Cyclic AMP Inhibits the Activity and Promotes the Acetylation of Acetyl-CoA Synthetase through Competitive Binding to the ATP/AMP Pocket*§

Received for publication, August 15, 2016, and in revised form, December 8, 2016 Published, JBC Papers in Press, December 14, 2016, DOI 10.1074/jbc.M116.753640

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Edited by Ruma Banerjee

The high-affinity biosynthetic pathway for converting acetate to acetyl-coenzyme A (acetyl-CoA) is catalyzed by the central metabolic enzyme acetyl-coenzyme A synthetase (Acs), which is finely regulated both at the transcriptional level via cyclic AMP (cAMP)-driven trans-activation and at the post-translational level via acetylation inhibition. In this study, we discovered that cAMP directly binds to Salmonella enterica Acs (SeAcs) and inhibits its activity in a substrate-competitive manner. In addition, cAMP binding increases SeAcs acetylation by simultaneously promoting Pat-dependent acetylation and inhibiting CobB-dependent deacetylation, resulting in enhanced SeAcs inhibition. A crystal structure study and site-directed mutagenesis analyses confirmed that cAMP binds to the ATP/AMP pocket of SeAcs, and restraints SeAcs in an open conformation. The cAMP contact residues are well conserved from prokaryotes to eukaryotes, suggesting a general regulatory mechanism of cAMP on Acs.

Acetyl-coenzyme A (acetyl-CoA) is a central metabolic intermediate that modulates the balance of anabolism and catabolism by functioning as the fuel for energy generation or as the precursor of lipogenesis for energy storage in many organisms (1). Acetyl-CoA is also deemed a second messenger for physiological regulation by supplying an acetyl group for protein acetylation (1, 2), an important mechanism of post-translational modification (PTM). Acetyl-coenzyme A synthetase (Acs) (EC 6.2.1.1) belongs to the acyl- or aryl-CoA synthetase family and catalyzes the biosynthesis of acetyl-CoA from acetate, coenzyme A (CoA), and ATP, and is well conserved from bacteria to mammals. The Acs of Escherichia coli scavenges environmental acetate with high affinity (≈10 mM in general) and converts it to acetyl-CoA during glucose limitation (3). In humans, Acs is implicated in disposing of alcohol in normal hepatocytes (1, 4) and maintaining cell growth under metabolic stress conditions in certain tumors (5, 6). Acs is also reported to be involved in chromatin regulation (7) and bacterial chemotaxis (8, 9) by supplying acetyl-CoA as the acetylation donor. Acs is strictly regulated at both transcriptional and post-translational levels in enteric bacteria. Under carbon limitation, cAMP-CRP (cAMP receptor protein) binds upstream of the acs proximal promoter (acsP2) and initiates transcription of acs (10). However, this cAMP-CRP-dependent activation may be inhibited by the nucleoid proteins IHF and FIS by competitive binding to the CRP-binding sites or other unknown steric hindrances (11). The activity of Acs is regulated by acetylation catalyzed by the protein acetyltransferase Pat and deacetylation catalyzed by the NAD+−dependent deacetylase CobB. Pat transfers an acetyl group from acetyl-CoA onto the Salmonella enterica Acs (SeAcs) Lys590 and blocks the first half of the catalytic reaction, acetyl-AMP formation (12), whereas CobB restores activity by specifically removing this acetyl group (13). cAMP is a master second messenger that is involved in rapid response to environmental nutritional changes in bacteria (14,
The acetate scavenging by Acs is an alternative pathway for acetyl-CoA production under a low-glucose condition, and cAMP regulates Acs in multiple ways. Besides the direct transcription activation of the *acs* gene by cAMP-CRP as described above (10), cAMP indirectly modulates Acs activity by activating transcription of the *pat* gene in the presence of non-phosphotransferase system carbon sources in enteric bacteria (16), or by activating Pat activity via binding to its allosteric site in mycobacteria (17, 18).

In this study, we discovered that cAMP inhibits the activity of SeAcs by directly binding to the enzyme and competing against one of its substrates, ATP. The binding of cAMP increases SeAcs Lys\(^{609}\) acetylation, which further inhibits SeAcs activity. The acetylation is apparently caused by concomitant promotion of Pat-dependent acetylation and inhibition of CobB-dependent deacetylation. We further determined a crystal structure of SeAcs complexed with cAMP and CoA, and the crystal structure not only confirms that cAMP binds to the conserved ATP/AMP binding pocket of SeAcs, but also suggests cAMP, in addition to CoA, restrains SeAcs in an open conformation. This structure provides possible scenarios for the mechanism that promotes SeAcs Lys\(^{609}\) acetylation. In addition, we found cAMP also inhibited *S. enterica* propionyl-CoA synthetase (SePrpE) and long-chain acyl-CoA synthetase (SeFadD), which are from the same protein family of acyl- or aryl-CoA synthetases. As the residues of cAMP-binding site are generally conserved in this family, we infer that cAMP possibly inhibits other acyl- or aryl-CoA synthetases in *S. enterica*.

