Allosteric Regulation of a Protein Acetyltransferase in *Micromonospora aurantiaca* by the Amino Acids Cysteine and Arginine*

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ACT domains (amino acid-binding domains) are linked to a wide range of metabolic enzymes that are regulated by amino acid concentration. Seventy proteins with ACT-GCN5-related N-acetyltransferase (GNAT) domain organization were found in actinomycetes. In this study, we investigate the ACT-containing GNAT acetyltransferase, Micau_1670 (MaKat), from *Micromonospora aurantiaca* ATCC 27029. Arginine and cysteine were identified as ligands by monitoring the conformational changes that occur upon amino acids binding to the ACT domain in the MaKat protein using FRET assay. It was found that MaKat is an amino acid-regulated protein acetyltransferase, whereas arginine and cysteine stimulated the activity of MaKat with regard to acetylation of acetyl-CoA synthetase (Micau_0428). Our research reveals the biochemical characterization of a protein acetyltransferase that contains a fusion of a GNAT domain with an ACT domain and provides a novel signaling pathway for regulating cellular protein acetylation. These findings indicate that acetylation of proteins and acetyltransferase activity may be tightly linked to cellular concentrations of some amino acids in actinomycetes.

The dynamic and reversible $N^\alpha$-lysine acetylation of proteins is now recognized as a ubiquitous and conserved post-translational modification from prokaryotes to eukaryotes. Recent studies have identified over 3,000 acetylated proteins, ranging from transcriptional factors and ribosomal proteins to many metabolic enzymes that are related to glycolysis, gluconeogenesis, the TCA cycles, and fatty acid, as well as nitrogen metabolism. This kind of post-translational modification has been emerging as an important metabolic regulatory mechanism in bacteria since the discovery of acetylation of the *Salmonella enterica* acetyl-CoA synthetase in 2002 (1). In the last decade, lysine acetylation of proteins has been reported in other microorganisms, including *Escherichia coli*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* (2, 3).

All acetylation/deacetylation systems in prokaryotes consist of protein acetyltransferases and deacetylases. Acetyltransferases catalyze the transfer of the acetyl group from the acetyl-coenzyme A (Ac-CoA) donor to a primary amine of small molecules and proteins that are involved in a wide variety of cellular processes. Protein acetyltransferases specifically control acetylation of different proteins under various physiological conditions. Protein deacetylases play a role in globally removing the acetyl group from acetylated proteins that respond to changes of cellular energy status via promptly sensing the level of intracellular NAD$^+$:NADH ratio.

Despite our growing understanding of reversible lysine acetylation, it remains unclear how many protein acetyltransferases and deacetylases are involved in this post-translational modification, how signals regulate the activity of the acetylation/deacetylation system, and how a limited number of acetyltransferases and deacetylases control the acetylation of so many metabolic enzymes. It is likely that the protein acetyltransferase enzymes are carefully regulated at the transcriptional and post-translational levels in response to changes of the intracellular signals that control the acetylation of specific proteins, which in turn mold the metabolic network.

This hypothesis is supported by previous research. In the enteric bacteria *E. coli*, the transcription of protein acetyltransferase PatZ is controlled at the transcriptional level in response to the intracellular cAMP signal. The catabolite activator protein (cAMP) complex binds to two sites in the *patZ* promoter.

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The abbreviations used are: Ac-CoA, acetyl-coenzyme A; GNAT, GCN5-related N-acetyltransferase; CFP, cyan fluorescence protein.
and induces the expression of genes that increase the level of acetylated proteins (4). The activity of protein acetyltransferases is also controlled by allosteric effects. In *M. tuberculosis* and *Mycobacterium smegmatis*, cAMP directly activates the protein acetyltransferases MsKat (Rv0998) and MsKat (MSMEG_5458) by binding to a cyclic nucleotide-binding domain that is fused to the N terminus of the catalytic GNAT domain (5, 6).

It was previously known that the protein acetyltransferases have two different domain organizations: multidomain and single-domain. The multidomain acetyltransferases can be regulated at the transcriptional and post-translational levels; however, the single-domain acetyltransferases are generally controlled at the transcriptional level. For example, the regulation of expression of the *B. subtilis* acuA gene is under the control of the global regulatory protein CcpA, which is affected by the quality of the carbon source that is available to the cell (7).

