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S-Adenosylmethionine (SAM) Regulates Antibiotic Biosynthesis in *Streptomyces* spp. in a Mode Independent of Its Role as a Methyl Donor

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Abstract *S*-Adenosylmethionine (SAM) is a ubiquitous biomolecule serving mainly as a methyl donor. Our recent studies revealed that SAM controls antibiotic production in *Streptomyces*. In this study, the functional mode of SAM was studied in *S. coelicolor* and *S. antibioticus* ATCC11891, employing *S*-adenosylhomocysteine (SAH), a methylation reaction product of SAM. Actinorhodin biosynthesis did not require SAM as a methyl donor, whereas SAH enhanced the actinorhodin biosynthesis up to the level comparable to SAM, and the most effective concentration of SAH was higher than that of SAM. In the case of oleandomycin that requires SAM for its biosynthesis, both SAM and SAH at the concentration as low as 100 mM showed comparable efficacy in enhancing the production; SAM at 1 mM concentration additionally stimulated to give a 5-fold enhancement of oleandomycin production. *In vitro* autophosphorylation of protein kinase AfsK was found to be activated by both SAM and SAH, as well as other structurally related compounds. Our studies demonstrate that SAM regulates antibiotic biosynthesis in a mode independent of its role as a methyl donor and suggest that SAM acts directly as an intracellular signaling molecule for *Streptomyces*.

Key words: Actinorhodin, autophosphorylation, oleandomycin, protein kinase, *S*-adenosylhomocysteine, *S*-adenosylmethionine, *Streptomyces coelicolor* A3(2), *Streptomyces antibioticus* ATCC11891

The genus *Streptomyces* has received a great deal of attention for many years because of its utility to produce a large variety of pharmaceuticals, including many clinically important antibiotics [3, 7, 16, 29]. The extensive studies on the regulation of antibiotic biosynthesis in *Streptomyces* have thus been driven by a vision that the basic understanding of the mechanism underlying the developmental processes of *Streptomyces* could improve the yield of industrially important natural products of *Streptomyces* origin [9, 17, 19].

Protein kinases play pivotal roles in the protein phosphorylation-dephosphorylation network for the regulation of various cellular events. In *S. coelicolor*, the eukaryotic-type serine/threonine protein kinase AfsK has been identified as an important regulatory component for initiation of secondary metabolism [24]. AfsK becomes active after sensing some internal or external signals and autophosphorylates its serine/threonine residues to phosphorylate AfsR, the transcriptional factor that binds to the promoter region of *afsS* [11], a transcriptional activator. The binding of AfsR stimulates the transcription of *afsS*, which through a yet unknown pathway leads to the induction of *act*II-ORF4, the pathway specific regulator gene for actinorhodin biosynthesis. The AfsK-AfsR-AfsS system is one of the best-characterized signal transduction cascades in *Streptomyces*, but it is still a mystery how and when afsK is activated.

S-Adenosylmethionine (SAM) is the major methyl donor in both prokaryotes and eukaryotes, participating in a wide range of biochemical processes [4]. The importance of SAM in cellular functions has been extensively studied for the methylation of nucleic acids, proteins, and small molecules [2]. SAM is synthesized from ATP and L-methionine by SAM synthase (SAM-s) (EC 2.5.1.6). Genes encoding SAM-s have been isolated from bacteria, fungi, plants, and animal systems [1, 6, 22, 28]. Functional studies on SAM-s in many species have suggested the essential role of SAM in cell growth and differentiation. For example, the limited expression of the SAM-s gene *metK* in *E. coli* results in decreased genomic methylation and hampers cell division [12, 27]. In fission yeast *Saccharomyces pombe*, if one of the two SAM-s genes, *sam*1, is weakly expressed, the cell exhibits reduced growth, mating, and sporulation [6]. Furthermore, no deletion of *sam*1 can be obtained in yeast, demonstrating a vital role of SAM for the cell survival of *S. pombe* [6].

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During the studies of antibiotic biosynthesis in *Streptomyces*, we found that accumulation of intracellular SAM activates actinorhodin biosynthesis in *S. lividans* [10], in which the production of actinorhodin is normally silent. The precocious production of actinorhodin in *S. lividans* was found to be related to the elevated transcription level of the pathway specific regulatory gene *act*II-ORF4 [10]. Overexpression of the SAM-s gene in *S. coelicolor* was also reported to enhance the transcription level of *act*II-ORF4 [13]. The mechanism underlying the SAM-mediated activation of actinorhodin biosynthesis is yet not clear; however, because of the role of SAM as the major methyl donor for a myriad of reactions in the cell, the effect of SAM was assumed to be originated from its participation in cellular methylation processes [13]. Nevertheless, we hypothesized that SAM may exert control on actinorhodin production as an intracellular factor without being a substrate of the methylation process.