### Results

**cAMP Binds to SeAcs Directly**—We measured the thermodynamic parameters of the proposed interaction between cAMP and non-acetylated SeAcs isolated from a *pat*-null *S. enterica* strain using the isothermal titration calorimetry (ITC) method to test whether cAMP binds directly to Acs. The result shows that cAMP directly interacted with SeAcs, with one cAMP-binding site for each SeAcs (blue in Fig. 1A). The dissociation constant (\(K_d\)) was estimated to be 164 \(\pm\) 11 \(\mu\)M, which is very close to the physiological concentration of cAMP in live bacterial cells under starvation conditions (19, 20), implicating a physiological relevance. We next measured the interaction between cAMP and acetylated SeAcs isolated from a *cobB*-null *S. enterica* strain, and the \(K_d\) was estimated to be 297 \(\pm\) 15 \(\mu\)M (red in Fig. 1A).

Given the 0.8-fold increase in \(K_d\) of cAMP binding to acetylated SeAcs compared with the non-acetylated enzyme, we investigated if acetylation of SeAcs also affects the binding of its natural substrate, ATP. The results indicate a similar 2.4-fold increase in \(K_d\) of ATP binding to the acetylated SeAcs (1.09 \(\pm\) 0.079 m\(\mu\); red in Fig. 1B) compared with the non-acetylated SeAcs (322 \(\pm\) 19 \(\mu\); blue in Fig. 1B). Therefore, both cAMP and ATP preferably bind to the non-acetylated SeAcs.

We next measured the acetylation level of Lys\(^{609}\), the primary site on SeAcs under acetylation regulation. There is a clear difference in the Lys\(^{609}\) acetylation level of SeAcs isolated from *pat*-null or *cobB*-null *S. enterica* cells (Fig. 2, A and B). As Lys\(^{609}\) participates in the catalytic reaction of SeAcs, it is not surprising that acetylation at Lys\(^{609}\) affects the binding of ATP to SeAcs.

The results also suggest that cAMP might bind to a location similar to that of ATP in SeAcs.

**cAMP Inhibits SeAcs by Competing with the Substrate ATP**—We measured SeAcs activity (isolated from *pat*-null *S. enterica* cells) in the presence or absence of cAMP to test whether binding of cAMP inhibits the activity of SeAcs. SeAcs catalyzes the formation of acetyl-CoA using CoA, acetate, and ATP as substrates. The potential inhibitory effect of cAMP on Acs was tested using a subsaturated concentration for each of the substrates. cAMP substantially inhibited SeAcs activity only in an enzymatic reaction system with a low concentration of ATP (Fig. 1C), suggesting cAMP inhibits SeAcs via an ATP-competitive mechanism.

To further confirm that cAMP inhibits SeAcs by competing against the substrate ATP, we measured the apparent \(K_m\) constants of ATP under various cAMP concentrations. The Lineweaver-Burk plot for cAMP shows a characteristic signature of a competitive inhibitor (Fig. 1D), in agreement with the above observation that cAMP barely inhibits SeAcs when ATP is saturated (Fig. 1C).

The inhibition constant (\(K_i\)) for cAMP-SeAcs was estimated as 185 \(\pm\) 25 \(\mu\)M by plotting the apparent \(K_m\) (toward substrate ATP) against cAMP concentration (inset of Fig. 1D), which is similar to the ITC estimated \(K_i\) (164 \(\pm\) 11 \(\mu\); blue in Fig. 1A).

**cAMP Promotes Lys\(^{609}\) Acetylation of SeAcs**—Acetylation of the catalytic lysine residue (Lys\(^{609}\) in SeAcs) is an efficient way to modulate Acs activity (12, 13). CAMP has been reported to enhance Acs acetylation by activating the lysine acetyltransferase Pat in mycobacteria (17, 18) via binding to the allosteric site in the cyclic nucleotide (cAMP)-binding domain of Pat. This domain is unique to mycobacteria species (17), and we speculate that the mechanism by which cAMP affects the Acs in enteric bacteria, such as SeAcs, must be different.

To determine whether direct binding of cAMP to SeAcs affects its Lys\(^{609}\) acetylation level, we performed *in vitro* enzymatic acetylation assays using purified non-acetylated SeAcs (from a *pat*-null *S. enterica* strain) and acetyl-CoA as the substrates. The acetylation assays were catalyzed by purified *S. enterica* protein acetyltransferase (SePat), with or without cAMP in the reaction system. The level of SeAcs acetylation was determined by Western blotting using an antibody that specifically recognizes acetylated Lys\(^{609}\) (Fig. 2A). As shown in Fig. 2C, SePat increases the acetylation level of SeAcs Lys\(^{609}\) *in vitro* (lane 4), and cAMP promotes Pat-dependent acetylation substantially (lane 7). The addition of cAMP to the acetylation assay system further decreases SeAcs activity, suggesting SeAcs acetylation promoted by cAMP also contributes to the inhibitory effect of cAMP on SeAcs. Because a cNMP-binding domain is absent in SePat (17), we infer that cAMP promotes SeAcs acetylation through direct binding to its ATP/AMP pocket as well.