The ACT domain is recognized as a structurally conserved motif that consists of four β strands and two α helices that are arranged in a βαββαβ fold. The amino acid-binding domains are mostly found in a variety of proteins that are directly or indirectly involved in amino acid and purine metabolism (8). The name “ACT” originates from three of the proteins that contain the ACT domain: aspartate kinase, chorismate mutase, and TyrA (prephenate dehydrogenase). In the Pfam database (v.27.0), 20,885 sequences containing ACT domains (PF01842) with 130 domain architectures were recently collected. In the InterPro database (v.47), a total of 107,722 ACT domain-containing proteins (IPR002912) were matched, most of which (95%) were discovered in bacteria. This domain is fused to a variety of different functional domains, thus producing 289 domain organizations. According to the hypothesis proposed by Aravind and Koonin (9), the ACT domain, as the conserved, evolutionarily mobile module, was suggested to be a provider of allosteric effect. The ACT domain was independently fused to a variety of enzymes, conferring ligand-induced allosteric regulation to these enzymes. The majority of these enzymes have not been studied in detail.

In this study, we found that 70 proteins contain the ACT-GNAT domain organization. These proteins have all been discovered in actinomycetales, indicating that this domain organization is unique to actinomycetales. It is perhaps expected that the binding of amino acids allosterically regulates the activity of the acetyltransferases with ACT-GNAT domain organization.

We further investigated the first ACT-containing GCN5-related acetyltransferase, Micau_1670, from *Micromonospora aurantiaca* ATCC 27029. *Micromonospora* species are ubiquitous in soils, sediments, and aquatic environments and in the rumen of cattle and the guts of termites; moreover, they have been recognized as important sources of antibiotics (e.g. aminoglycoside antibiotics, gentamicin, netaminic, lomavitcins A and B, tetrocarcin A, and the LL-E33288 complex) (10). Arginine and cysteine were identified as ligands by monitoring the conformational changes that occur upon amino acids binding to the ACT domain in the Micau_1670 protein using a FRET biosensor. We observed that the two amino acids stimulated the activity of protein lysine acetyltransferase Micau_1670 on acetylation of acetyl-CoA synthetase (Micau_0428), MaAcS. Furthermore, the important residues implicated in binding of Cys or Arg in the ACT domain of Micau_1670 were investigated. These findings indicate that acetylation of proteins and acetyltransferase activity may be tightly linked to cellular concentrations of some amino acids in actinomycetales.

**EXPERIMENTAL PROCEDURES**

**Cloning, Overexpression, and Purification of Proteins**—The Micau_1670 and Micau_0428 genes were amplified by PCR from the genomic DNA of *M. aurantiaca* ATCC 27029 using two primer pairs (5′-TAAGAATTCATGAGCGAGGCATTGGCGCAACT/TAAAGCTTTCAGTCCTCGGACTTCCCG-3′ and 5′-TAAGAATTCATGGCGCTCTGCGGGAATC/TAAAGCTTACTGACCCGTGCG-3′). After restriction digestion with HindIII and EcoRI, the genes coding for Micau_1670 and Micau_0428 were cloned into pET-28a to generate PET28a-Micau_1670 and PET28a-Micau_0428. The clones were confirmed by sequencing. The proteins were expressed using the *E. coli* BL21 (DE3) strain. A single colony was selected to begin a 3-ml overnight culture, which was then used for inoculation in 50 ml of Luria Bertani medium that was supplemented with 1% kanamycin. The cells were grown at 37 °C and then induced with 0.7 mM isopropyl-β-D-thiogalactoside at 20 °C overnight. Cells were harvested by centrifugation and resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and incubated on ice for 15 min. The cells were sonicated in PBS buffer, and cell debris was removed by centrifugation at 8,000 rpm for 20 min. The supernatant was purified with a nickel-nitrilotriacetic acid-agarose column (Merck), and bound protein was pre-equilibrated with the binding buffer. After discarding the flow through, the column was washed with 10 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) to remove the hybrid proteins, and bound proteins were eluted using a linear gradient from 20 to 250 mM imidazole in 50 mM NaH2PO4 and 300 mM NaCl, pH 8.0. The fractions were analyzed by SDS-PAGE, and those containing the desired protein were pooled and dialyzed against buffer P (37 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 5% glycerol, pH 7.9). The His tag of Micau_0428 was digested by thrombin (4°C overnight). The protein was then concentrated using an Amicon Ultra-4 30,000 cutoff centrifugal device (Millipore, Billerica, MA). Protein concentration was monitored by the BCA method using buffer P as the control, and the amount of protein after concentration was also analyzed by SDS-PAGE.

