium leprae* AC (MlepAC), CAA19149; *Sinorhizobium melioti* AC-1 (SmeliAC1), P19485; *S. melioti* AC-2 (SmeliAC2), Q52915; *S. melioti* AC-3 (SmeliAC3), Q9Z3Q0; *Mesorhizobium loti* AC-3 (MlotiAC3), BAB50205; *Myxococcus xanthus* CyaA (MxanCyaA), BAC00918; *M. xanthus* CyaB (MxanCyaB), BAD98264; *Rhizobium etli* CyaA (RetlCyaA), AAM66143; *R. etli* CyaB (RetlCyaB), AAM66145; *Anenome cylindrica* A (AcylACA), P43524; *E. coli* ACA (EcoliACA), CAA47280.

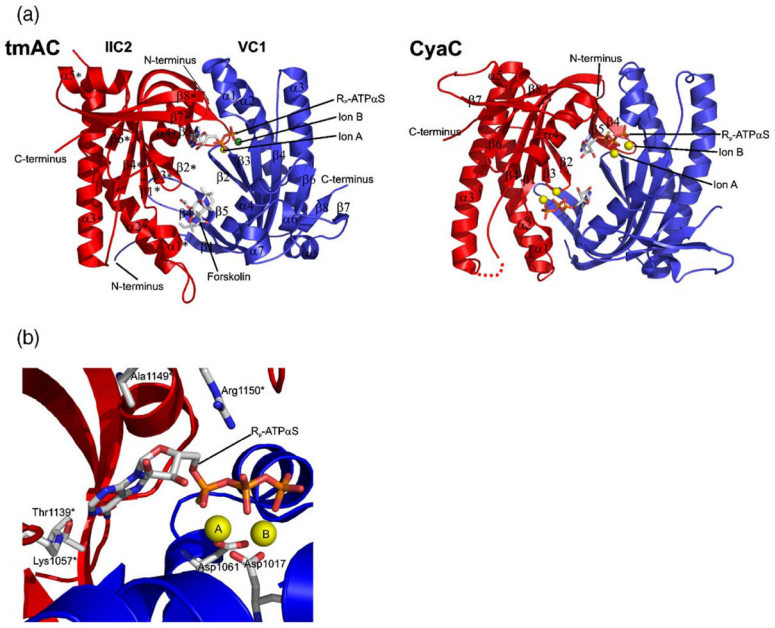

Figure 4.

Structure of class III catalytic units. (a) Overall structures of the catalytic domains of a mammalian tmAC (left) and the cyanobacterial sAC-like enzyme CyaC (right), both in complex with ATP analogs. The two subunits of the catalytic cores are colored red and blue, respectively, and secondary structure elements are labeled according to Steegborn *et al.*,[38] and Tesmer *et al.*[40] (b) Active site of the sAC-like cyclase CyaC in complex with the ATP analog Rp-ATPαS and two magnesium ions A and B (yellow spheres). The ion coordinating Asp residues 1017 and 1061, the residues recognizing the adenine ring (1139* and 1057*), the phosphate binding Arg (1150*), and the Ala conserved in sACs and replaced by Ser in tmACs (1149*) are labeled. This Figure was generated with PyMol.[134]