Lysine acetyltransferase Pat and deacetylase CobB exist normally as a pair to modulate cellular protein acetylation levels in most bacteria (21). To test if cAMP also affects deacetylation of SeAcs by SeCobB, we performed *in vitro* enzymatic deacetylation assay using purified acetylated SeAcs from a *cobB*-null *S. enterica* strain as the substrate, NAD\(^+\) as a coenzyme, and purified *S. enterica* Sir2-like lysine deacetylase (SeCobB) as the
cAMP directly inhibits Acs activity

FIGURE 1. cAMP directly binds to SeAcs and inhibits its activity by competing with substrate ATP. A, representative real-time ITC heat change (upper and middle panels) and the integration (lower panel) of each titration of cAMP to SeAcs or acetylated SeAcs (ac-SeAcs). B, representative real-time ITC heat change (upper and middle panels) and the integration (lower panel) of each titration of ATP to SeAcs or ac-SeAcs. C, cAMP inhibits SeAcs at a low concentration of ATP (1 mM CoA, 20 μM ATP, and 20 mM acetate), but not at either a low concentration of acetate (1 mM CoA, 1 mM ATP, and 20 mM acetate), or a low concentration of CoA (20 μM CoA, 1 mM ATP, and 20 mM acetate). The SeAcs activity in the absence of cAMP was treated as 100%. D, the representative Lineweaver-Burk plot shows cAMP inhibits SeAcs by competing with the substrate ATP. The inset panel is a Dixon plot of apparent K_m versus cAMP concentration. The K_i of cAMP is estimated from the linear regression. The experiments were repeated in triplicate, and the data are presented as mean ± S.D., ***, p < 0.001.

catalytic enzyme. The result in Fig. 2D confirmed that SeCobB was able to remove the acetyl group on Lys^{609} of SeAcs (lane 4), and rescued SeAcs activity. However, decrease in the acetylation level as well as the recovery of SeAcs activity by CobB treatment was inhibited remarkably in the presence of cAMP (lane 7). Because cAMP does not bind to SeCobB (data not
shown), we infer that cAMP inhibits CobB-dependent deacetylation by directly binding to SeAcs.

**cAMP Binds to the ATP/AMP Binding Pocket of SeAcs**—To understand the structural basis of SeAcs inhibition by cAMP, we determined the crystal structure of SeAcs complexes with cAMP, CoA, and acetate (Fig. 3, Table 1) at 1.65-Å resolution. The structure defined the binding site of cAMP on SeAcs, and the effects of cAMP and CoA binding on SeAcs conformation. The simulated annealing omit density map shows unambiguous electron density of cAMP within the ATP/AMP binding pocket of SeAcs (Fig. 3, A and E), in agreement with our observation that cAMP inhibits SeAcs by competing with the substrate ATP (Fig. 1, C and D). The map also shows unambiguous electron density for adenosine and diphosphate moieties of CoA within the adjacent CoA binding pocket (right in Fig. 3A). However, no electron density for acetate was observed within the acetate-binding site of SeAcs, probably due to effect of steric hindrance by cAMP.

Our crystal structure revealed that in the presence of cAMP and CoA, the C-terminal domain of SeAcs adopted an open conformation (red in Fig. 3B), exposing Lys<sup>609</sup> to the solvent. Lys<sup>609</sup> plays an important role in the first half of the reaction catalyzed by SeAcs and is subject to negative regulation by acetylation (13). We compared the “open” conformation in our crystal structure of SeAcs complex with the previously reported open conformation in a crystal structure of SeAcs in complex with CoA, acetate, and AMP (Protein Data Bank (PDB) code 2P2F; Fig. 3D); and the two open conformations are essentially identical. The Lys<sup>609</sup> residue is located at the same position, and the side chains of Lys<sup>609</sup> are both partially disordered in the two structures (Fig. 3C).

We also compared our open conformation with a previously determined “closed” conformation of a crystal structure of yeast Acs in complex with AMP. We observed a large rotation of 100° in the Acs C-terminal domain, which includes Lys<sup>675</sup> (Lys<sup>609</sup> in SeAcs), in the closed conformation (Fig. 3D), as a result Lys<sup>675</sup> is buried into the active center. The exposed conformation of Lys<sup>609</sup> in our current structure would allow Lys<sup>609</sup> to be more accessible for acetylation, consistent with the observation that the acetylation of SeAcs was remarkably increased in the presence of cAMP (Fig. 2C).

Contacts between cAMP and residues of SeAcs are shown in Fig. 4, A and B. The adenine moiety of cAMP makes the same interactions with SeAcs as the adenine moiety of AMP makes in the crystal structure of SeAcs in complex with AMP, CoA, and acetate (PDB 2P2F; Fig. 3E) (22). These interactions are also the same for the adenine moiety of 5′-propyl-AMP in the crystal structure of SeAcs in complex with 5′-propyl-AMP and CoA (PDB 1PG4; Fig. 3E) (23). These interactions include: stacking effects of the adenine base to side chain atoms of Trp<sup>313</sup> and Ile<sup>312</sup> from one side and main chain atoms of Val<sup>1186</sup>, Glu<sup>388</sup>, and Pro<sup>399</sup> from the other side; one H-bond through the N6 atom of
cAMP Directly Inhibits Acs Activity