**Generating the Constructs Used for FRET Analysis**—To construct FRET biosensors, the genes coding for Micau_1670 and Micau_1670(AACT) were all cloned into PET28a-YFP-CFP that was digested with EcoRI and HindIII to generate recombinant plasmid PET28a-YFP-Micau_1670-CFP and PET28a-YFP-Micau_1670(AACT)-CFP. Micau_1670(AACT) represented the region that encompasses residues 151–362 of Micau_1670. The constructs were confirmed by DNA sequencing. The proteins are expressed in the same way as described above. The FRET biosensors were constructed according to the procedures that was previously reported by our laboratory (11).
Site-directed Mutagenesis and Purification of Micau_0428 and Micau_1670—The D11A, L16A, and D45A mutations were separately introduced into the recombinant plasmid PET28a-Micau_1670, as well as PET28a-YFP-Micau_1670-CFP, using a QuikChange mutagenesis kit (TransGen, Beijing, China). The K619Q mutation for PET28a-Micau_0428 was introduced in the same way. All mutations were confirmed by DNA sequencing. The expression and purification of the mutants were performed following the same procedure described above.

In Vitro Protein Acetylation Assays—The protein concentrations of the samples were determined using the BCA protein assay kit (Pierce) with BSA as the standard. Assays were carried out in a 100-μl total reaction volume. MaAcs (Micau_0428) (1.5 μM) was incubated in the presence of Micau_1670 (0.2 μM), acetyl-CoA (60 μM), and Cys or Arg (1 mM) at 37 °C for 60 min. The reaction contained 0.05 M HEPES buffer (pH 7.5). After reaction, the acetylated MaAcs was isolated from the reaction mixture by gel filtration (adding nickel beads to the reaction and the blot was washed with TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) three times (once every 10 min), and bound antibody was detected by enhanced chemiluminescence.

RESULTS

MaKat Containing ACT-GNAT Domain Organization Is a Protein Acetyltransferase—In the InterPro database (v.47), 70 proteins containing the domain organization of ACT-GNAT were discovered in actinomycetales. The N-terminal ACT domain is fused to a catalytic GNAT domain (Fig. 1A). A phylogenetic analysis of the full-length sequences of all ACT-GNAT proteins is shown in Fig. 2. Additionally, multiple alignment of eight proteins was performed using ClustalX (Fig. 1B) and revealed that these proteins contain the conserved βαββαββ fold of the ACT domain. In particular, β1, α1, β2, and β3, as the core of the ACT domain, are the most highly conserved motifs that are important for amino acid recognition and binding. Four ACT-GNAT proteins were found in Micromonospora (Micau_1670, MCAG_04541, ML5_1930, and MILUP08_41772). The high conservation among ACT domains of Micromonospora proteins indicates that they would have similar ligand binding. In this work, we studied the acetyltransferase Micau_1670 as a representative model of the ACT-GNAT enzymes.

The ACT domain of Micau_1670 was also compared with the representative ACT domains, including those whose structures have been determined, such as AHAS/ILVN, ASPK, PGDH, Glyc, PHE, PURU, GLND, and SPOT (Fig. 1C). The multiple alignments revealed that the ACT domains have low conservation in the sequence. However, all have the conserved fold of secondary structures, which are sequentially labeled β1, α1, β2, β3, α2, and β4. The crystal structure studies of ACT domains with ligands illustrated that the amino acids tend to bind at the interfaces between ACT domains, and there appears to be a correlation of the ligand binding sites with specific glycine residues that is located in loops between the first strand, β1, and the first helix, α1 (14). As shown in Fig. 1B, ACT-GNAT acetyltransferases indeed contain a conserved glycine residue (Gly-14 of Micau_1670) at this location.