In this study, we evaluated and compared the efficacies of SAM and *S*-adenosylhomocysteine (SAH), the demethylated SAM, related to actinorhodin biosynthesis in *S. coelicolor*. We found that SAH also stimulated actinorhodin biosynthesis in *S. coelicolor* in the same manner as SAM. The promoter activity of *afsS* was also strongly induced by both SAM and SAH, suggesting that both compounds induced a common cellular event(s). Biosynthesis of oleandomycin, which requires SAM-dependent methylation in the biosynthesis process [18], was also promoted by both SAH and SAM. Autophosphorylation of protein kinase AfsK was found to be activated by both SAM and SAH, as well as other adenosine derivatives.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used are shown in Table 1. *S. coelicolor* A3(2) was cultured on R2YE liquid medium [8]. YEME medium [8] was used for seed culture medium of *S. antibioticus* ATCC11891. For oleandomycin production, the basal medium was used as previously described [26] with supplementation of 55 mM fructose as carbon source and 15 mM aspartic acid as nitrogen source. SAM or SAH was added 12 h after inoculation in the lag phase of cell growth, and strains were cultured at 28°C for 5 days and samples were taken for determination of actinorhodin production [8] and antibacterial activities (see below).

Determination of Antibacterial Activities of Oleandomycin

Antibacterial activities were determined by the agar disc diffusion method [26]. The culture broth of *S. antibioticus* ATCC11891 was applied to 8 mm paper discs on 1% nutrient agar (Sigma) spread with an overnight culture of *M. luteus* as the test organism [26]. To compare the antibacterial activities of oleandomycin in SAM and SAH treated samples, a serial-dilution method was applied as previously described [5].

Overexpression and Purification of Thioredoxin (TRX) Fusion AfsK

An overnight culture (3 ml) of *E. coli* BL21 harboring pTRX-AfsK [25] was inoculated in 150 ml of LB broth. The culture was maintained at 37°C until OD_{600} attained 0.5 to 0.7 before adding isopropyl-β-D-thiogalactopyranoside (IPTG) at the final concentration of 1 mM and was further incubated at 37°C for 4 h. TRX-AfsK was obtained as an insoluble form, and therefore, was solubilized with 6 M urea, purified, and refolded by FPLC according to the method previously described [23].

In Vitro Autophosphorylation Assay

In vitro autophosphorylation assay of AfsK was performed using the method described by Umeyama and Horinouchi [25] with some modifications. The standard phosphorylation reaction mixture contained 10 mM Tris-HCl (pH 7.2), 10 mM $MnCl_2$, 10 mM $MgCl_2$, 2 μCi (74 kBq) of [γ-^{32}P]

Table 1. Bacterial strains and plasmids used in this study.

Strains	Characteristics	Reference
S. coelicolor A3(2)	For study of actinorhodin production	[11]
S. antibioticus ATCC11891	Oleandomycin producer	[26]
Micrococcus luteus ATCC10240	Antibacterial activity test strain for oleandomycin	[26]
E. coli BL21 (DE3)	For overexpression of TRX-AfsK	[25]
E. coli DH5α	To preserve and propagate pTRX-AfsK	[10]
E. coli ET12567	For isolation of demethylation of DNA for transformation of *S. coelicolor* A3(2)	[14]
Plasmids		
pTRX-AfsK	*afsK* coding sequence inserted in EcoRI and HindIII sites of pET32a(+) for production of TRX-AfsK	[25]
pJWS 1003	*afsS* promoter region fused with *xylE* in pIJ4083 for transcription analysis of *afsS*	[8, 20]

ATP, and 4 μg of AfsK. The assay was conducted in a 20 μl reaction mixture. The compounds used in the assay included adenosine 3',5'-cyclic monophosphate; N^6, 2'-*O*-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt; 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt; 8-Azidoadenosine 3',5'-cyclic monophosphate; Cytosine β-D-arabinofuranoside hydrochloride; Cytidine 5'-diphosphoethanol-amine sodium salt; 2'-3'-5'-Tri-*O*-acetylcytidine hydrochloride; SAH and SAM. The reaction was started by adding [γ-^{32}P] ATP and incubated at 30°C for 5 min. The reaction was subsequently quenched by adding 5-fold volume of SDS sample buffer, subjected to a 12% SDS-PAGE, and visualized with a Fuji FLA-3000 image analyzer.