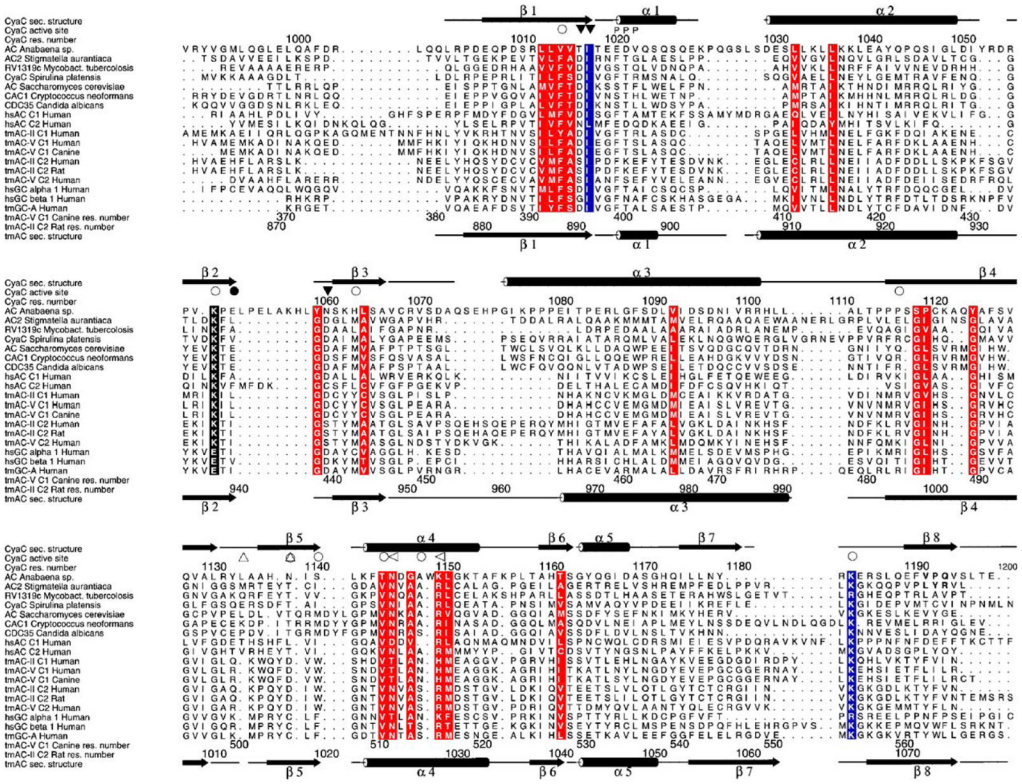

Figure 5.

Structure-based sequence alignment of representative members of nucleotidyl cyclase class III. Included are bicarbonate responsive cyclases (top 9 sequences) from bacteria, fungi, and human; the human G protein-regulated tmACs II and V and the structurally characterized tmACs from rat and dog; and two guanylyl cyclases, the homodimeric transmembrane receptor GC-A and the heterodimeric soluble guanylyl cyclases sGC1 (bottom three sequences). Secondary structure elements of CyaC are shown on top and those of tmAC II C_2 at the bottom. Ion binding residues (▽) and residues binding the substrate (○) or the transition state (◁) are labeled with filled and empty symbols indicating C_1 and C_2 residues, respectively. CyaC Thr1139*, which is characteristic for sAC enzymes and replaced by Asp in tmACs and Cys in GCs, is indicated (△). Homologous regions are shaded red, and highly conserved positions are shown in blue. Residue numbering on the top refers to CyaC and at the bottom to human tmAC VC_1 and IIC_2, respectively. The Figure was generated with Alscript.[135]

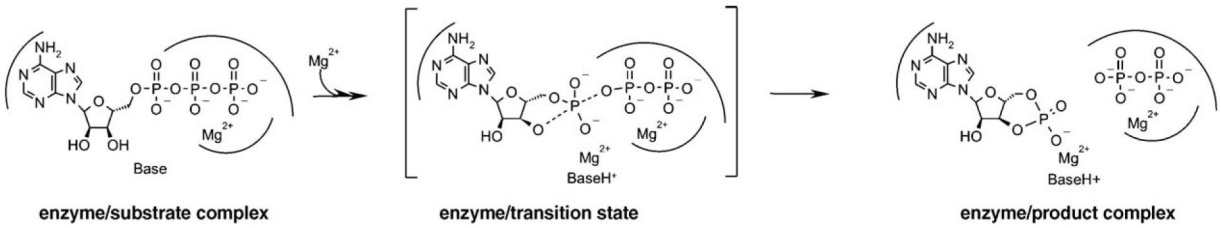

Figure 6.