In prokaryotes, some acetyltransferases for protein acetylation have been investigated, including SePat in S. enterica (15), EcPatZ in E. coli (4), RpPat in Rhodopsedomonas palustris (16), MtKat in M. tuberculosis, MsKat in M. smegmatis (6), and SLPatA in Streptomyces liydivans (17). These protein acetyltransferases containing a GNAT domain can acetylate acetyl-CoA synthetases. Comparison of the amino acid sequence of the GNAT domain in Micau_1670 (named for MaKat) with other representative GNAT domains in various proteins is shown in Fig. 3A.

The results of this study reveal that MaKat contains some conserved pivotal residues in motif A of the GNAT domain.
Motif A, as the core of the GNAT domain, is the most highly conserved motif and generally has an (R/Q)XGX(G/A) sequence that is important for acetyl-CoA recognition and binding. The Q_{269}G,G sequence is indeed found in motif A of MaKat. Interestingly, GNAT domain contains a nearly invariant Glu residue (Glu-269 in MaKat, Glu-235 in MtKat, or Glu-234 in MsKat) in the cleft between motifs A and D. This most conserved amino acid provides an active site that serves as a key base to deprotonate the lysine residue of the acetyl acceptor. Furthermore, a phylogenetic analysis with the GNAT domain sequence of these acetyltransferases showed MaKat clusters with MsKat, MtKat, and SPlat (Fig. 3B), thus indicating that MaKat is able to acetylate acetyl-CoA synthetase in M. aurantiaca as MsKat, MtKat, and SPlat that occur in other actinomycete species.

Moreover, we confirmed that MaKat directly acetylates acetyl-CoA synthetase MaAcs (Micau_0428) in vitro by incubating purified MaKat with Ac-CoA and recombinant Micau_0428 of M. aurantiaca. As shown in Fig. 3C, this result strongly indicates that MaKat has protein lysine acetyltransferase activity for acetylation of acetyl-CoA synthetase. To test the effect of acetylation on enzyme activity, MaAcs was incubated with MaKat in the presence or absence of acetyl-CoA for 2 h. In the presence of both acetyl-CoA and MaKat, MaAcs activity was reduced, indicating that MaKat lysine acetylation effectively decreases MaAcs activity (Fig. 3D). Time-dependent inactivation of MaAcs by MaKat acetylation was also investigated. In Fig. 3E, it is observed that MaAcs gradually lost its activity during acetylation by the MaKat enzyme.

Conformational Changes of MaKat Induced by Cys and Arg Are Monitored by FRET—It was known that the ACT domain is involved in binding of specific amino acids and potentially provides allosteric regulation via transmission of finely tuned conformational changes, leading to the change of the activity of the catalytic domain. The ACT-GNAT domain organization of MaKat strongly indicates that MaKat has protein acetyltransferase activity with allosteric regulation altering its activity by binding to one or several specific amino acids. First, we tried to utilize genetically encoded FRET-based biosensors to screen the putative amino acid ligands that can change the configuration of MaKat among all 20 L-amino acids that exist in nature. The intact MaKat protein from M. aurantiaca was used as the binding unit, and it was sandwiched directly with enhanced CFP and YFP. Conformational change in the MaKat protein is likely to lead to a change in FRET efficiency between CFP and YFP in this sandwich configuration. Two FRET-based biosensors were constructed by flanking the ACT-GNAT domain (intact MaKat protein) and GNAT domain (MaKat protein without ACT domain, as a control) with CFP and YFP to investigate the conformational change of the ACT domain in response to amino acids (Fig. 4A). Two biosensors were excited at 440 nm, and their emission spectra showed two peaks at 478 and 528 nm, which correspond to CFP and YFP. As shown in Fig. 4B and 4C, the addition of Arg, Cys, and Asn resulted in apparent changes of the 478/528 ratios of the CFP and YFP emission intensities between two biosensors. The changes in the 478/528 ratio induced by amino acids indicate that conformation changes that result from the ligand-binding ACT domain are translated into a change in FRET efficiency.