FIGURE 3. Crystal structure of SeAcs in complex with cAMP and CoA. A, simulated-annealing omit F–F electron density map (green) contoured at 3.0 σ for cAMP (left) and CoA (right) molecules. Blue, orange, and red represent nitrogen, phosphorus, and oxygen atoms, respectively. Cyan and green represent carbon atoms of cAMP and CoA, respectively. B, overall structure of SeAcs complexed with cAMP (cyan) and CoA (green). White and red ribbons represent the N-terminal and C-terminal domains of SeAcs, respectively; red sphere, Lys609; cyan sphere, cAMP; green sphere, CoA. C, superimposition of our SeAcs-cAMP-CoA crystal structure (red and gray) with the crystal structure of SeAcs complexes with AMP, CoA, and acetate (PDB code 2P2F; cyan). D, superimposition of our SeAcs-cAMP-CoA crystal structure (red and gray) with the crystal structure of yeast Acs in complex with AMP (PDB code 1RY2; green). E, close-up view of the cAMP (cyan) binding pocket with superimposed propyl-AMP (red) from the crystal structure of SeAcs complexed with propyl-AMP, CoA, and acetate (PDB code 1PG4), and with superimposed AMP (purple) from the crystal structure of SeAcs in complex with AMP (PDB code 2P2F).

the adenine with Asp411; and one water-mediated H-bond through the N3 atom of the adenine with Asp500 (Fig. 4, A and B). The ribose moiety of cAMP makes three H-bonds through its 2′-OH group with Asp411, Gln415, and Arg515, respectively, and one H-bond through its 5′-O atom with Asn521. The phosphate group of cAMP contacts SeAcs by one H-bond with Thr416, one salt-bridge bond with Arg426, and Van der Waals interactions with Thr416 and Glu417.

cAMP binds to the highly conserved ATP/AMP binding pocket of Acs and shares most contact residues with AMP (Figs. 3E and 4, A and B). Therefore it is not surprising that most contact residues of cAMP are conserved among bacterial species, yeast, and humans (Fig. 4C). We thereby propose that the regulatory effect of cAMP on Acs is possibly an important mechanism that has been preserved during evolution.

Trp413, Gln415, Asp411, and Arg526 in SeAcs Are Determinants for cAMP Binding and Promotion of SeAcs Acetylation—Although the SeAcs crystal structure shows 14 total residues interact with cAMP either by H-bonds or Van der Waals forces (Fig. 4), it is still unknown which site(s) is critical. To further define the determinants of cAMP binding, we performed ITC experiments analyzing the binding affinity of cAMP to SeAcs derivatives bearing alanine substitution of residues that are involved in side chain interactions. As shown in Table 2, the SeAcs derivatives W413A, Q415A, and D500A lost their ability to bind cAMP; whereas D411A, R515A, and N521A displayed decreased affinity; and T416A displayed a similar affinity to wild-type SeAcs.

To verify that cAMP promotes SeAcs Lys609 acetylation through direct binding of cAMP to the ATP/AMP pocket in SeAcs, we performed similar in vitro Pat-dependent acetylation and CobB-dependent deacetylation assays using SeAcs derivatives W413A, Q415A, and D500A, which lost their cAMP binding ability. The W413A, Q415A, and D500A derivatives of SeAcs isolated from pat-null S. enterica cells were acetylated in vitro by SePat to a similar level to wild-type SeAcs (Fig. 5A, lanes 2 versus 3, 5 versus 6, 8 versus 9, and 11 versus 12). The W413A, Q415A, and D500A SeAcs derivatives isolated from cobB-null S. enterica cells were deacetylated by SeCobB in vitro to a similar level of undetectable acetylation to wild-type SeAcs (Fig. 5B, lanes 2 versus 3, 5 versus 6, 8 versus 9, and 11 versus 12). However, in contrast to wild-type SeAcs (Fig. 5, A and B, lanes 1 versus 2), cAMP failed to affect either Pat-catalyzed acetylation (Fig. 5A, lanes 4 versus 5, 7 versus 8, and 10 versus 11) or CobB-catalyzed deacetylation (Fig. 5B, lanes 4 versus 5, 7 versus 8, and 10 versus 11) of the Lys609 on these SeAcs derivatives. As W413A, Q415A, and D500A derivatives of SeAcs were defective in cAMP binding (Table 2), the above results provide further support that the modulation of SeAcs acetylation by cAMP is dependent on direct cAMP binding.

cAMP Generally Inhibits Other Acyl- or Aryl-CoA Synthetases—Acs belongs to a large acyl- or aryl-CoA synthetase protein family (EC 6.2.1-), which all contain a two-domain architecture and harbor a conserved ATP/AMP binding pocket (24). To explore whether cAMP might inhibit other members of the acyl- or aryl-CoA synthetase family in S. enterica, we tested the effect of cAMP against two other acyl-CoA synthetases from S. enterica, SePrpE and SeFadD. The potential inhibitory activities were measured in the presence of subsaturation ATP concentration. The activities of SePrpE and SeFadD were inhibited by cAMP by 80 and 70%, respectively (Fig. 6A). These results support our hypothesis that cAMP possibly regulates other members of the acyl- or aryl-CoA synthetase family in S. enterica.

We aligned the protein sequences of all available acyl- or aryl-CoA synthetases (1190 records) from the UniPortKB data-
base, and found that 13 of the 14 cAMP contact residues are generally conserved (Fig. 6B). We speculate that the regulation by cAMP might be a more widespread occurrence in this protein family in organisms apart from S. enterica.