Promoter-Probe Assay to Access Transcription of *afsS*

The promoter region of *afsS* was amplified by PCR using the forward primer of 5'-TAT GAATTC ACCCGGCCGT-CGACCGGCGG-3' (EcoRI site is boxed) and the reverse primer of 5'-TAT GGATCC GGACTTCGCTCCTCATGG-GTCGTGAC-3' (BamHI site is boxed) from genomic DNA of *S. coelicolor*. After confirmation of the nucleotide sequence, the PCR product (224 bp) was digested by EcoRI and BamHI and ligated into pIJ4083. The ligation mixture conceivably contained pJWS1003 (pIJ4083/*afsS*p*-*xylE*) and was directly used to transform *S. coelicolor* according to the method of OH and Chater [14]. The transformant containing pJWS1003 was cultured in R2YE liquid medium containing 5 μg/ml thiostrepton. SAM and SAH were added into the culture medium at the final concentration of 10 μM at 12 h post-inoculation. Catechol 2,3-dioxygenase activity was quantified as previously described [8, 20]. The specific activity was calculated as follows [8]:

$$\text{mU catechol dioxygenase [nmol/min]} = \frac{30.03 \times \Delta A_{375}}{\text{time[min]}}$$

RESULTS

Both SAM and SAH Increased Actinohordin Biosynthesis in *S. coelicolor* A3(2)

In the previous studies, we found that exogenous feeding of SAM at the concentration as low as 2 μM stimulated actinorhodin production in *S. coelicolor* A3(2). In this study, in order to test whether SAM-dependent methylation was involved in the actinorhodin biosynthesis, we employed SAH, the demethylated product of SAM, for its effect on actinorhodin biosynthesis. As shown in Fig. 1, SAH displayed stimulatory effect on actinorhodin production when it was added at the concentrations ranging from 10 to 100 μM. Although its optimum concentration was higher than SAM, SAH at 100 mM stimulated actinorhodin production more than 2-fold. This result suggested that the

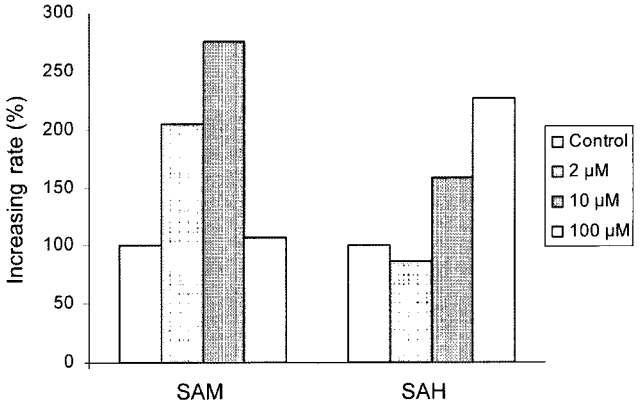

Fig. 1. Effects of SAM and SAH on actinorhodin biosynthesis in *S. coelicolor* A3(2).

Actinorhodin production was assessed in *S. coelicolor* A3(2) cultured in R2YE liquid medium. SAM and SAH were added at concentrations of 2, 10, and 100 μM at 12 h post-inoculation. Samples were taken after 5-day culture at 28°C. The production level of non-treated culture was set as 100% to evaluate the increase by SAM and SAH.

SAM-activation of actinorhodin biosynthesis is not related to its role as a methyl donor.

Autophosphorylation of AfsK was Activated by SAM and SAH

We further studied the molecular mechanism of SAM-mediated activation of actinorhodin biosynthesis by examining a series of mutants that are defective in actinorhodin biosynthesis. SAM activation of actinorhodin biosynthesis was found to be greatly impaired in the *afsK* disruptant (the *afsK* disruptant used in this study was a generous gift from Dr. Horinouchi Sueharu, Tokyo University), suggesting the important role of the *afsK* locus in delivering the SAM signal. Subsequent studies showed that SAM stimulated *in vitro* autophosphorylation of AfsK at the concentration as low as 20 nM (Zhao *et al.*, unpublished data). In this study, the effect of SAH on the *in vitro* autophosphorylation was also studied. As shown in Fig. 2, SAH stimulated *in vitro* autophosphorylation of AfsK at the concentration of 20 nM and 200 nM, and the stimulatory effect was comparable to

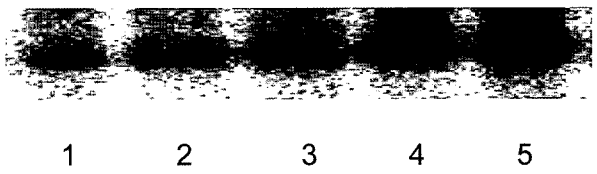

Fig. 2. *In vitro* autophosphorylation of AfsK by SAM and SAH.