To further investigate whether amino acids regulate the activity of MaKat, Western blotting was conducted to detect the acetylation level of MaAcs with anti-acetyl-lysine antibody in the presence or absence of amino acids. As shown in Fig. 4D, Arg and Cys were able to increase the rate of acetylation of MaKat, which is consistent with the results obtained from the FRET method. However, no similar effect was observed for asparagine.
All proteins containing the domain organization of ACT-GNAT were discovered in actinomycetales using the InterPro database (v.46). A phylogenetic analysis with the full-length sequences of all ACT-GNAT proteins is presented.  

FIGURE 2. Phylogenetic analysis. All proteins containing the domain organization of ACT-GNAT were discovered in actinomycetales using the InterPro database (v.46). A phylogenetic analysis with the full-length sequences of all ACT-GNAT proteins is presented.
MaKat Could Function as an Amino Acid-regulated Protein Acetyltransferase—Bioinformatics analysis and Western blotting assay demonstrated that MaKat is a protein lysine acetyltransferase and can acetylate the acetyl-CoA synthetase MaAcs. As seen in Fig. 6A, acetylation of MaAcs was observed when incubated with MaKat and acetyl-CoA, and acetylation increased in the presence of Cys or Arg. The Cys/Arg-binding ACT domain fused to the acetyltransferase domain in MaKat, and an increase in acetylation level of MaAcs in the presence of Cys or Arg indicates that the ACT domain may allosterically regulate the activity of the GNAT domain, and MaKat could function as an amino acid-regulated protein acetyltransferase. Furthermore, in the presence of Cys or Arg, MaKat activity exhibits a ligand concentration-dependent increase for protein acetylation (Fig. 6B). The concentrations of amino acid ligands that would saturate acetylation of protein were in the submicromolar range, which is comparable with the EC_{50} or IC_{50} concentrations that were obtained from the FRET assay.

The previously reported bacterial acetyltransferases exhibit specificity of the lysine site for protein acetylation. There is a proposed acetylation motif (PXXXXGK) that is found in AMP-forming acyl-CoA synthetases (14). GNAT acetyltransferases recognize this motif and acetylate the last lysine residue of PXXXXGK, such as Lys-609 of SeAcs from S. enterica, Lys-606 of RpAcs from R. palustris, Lys-617 of MtAcs from M. tuberculosis, and Lys-549 of BsAcs from B. subtilis (Fig. 7A).
To confirm the site of acetylation, we created substitution mutations at the Lys-619 position to generate K619Q variant of MaAcs. Glutamine abolishes the positive charge and serves as a structural mimic for acetyl-lysine. MaAcsK619Q and MaAcsWT were incubated with the MaKat enzyme and Ac-CoA. Western blotting was conducted to detect the acetylation level of two MaAcs enzymes with anti-acetyl-lysine antibody. As shown in Fig. 7, acetylation of only MaAcsWT was observed. No acetylation was observed at MaAcsK619Q, indicating that MaKat modified only conserved the lysine residue Lys-619 of the active site in MaAcs.

To test the effect of Cys/Arg on the MaKat enzyme activity, we determined the initial rate of acetylation of MaAcs in the absence and presence of Cys/Arg. The acetylation reaction of MaKat was monitored by a coupled enzymatic assay where the amount of CoA that is liberated following acetylation is measured by formation of reduced NADH from NAD⁺ by pyruvate dehydrogenase (12, 13). As shown in Fig. 8, MaKat could acetylate MaAcs in the absence of amino acid ligand (4.6 ± 0.9 nmol of NADH formed/min/ml), and the rate of acetylation was increased 208 and 150% in the presence of Cys (9.6 ± 1.5 nmol of NADH formed/min/ml) and Arg (6.9 ± 1.2 nmol of NADH formed/min/ml), respectively.