**Discussion**

This study establishes that cAMP modulates Acs activity through a novel mechanism. We show that cAMP occupies the ATP/AMP binding pocket of SeAcs, inhibiting SeAcs activity by competitive binding against its natural substrate ATP. We found that cAMP cooperates with Pat/CobB to regulate SeAcs acetylation and activity. Finally, we observed a similar inhibitory effect of cAMP against SePrpE and SeFadD, both harboring the ATP/AMP-binding domain and forming AMP during catalysis, and thus, this novel regulatory mechanism is proposed to be extendable to acyl- or aryl-CoA synthetase family enzymes, subject to future confirmation.

CAMP adjusts cellular anabolism and catabolism in some bacteria to allow adaptation to an environment with a non-preference carbon source. CAMP activates/represses the transcription of about 200 genes by directly binding to and allosterically activating CRP (25). One of them is the gene that encodes Acs, which converts acetate to acetyl-CoA to fuel the life when glucose is absent and the environmental concentration of acetate is low. This reaction is costly and is deemed to be regulated by the ATP/AMP-binding domain and forming AMP during catalysis, and thus, this novel regulatory mechanism is proposed to be extendable to acyl- or aryl-CoA synthetase family enzymes, subject to future confirmation.

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Previous studies have revealed activity-based negative regulation of cAMP upon Acs through PTM. CAMP coordinates expression of yfiQ genes in E. coli, which encodes the lysine acetyltransferase Pat (16). Pat completely abolishes the activity of Acs by adding an acetyl group to the catalytic lysine residue in Acs active center (13). CAMP also allosterically activates the activity of the protein acetyltransferase Pat in Mycobacterium tuberculosis and Mycobacterium smegmatis by targeting the Pat cNMP-binding domain, hence inhibiting the activity of AcS (17, 18). The new cAMP regulation mechanism identified in this study provides an alternative means for directly sensing the cellular ATP/cAMP levels, and also a rapid way to rescue energy deficiency by inhibiting Acs activity. We found binding of cAMP could influence the acetylation potential of Acs by promoting Pat-dependent acetylation and inhibiting CobB-dependent deacetylation concomitantly, thus enhancing the PTM inhibition of Acs activities.

SeAcs enzymatic activity is subject to post-translational regulation by acetylation of the catalytic Lys609 residue (13). Lys609 is located at the C-terminal domain of SeAcs, which adopts open and closed conformations by rotating −100° relative to the N-terminal domain (22, 23, 27). The C-terminal domain of SeAcs is proposed to fold into a closed conformation during the first half-reaction (converting ATP and acetate into acetyl-AMP), in which Lys609 inserts into the active center and makes critical contacts with the substrates (27). The C-terminal domain adopts an open conformation in the second half-reaction (converting acetyl-AMP and CoA into acetyl-CoA and AMP), in which Lys609 moves out from the active center and is exposed to solvent (27).

The two conformations have been captured in the crystal structures of SeAcs complexed with CoA, acetate, and AMP (open conformation; PDB code 2P2F) (22), and the yeast Acs in complex with AMP (closed conformation; PDB code 1RY2) (27), respectively. It is proposed that binding CoA triggers the conformation change (22). In the current crystal structure of SeAcs in complex with cAMP and CoA, the C-terminal domain of SeAcs is held to an open conformation, which exposes Lys609 to the protein surface. A recently reported crystal structure of SeAcs in complex with SePat (28) revealed that SeAcs adopts an open conformation during Lys609 acetylation exactly as that of our crystal structure, which may account for enhanced acetylation by SePat via cAMP binding. However, it is puzzling why CAMP also inhibits the SeCobB catalyzed deacetylation of SeAcs. Because no crystal structures of either bona fide acetylated SeAcs or SeAcs complexed with SeCobB are available, it is unclear how Lys609 acetylation affects SeAcs conformation and what conformation of SeAcs would adopt during deacetylation. We speculate that SeCobB might favor a slightly different conformation of SeAcs from the open one observed in our structure, and the presence of CAMP hinders the conformation flexibility.

This study also broadens our knowledge of the complexity of CAMP signaling. This second messenger was initially discovered as a regulator for carbohydrate metabolism in bacteria (29, 30), and then increasingly recognized as an important factor involved in other biological processes such as cell division, cell motility, and microbial virulence (31–33). CAMP has been

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**TABLE 1**

**Statistics of crystal structure of SeAcs in complex with cAMP and CoA**

| Data collection | SeAcs-cAMP-CoA |
|----------------|----------------|
| Space group | P 2₁ |
| Cell dimensions | a, b, c (Å) | 60.2, 144.3, 71.7 |
| α, β, γ (°) | 90.0, 91.7, 90.0 |
| Resolution (Å) | 40.00–1.65 (1.68–1.65)* |
| Rmerge or Rmerge | 0.146 (0.616)* |
| I/| |
| Complementality (%) | 1.000 (1.000)* |
| Completeness (%) | 3.3 (3.3)* |
| CC1/2 in highest shell | 0.630 |