Purified TRX-AfsK was incubated with [γ-^{32}P] ATP. SAM and SAH were added to the reaction mixture at the concentrations of 20 nM and 200 nM. The reaction was performed at 30°C for 5 min and quenched by adding 5-fold excess of SDS sample buffer, and then subjected to SDS-PAGE and analyzed by autoradiography. Lane 1, control; lanes 2 and 3, SAM at 20 nM and 200 nM, respectively; lanes 4 and 5, SAH at 20 nM and 200 nM, respectively.

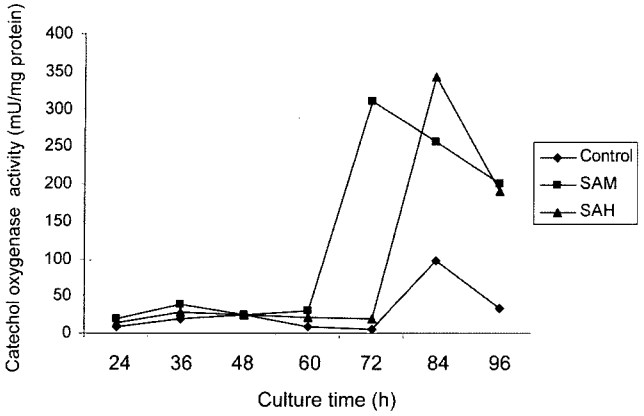

Fig. 3. Regulation of promoter activiy of *afsS* by SAM and SAH.

Promoter activity of *afsS* was detected by quantitative catechol dioxygenase assays. Cells were cultured in R2YE liquid medium. SAM and SAH at the final concentrations of 10 μM and 100 μM were added 12 h after inoculation. Samples were taken at 12-h intervals. The time points indicate the culture time. Symbols: ◆, control; ■, 10 μM SAM; ▲, 10 μM SAH. Independent experiments were repeated three times, and reproducible results were obtained. The results here show a representative experiment.

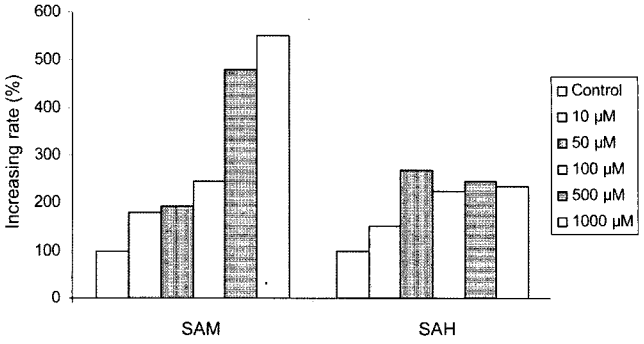

Fig. 4. Effects of SAM and SAH on oleanodomycin biosynthesis.

The YEME culture of *S. antibioticus* ATCC11891 was used to inoculate the production medium [15], and SAM and SAH were supplied at concentrations of 10 μM, 50 μM, 100 μM, 500 μM, and 1,000 μM, 12 h after starting the main culture.

that of SAM. This result further substantiated that the effect of SAM is independent of its typical role in methylation processes.

Promoter Activity of *afsS* was Stimulated by SAM and SAH

In the AfsK-AfsR-AfsS signal transduction system, the activation of protein kinase AfsK by autophosphorylation results in the enhancement of DNA binding activity of AfsR, which then leads to the induction of *afsS*. Therefore, the promoter activity of *afsS* was chosen to further test whether SAM and SAH have any common effect on the AfsK-AfsR-AfsS signal transduction system. Based on the effects of SAM and SAH on actinorhodin biosynthesis in *S. coelicolor* A3(2), the concentrations of SAM and SAH chosen were 10 μM and 100 μM, respectively, in the following experiment. As shown in Fig. 3, when added at the final concentration of 100 μM, SAH stimulated the promoter activity of *afsS* in a manner similar to SAM, although SAM induced the promoter activity of *afsS* 12 h earlier than SAH. In both cases, the dramatic induction of *afsS* promoter activity was accompanied with initiation of actinorhodin biosynthesis.