We further determined the kinetic parameters of the MaKat catalyzed acetylation reaction of MaAcs in the absence and presence of Cys/Arg. We used a coupled enzymatic assay to

FIGURE 4. Screening of MaKat ligands. A, the whole sequence of Micau_1670 (amino acids 1–361) and the truncated sequence of Micau_1670 (amino acids 150–361) were sandwiched in the FRET pair YFP/CFP. B, the basal FRET ratio was normalized, and the ratio was calculated to evaluate the specific amino acid response to two sensors. C, comparison of the emission ratio between two sensors in the presence of Cys and Arg. D, the effect of amino acids on enzyme activity of MaKat. The reaction involves MaAcs (1.5 μM), MaKat (0.2 μM), acetyl-CoA (60 μM), and one of the 20 amino acids. Basal mean was observed by adding PBS buffer.
continuously monitor the acetylation reaction. The resulting data were fitted using the Michaelis-Menten kinetics model. The results are shown in Table 1. The $K_m$ and $k_{cat}$ values of $Ma$Kat for Ac-CoA are $4.2 \mu M$ and $0.02 \text{s}^{-1}$, respectively. Similarly, the $K_m$ and $k_{cat}$ values for $Ma$Acs are $21.6 \mu M$ and $0.03 \text{s}^{-1}$. $Ma$Kat exhibited a $K_m$ value of $4.2 \mu M$ for Ac-CoA substrate, which was slightly higher compared with that of yeast GCN5 HAT ($K_m$ of $2.5 \mu M$) (18), although it was 5-fold lower than that of $B. subtilis$ AcuA ($K_m$ of $22 \mu M$) (19). The $k_{cat}$ value of $Ma$Kat for Ac-CoA was only $0.02 \text{s}^{-1}$, which is 85-fold lower than the $k_{cat}$ value ($1.7 \text{s}^{-1}$) of yeast GCN5 HAT and 15-fold lower than the $k_{cat}$ value ($0.3 \text{s}^{-1}$) of $B. subtilis$ AcuA. As seen in Table 1, Cys or Arg was able to increase the $k_{cat}$ value of $Ma$Kat for Ac-CoA and $Ma$Acs by 2–2.5-fold and showed no significant effect on $K_m$ values.

Identification of Residues in the ACT Domain That Are Associated with Cys and Arg Binding—The ACT domain is an amino acid-binding domain, which is involved in the allosteric regulation of prokaryotic amino acid metabolism. In a recent study, it was found that most of the ACT domains bind to only one amino acid. However, some ACT domains can bind to more than one amino acid. In Arabidopsis thaliana, AK-HSDH I and AK-HSDH II are inhibited by Thr (AK-HSDH II is also inhibited by Leu) and activated by five amino acids (Ala, Cys, Ile, Ser, and Val) (20–22). AK1 is synergistically inhibited by lysine and $S$-adenosylmethionine (23, 24). In E. coli, formyl FH4 hydrolase...
is positively regulated by Met and negatively regulated by Gly (25).

The first ACT domain determined was *E. coli* D-3-phosphoglycerate dehydrogenase, which folds with a ferredoxin-like topology, and the most conserved portion of the ACT domain is the region at the interface between the first strand (1) and the first helix (1) (9). According to previous studies (21, 22, 26), the important residues may be found at the end of the first strand (1) of the conserved fold. Comparison of the conserved amino acid residues among the ACT domains and prediction of the secondary structure (Fig. 1B) showed that *Ma* Kat also contained the conserved topology. It has been suggested that the hydrogen-bonding network may play an important role in the binding of specific amino acids. To investigate the important residues implicated in binding of Cys or Arg in *Ma* Kat, the structure of the ACT domain of *Ma* Kat was modeled using the ACT domain of formyltetrahydrofolate hydrolase (PURU) from *Thermus thermophilus* as a model (RCSB identifier rcsb095976 and Protein Data Bank code 3W7B). As shown in Fig. 9 (A and B), the residues implicated in hydrogen bonding with Cys (Asp-11, Arg-12, Gly-14, Tyr-15, Leu-16, and Asp-45) and Arg (Asp-11, Leu-16, Ala-40, and Asp-45) are mostly located in the first strand (1) and the first helix (1) of the *Ma* Kat ACT domain. Gly-14 is the most conserved residue in the loop and has an important role in maintaining the stabilization of the topology.