**Refinement**

| Resolution (Å) | 40.00–1.65 |
| No. reflections | 144575 |
| Rwork/Rfree | 0.169/0.207 |
| No. atoms | Protein 9998 |
| Ligand/ion | 138 |
| Water | 1113 |
| B-factors (Å²) | Protein 17.2 |
| Ligand/ion | 29.6 |
| Water | 25.3 |
| Root mean square deviations | Bond lengths (Å) 0.007 |
| Bond angles (°) | 1.148 |

* Highest resolution shell is shown in parentheses.
implicated into multiple events in humans, ranging from metabolism (34) to memory formation (35) and innate immunity (36). The major outcome of cAMP signaling is regulating gene expression, either by directly modulating transcription through cAMP-CRP in bacteria, or by indirectly controlling gene expression through protein kinase A as an intermediate in eukaryotes (37). Besides gene expression, cAMP also controls gating of some ion channels (38) and activates guanine exchanging factors (39). For all the target proteins discussed above, cAMP binds to a conserved cNMP-binding domain (40) shared by all of them, characterized by a barrel flanked by helixes folded from 120 continuous residues. The cAMP binding pocket of Acs discovered in our crystal structure exhibits a typical Rossmann-fold (Fig. 3), six sheets and three helixes form the cAMP binding pocket), which is radically different from the fold of the conserved cNMP-binding domain. Moreover, this new cAMP binding pocket is well conserved based on our sequence alignments of Acs from bacteria, yeast, and humans (Fig. 4C). Therefore, we propose that the regulatory mechanism of cAMP on Acs is evolutionally conserved from bacteria to mammals.

The newly identified cAMP binding pocket is also generally conserved in other acyl- or aryl-CoA synthetases (Fig. 6B), as implicated by the sequence alignment of 1190 acyl- or aryl-CoA synthetase family members across multiple species. We tested cAMP inhibitions of two other family members from S. enterica experimentally, and the inhibition effects upon SePrpE and SeFadD were similar to that for SeAcs. We infer that the regulatory mechanism of cAMP on Acs might be extendable to other family members of acyl- or aryl-CoA synthetases in bacteria.

### TABLE 2

| SeAcs Derivatives | K<sub>d</sub> (μM) |
|-------------------|------------------|
| WT                | 164 ± 11         |
| W413A             | ND               |
| Q415A             | ND               |
| D500A             | ND               |
| R526A             | 200 ± 50         |
| T416A             | 1500 ± 300       |
| D411A             | 1400 ± 300       |
| N521A             | 590 ± 50         |

* ND, not detectable.

### FIGURE 4

**Detail interactions between SeAcs and cAMP.** A, stereo-presentation of detail interactions between SeAcs and cAMP. White ribbon, SeAcs backbone; white stick, SeAcs carbon atoms; cAMP is colored as in panel A. B, schematic presentation of the possible interaction between SeAcs and cAMP. Red dashed lines, H-bonds; blue arcs, Van der Waals interactions. C, sequence alignment of the cAMP-binding pocket in Acs enzymes from different species. The number at the beginning of each line indicates the residue position relative to start of each protein sequence. Residues involved in interactions with cAMP are denoted with colored dots (red, H-bond interaction; black, Van der Waals interaction). Residues conserved in different species are shaded red.

### FIGURE 5

**cAMP has no effect on acetylation promotion of mutant SeAcs.** A, cAMP activates SePat-dependent acetylation of Lys<sup>609</sup> on wild-type SeAcs but has no effect on mutant SeAcs in vitro. B, cAMP inhibits SeCobB-dependent deacetylation of Lys<sup>609</sup> on wild-type SeAcs but has no effect on mutant SeAcs in vitro. Top band, acetylation detection by Western blotting using anti-SeAcs Lys<sup>609ac</sup> antibody; bottom band, Coomassie Brilliant Blue-stained proteins on SDS-PAGE gels are shown as a loading control. Each experiment was independently repeated three times.
The regulation of Acs by cAMP binding is yet to be characterized in vivo. However, the $K_d$ of cAMP measured in this study is comparable with the physiological concentration found in bacterial cells under glucose depletion conditions (19, 20), and then inoculated to 250 ml LB. Protein expressions were induced with isopropyl $\beta$-D-thiogalactoside to a final concentration of 0.5 mM overnight at 18 °C. Cells were harvested and resuspended in lysis buffer containing 50 mM Tris (pH 7.5), 300 mM KCl, 10% (v/v) glycerol, and 1 mM PMSF, and disrupted using an Emulsiflex-C5 cell disruptor (AVESTIN, Inc., Ottawa, Canada). Cellular debris was removed by centrifuging at 13,000 $\times$ g for 30 min at 4 °C. The recombinant proteins were purified with affinity chromatography using a nickel-nitrilotriacetic acid Superflow column (Qiagen) and dialyzed into a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, and 10% (v/v) glycerol. After purification, the target proteins with sufficient purity (above 95%) were quantified and kept frozen at $-80^\circ$C. The variants of SeAcs were constructed via PCR site-directed mutagenesis (Agilent, Inc.) and then expressed in S. enterica G2466 and purified in the same way as that of wild-type SeAcs. Specially, SeAcs used in Pat-dependent acetylation assay and enzymatic inhibition assay was produced from the pat-null S. enterica G2466 strain, whereas SeAcs used in CobB-dependent deacetylation assay was produced from cobB-null S. enterica G2466 strain.