Oleandomycin Biosynthesis was Enhanced by SAM and SAH

Oleandomycin biosynthesis requires SAM as the methyl donor. The effect of SAM and SAH on oleandomycin production was investigated to see whether there was any difference in the stimulatory effects between SAM-independent and SAM-dependent biosynthetic pathways, and also whether the stimulation mechanism of oleandomycin by SAM is different from that of actinorhodin biosynthesis. We found that SAM activated oleandomycin biosynthesis in a dose-dependent manner at the concentrations range from 10 to 1,000 μM (Fig. 4). Although SAH is a potent inhibitor for the SAM-dependent methylation process, SAH also showed the stimulatory effect at the concentration range of 10 to 1,000 μM with optimum concentration at 50 μM. At the concentration as high as 500 μM, the stimulatory effect of SAH was less significant than that of SAM (Fig. 4).

Activation of Autophosphorylation of AfsK by Structually Related Compounds of SAM

Since both SAM and SAH contain an adenosine group in their structures, we asked a question of whether other adenosine derivative compounds also activate the autophosphorylation of AfsK. Therefore, we tested several adenosine-containing compounds for their effects, using the *in vitro* autophosphorylation assay. Interestingly, of the seven compounds examined, four showed stimulatory effects on the *in vitro* autophosphorylation of AfsK. Furthermore,

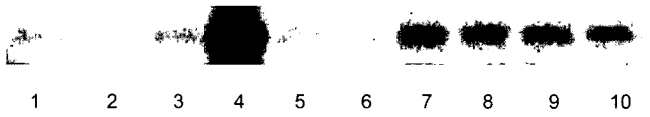

Fig. 5. Activation of autophosphorylation of AfsK by adenosine derivative compounds.

In vitro autophosphorylation of AfsK was performed as described in Materials and Methods. 1, Non-treated control; 2, adenosine 3',5'-cyclic monophosphate; 3, N^6, 2'-*O*-Dibutyryladenosine; 4, 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt; 5, 8-Azidoadenosine 3',5'-cyclic monophosphate; 6, Cytosine β-D-arabinofuranoside hydrochloride; 7, Cytidine 5'-diphosphoethanol-amine sodium salt; 8, 2'-3'-5'-Tri-*O*-acetylcytidine hydrochloride; 9, SAH; 10, SAM. The final concentration of the compounds in the reaction mixture was 200 nM.

one compound, 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate, showed the highest activation effect on autophosphorylation of AfsK (Fig. 5).

DISCUSSION

In this study, the mechanism by which SAM stimulates actinorhodin and oleandomycin biosynthesis was shown to be independent of its role as a methyl donor. SAH, the demethylated product of SAM, showed similar stimulatory effects on acinorhodin (Fig. 1) and oleandomycin biosynthesis (Fig. 4). When *in vitro* autophosphorylation of AfsK was tested, SAH also promoted *in vitro* autophosphorylation of AfsK as SAM did (Fig. 2). Furthermore, both SAM and SAH induced the transcription of *afsS* (Fig. 3), the product of which can stimulate actinorhodin biosynthesis by enhancing the transcription of the pathway specific regulatory gene *act*II-ORF4. The stimulatory effect of SAH on autophosphorylation of AfsK seems to be intriguing; SAH displayed a stronger effect on the activation of AfsK autophosphorylation than SAM (Fig. 2); however, the effective concentration of SAH was higher than SAM in the activation of actinorhodin production. We speculate that the exogenously added SAH was rapidly removed by SAH dehydrolase, since SAH is a potent inhibitor for cellular methylation processes and its accumulation is toxic to cells [21]; therefore, it may be eliminated in order to remove its negative effect on cellular metabolism. In the case of oleandomycin biosynthesis, SAM enhanced oleandomycin biosynthesis more strongly (about 5-fold) than SAH at the high concentration (100 μM to 1 mM), thereby providing methyl groups to oleandomycin biosynthetic enzymes. However, the fact that SAH also exhibited a moderate stimulatory effect (about 2-fold), when applied at relatively low concentration (10–100 μM), indicates that SAM also exerted control on oleandomycin biosynthesis in a mode independent of its role as a methyl donor. The finding that both SAM and SAH can activate the *in vitro* autophosphorylation of AfsK further supports this notion; that is, either DNA, RNA, protein methylation, or some other SAM-dependent methylation process is unlikely to be involved for the effect of SAM on antibiotic biosynthesis. Our previous proteomic studies have revealed that exogenous feeding of SAM induced expression levels of some oligopeptide transporters, including BldK, which is a regulatory component of the *bld* cascade for the cell differentiation process in *S. coelicolor* [15]. We propose that the action of SAM as an intracellular factor to modulate antibiotic biosynthesis involves multiple regulatory components and is not confined to its well-known role as a methyl donor. Further studies are needed to explore the molecular events underlying the activation of biosynthesis of actinorhodin and oleandomycin.

Acknowledgments

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