Catalytic mechanism of class III AC enzymes. Schematic view of the two-ion catalyzed reaction, which starts with the substrate ATP and yields the products cAMP and pyrophosphate. The reaction proceeds through a magnesium-stabilized penta-covalent transition state with an in-line arrangement of the incoming and the leaving group.

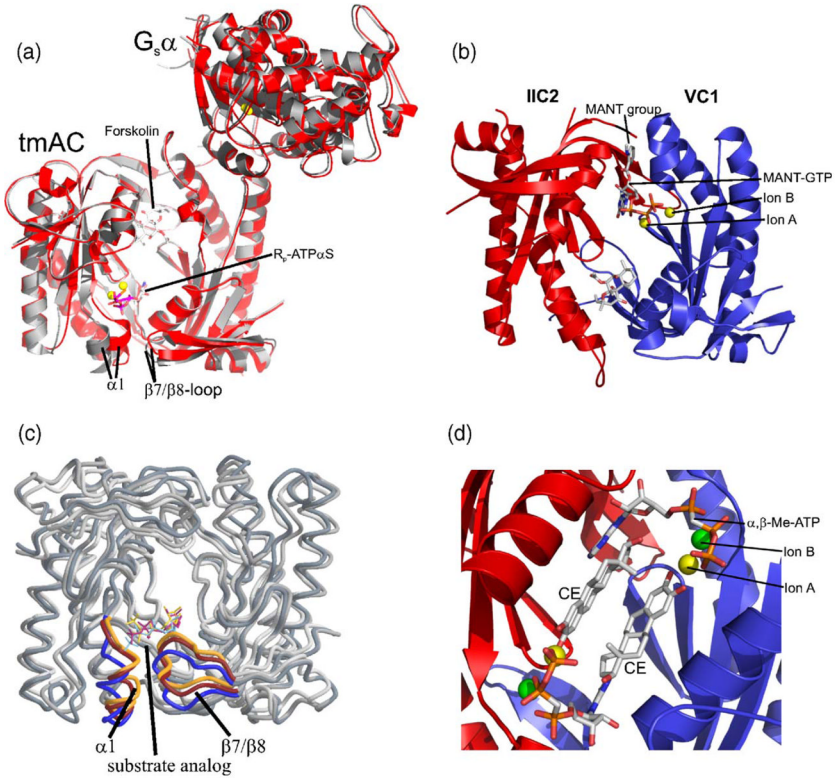

Figure 7.

Modulation of class III AC catalytic activity. (a) Complex of the tmAC catalytic core with $G_s\alpha$. Displayed is an overlay of the tmAC/$G_s\alpha$ complex in the absence (grey) and in the presence (red) of the ATP analog Rp–ATPαS, showing the large movement of α1 upon ligand binding, accompanied by a smaller shift of the β7/β8 loop. (b) Active site of a tmAC occupied by two Mg^{2+} (yellow spheres) and the inhibitor MANT–GTP. The MANT fluorophor extends from the active site into the dimer interface patch formed by α1 and the β7/β8 loop, the structural elements, which are responsible for the open–closed transition of the enzyme. (c) Bicarbonate inducible closure of the sAC active site. Overlay of the sAC–α,β-Me-ATP structure (open state; darkest gray, α1 and β7–β8 in blue), the sAC–Rp-ATPαS complex (partially closed; middle gray and red), and the bicarbonate-soaked Rp-ATPαS structure (closed; lightest gray and yellow). This Figure was reproduced from Steegborn *et al.*[38] (d) Active site region of CyaC in complex with the substrate analog α,β-Me-ATP and the non-competitive inhibitor catechol estrogen (CE). The inhibitor is bound next to the active site and acts as chelator on the catalytic magnesium ion (yellow sphere), thereby distorting the active site and the substrate analog (green sphere: calcium). Two inhibitor molecules are bound due to the symmetry of the homodimeric sAC homolog CyaC used in this study. This Figure was generated with PyMol[134] and Setor (c).[136]