SeAcs for Crystallization—A single colony of BL21(DE3) transformed with plasmid Ph 3 (pET22b-tac-ScAcs) was used to inoculate 20-ml LB broth containing 100 $\mu$g/ml of ampicillin, and cultures were incubated overnight at 37 °C with shaking. LB broth (1 liter) was inoculated with a 1:100 ratio, incubated at 37 °C for 16 h, and then harvested and resuspended in lysis buffer containing 50 mM Tris (pH 7.5), 300 mM KCl, 10% (v/v) glycerol, and 1 mM PMSF, and disrupted using an Emulsiflex-C5 cell disruptor (AVESTIN, Inc., Ottawa, Canada). Cellular debris was removed by centrifuging at 13,000 $\times$ g for 30 min at 4 °C. The recombinant proteins were purified with affinity chromatography using a nickel-nitrilotriacetic acid Superflow column (Qiagen) and dialyzed into a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, and 10% (v/v) glycerol. After purification, the target proteins with sufficient purity (above 95%) were quantified and kept frozen at $-80^\circ$C. The variants of SeAcs were constructed via PCR site-directed mutagenesis (Agilent, Inc.) and then expressed in S. enterica G2466 and purified in the same way as that of wild-type SeAcs. Specially, SeAcs used in Pat-dependent acetylation assay and enzymatic inhibition assay was produced from the pat-null S. enterica G2466 strain, whereas SeAcs used in CobB-dependent deacetylation assay was produced from cobB-null S. enterica G2466 strain.
cAMP Directly Inhibits Acs Activity

| E. coli strains | Supplements in LB medium | Growth condition | cAMP | References |
|----------------|---------------------------|-----------------|------|-------------|
| ATCC 8739     | 1% Glucose                | Pre-logarithmic phase | 0.5 µM | 19          |
| ATCC 8739     | 1% Glucose                | Incubation for 60 min | 170 | 19          |
| W3110         | 0.04% Glucose and 0.2% lactose | Depletion of glucose | 15 | 48          |
| MC4100        | 0.02% Glucose             | Depletion of glucose | 500 | 49          |
| MC1000        | 0.02% Glucose             | Depletion of glucose | 125 | 20          |
| CHE9          | 0.2% Glucose-6-P          | Mid-logarithmic phase | 0.38 | 50          |
| CHE9          | 0.2% Glucose              | Mid-logarithmic phase | 0.39 | 50          |
| CHE9          | 0.2% Glycerol             | Mid-logarithmic phase | 1.3 | 50          |
| CHE9          | 0.2% Glycerol to 0.2% glucose | Incubation for 20 min | 0.1 | 50          |

37 °C with shaking until A600 = 0.8. Protein expression was induced by addition of isopropyl β-D-thiogalactoside to 1 mM, and cultures were incubated for 3 h at 37 °C. Cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris, pH 7.7, 500 mM NaCl, 5% glycerol, and 5 mM 2-mercaptoethanol), and lysed using an Avestin EmulsiFlex-C5 cell disrupter (Avestin, Inc.). The supernatant after centrifugation was washed with 20 ml of lysis buffer containing 0, 5, and 10 mM acid-agarose (Qiagen, Inc.). The column was sequentially loaded onto a column packed with 1 ml of nickel-nitrilotriacetic acid-agarose (Qiagen, Inc.). The initial velocities were analyzed using Prism 5 (GraphPad Software Inc.), and the apparent Kᵦᵣ values in the presence of inhibitors were estimated by non-linear regression using the Michaelis-Menten equation. The mode of cAMP inhibition was determined by a Lineweaver-Burk plot and the substrate binding affinity Kᵨ was estimated (intercept on x axis) in a Dixon plot with an apparent Kᵦᵣ toward ATP against the concentration of cAMP as described in Ref. 44. Each point was measured in triplicate.

In Vitro SeAcs Acetylation and Deacetylation Assay—Pat-dependent SeAcs acetylation assays were carried out as follows: reaction mixtures (100 µl) containing 50 mM Tris (pH 7.5), 200 µM TCEP, 200 µM acetyl-CoA, 0.5 µM SePat, 1 µM SeAcs, and in the presence or absence of 1 mM CAMP were incubated at 37 °C for 30 min. The acetylation level of SeAcs Lys609 was measured by Western blotting using a monoclonal antibody specific to the acetylated SeAcs Lys609. Meanwhile, the activity of treated SeAcs was detected by adding 10 µl of acetylation reaction mixtures (containing 2 pmol of SeAcs) to the enzymatic reaction mixtures (90 µl) containing 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 1 mM TCEP, 0.02 µM SeAcs, 5 units of myokinase, 1 unit of pyruvate kinase, 1.5 units of lactate dehydrogenase, 3 mM phosphoenolpyruvate, 0.1 mM NADH, 1 mM CoA, 1 mM ATP, and 20 mM acetate. CAMP (1 mM) was additionally supplied in the enzymatic reaction mixture if it was present in the acetylation reaction mixtures.

CobB-dependent SeAcs deacetylation assays were carried out as follows: reaction mixtures (100 µl) containing 50 mM Tris (pH 7.5), 200 µM TCEP, 1 mM NAD⁺, 0.3 µM SeCobB, and 1 µM SeAcs and in the presence or absence of 1 mM CAMP, were incubated at 37 °C for 60 min. The acetylation level of SeAcs Lys609 was measured by Western blotting. Meanwhile, the activity of treated SeAcs was detected by adding 10 µl of deacetylation reaction mixtures (containing 2 pmol of SeAcs) to the enzymatic reaction mixtures as described above.

Western Blotting—Standard Western blotting procedures were followed for protein acetylation analysis. Proteins were separated by SDS-PAGE, transferred onto NC membrane (Millipore), subjected to immunoblotting with the homemade antibody specific to the acetylated SeAcs Lys609, and detected by ImageQuant LAS 4000 (GE Healthcare). Coomassie Blue staining (SDS-PAGE) was used for loading controls.

In Vitro SePrpE, SeFadD Enzymatic Assay—The SePrpE and SeFadD enzymatic activities were measured by a similar spectrophotometric-coupled assay as described above. Reaction mixtures (90 µl) contain 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 1 mM TCEP, 10 mM SePrpE or 800 mM SeFadD, 5 units of myokinase, 1 unit of Fisher Inc.). The initial velocities were analyzed using GraphPad Software Inc., and the apparent Kᵦᵣ values in the presence of inhibitors were estimated by non-linear regression using the Michaelis-Menten equation. The mode of cAMP inhibition was determined by a Lineweaver-Burk plot and the substrate binding affinity Kᵨ was estimated (intercept on x axis) in a Dixon plot with an apparent Kᵦᵣ toward ATP against the concentration of cAMP as described in Ref. 44. Each point was measured in triplicate.
pyruvate kinase, 1.5 units of lactate dehydrogenase, 3 mM phosphoenolpyruvate, 0.1 mM NADH, and cAMP (1 mM for determining ratio of inhibition). Reaction mixtures (90 μl) were incubated at 37 °C for 2 min, and initiated by adding 10 μl of substrate mixture (final concentration: 1 mM CoA, 20 mM sodium propionate, and 20 μM ATP for SePrP; 1 mM CoA, 0.8 mM oleic acid, and 80 μM ATP for SeFadD). The reactions were continually monitored by measuring absorbance at A415 every 2 s for 10 min using a microplate reader (Thermo Multiskan FC, ThermoFisher Inc.).

Isothermal Titrination Calorimetry—ITC experiments were carried out using a Microcal iTC200 microcalorimeter (GE Healthcare). SeAcs and SeAcs derivatives were diazylated against a buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, and 1 mM TCEP and concentrated to 100 μM by ultrafiltration. cAMP or ATP was dissolved in the same buffer to the concentration of 4 mM. During the experiments, 200 μl of 100 μM SeAcs or SeAcs derivatives was titrated with 2 μl of 4 mM cAMP for 20 steps. The resulting titration curves were fitted with MicroCal ORIGIN software. The dissociation constant K_d was estimated by non-linear regression with ORIGIN 7.0 software (OriginLab Inc.).

SeAcs Crystallization—SeAcs (180 μM) was incubated with cAMP (2 mM), coenzyme A (1 mM), and acetate (1 mM), and incubated 1 h at room temperature. Crystal growth conditions of the complex were screened with commercial solutions (Emerald Biosystems, Inc. and Hampton Research, Inc.) using a sitting-drop vapor-diffusion technique (drop: 0.2 μl of protein complex plus 1 μl of reservoir solution; reservoir: 60 μl of commercial screening solution; 22 °C). Small shell-like crystals appeared within 3 days during screening, and were able to reach a dimension of 0.2 × 0.4 × 0.1 mm when growing in drops of larger size (2 μl: 1 μl of protein complex plus 1 μl of reservoir) using hanging-drop vapor diffusion with reservoir (0.2 mM KH_2PO_4, pH 4.8, 20% PEG3350). Crystals were harvested by transferring into reservoir solution containing 12.5% (v/v) (2,3-butane-diol (Sigma), and then flash-cooled in liquid nitrogen.

Structure Determination—Diffraction data were collected from cryo-cooled crystals at SSRF beamline BL17U1 (51). Data were processed using HKL2000 (45). The structure was solved by molecular replacement with Molrep (46) using the crystal structure of SeAcs (PDB code 1PG4) (23) as the search model, and refined with Phenix (47) and Coot (47). cAMP and coenzyme A were built into the model during the late stage of refinement. The final SeAcs-cAMP-CoA model, has been refined to R_work and R_free of 0.169 and 0.207, respectively.

Author Contributions—X. H. conceived the study, performed most in vitro biochemical experiments and data analysis; L. S. purified and crystallized SeAcs-cAMP-CoA, and determined the crystal structure; P. L., Q. W., X. C., J. W., and M. W. assisted in data analysis; W. Z., Y. Z., and G. Z. planned experiments, and wrote the manuscript. All the authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank the staff of beamline BL17U1 at the Shanghai Synchrotron Radiation Facility, Shanghai, People’s Republic of China, for assistance during data collection. We thank Dr. Andrew M. Gillick for pAC10 construction.

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