To investigate the effect of residues on binding of amino acids, we mutated residues Asp-11, Leu-16, and Asp-45 to Ala. Circular dichroism assays showed that these mutants do not perturb the ACT structure of *Ma* Kat (data not shown). The conformational changes of the wild type and mutant proteins induced by Cys and Arg were monitored by FRET biosensor assay (Fig. 9C). Mutate aspartic acid 11 to alanine reduced the conformational change induced by Arg, whereas L16A reduced the conformational change that is induced by Arg and Cys.

**FIGURE 7. MaKat acetylates Lys-619 of the MaAcs enzyme.** A, sequence alignment of acetyl-CoA synthetases (Acs). A conserved motif called PKTRSGK in Acs was found in MaAcs (Micau_0428). B, Western blot analysis of MaAcs acetylated by MaKat. Wild type MaAcs or MaAcs<sup>619K</sup> mutant was incubated with various components as indicated at 37 °C for 60 min, followed by SDS-PAGE analysis. The reaction was performed in the presence or absence of Cys (1 mM), as well as Arg (1 mM).

**FIGURE 8. Initial rates of the acetyltransferase activity of MaKat.** The acetyltransferase activity of MaKat was measured using a coupled enzymatic assay. In the presence or the absence of two amino acids, the initial rate of formation of NADH is shown.

**TABLE 1**

Kinetic analysis of MaKat on MaAcs

| Enzyme | Substrate | \( k_{\text{cat}} \) | \( k_{\text{cat}}/K_{\text{m}} \) |
|--------|------------|-----------------|-----------------|
| MaKat  | Ac-CoA     | 4.2 ± 0.3 \( \mu \text{M}^{-1} \text{s}^{-1} \) | \( 0.02 ± 0.003 \times 10^3 \) |
| MaAcs  | Ac-CoA     | 21.6 ± 0.9 \( \mu \text{M}^{-1} \text{s}^{-1} \) | \( 0.03 ± 0.003 \times 10^3 \) |
| MaKat (Cys) | Ac-CoA | 4.1 ± 0.7 \( \mu \text{M}^{-1} \text{s}^{-1} \) | \( 0.04 ± 0.041 \times 10^3 \) |
| MaAcs | Ac-CoA | 14.8 ± 1.3 \( \mu \text{M}^{-1} \text{s}^{-1} \) | \( 0.05 ± 0.001 \times 10^3 \) |
| MaKat (Arg) | Ac-CoA | 4.3 ± 0.6 \( \mu \text{M}^{-1} \text{s}^{-1} \) | \( 0.05 ± 0.001 \times 10^4 \) |
| MaAcs | Ac-CoA | 17.3 ± 1.5 \( \mu \text{M}^{-1} \text{s}^{-1} \) | \( 0.05 ± 0.001 \times 10^4 \) |
The acetylation activity of the wild type and mutant proteins in the presence of Cys or Arg was also determined using a coupled acetyltransferase enzyme assay and Western blot analysis. As shown in Fig. 9D, no increase in the rate of acetylation of MaAcs in the presence of Arg was observed in the D11A mutant protein. This is consistent with the FRET ratio that revealed that the smallest conformational change is induced by Arg. The presence of two amino acids did not result in an increase in the initial rate of the L16A mutant (Fig. 9E), which can be correlated with the lower change in FRET of that was induced by Arg and Cys. Two amino acids were able to enhance acetylation of MaAcs by the D45A mutant to a similar extent, as seen in the wild type (Fig. 9F). In agreement with this, the FRET ratios of the D45A mutant demonstrated similar conformational changes as the wild type in the presence of Cys and Arg. These results revealed that these amino acids are critical for binding in the region between β1 and α1.

**DISCUSSION**

Growing evidence has indicated that metabolic pathways are coordinated through reversible acylation of metabolic enzymes in response to the nutritional status of cells to maintain homeostasis. Approximately 78 acetyltransferases containing putative GNAT domains are found in *M. aurantiaca*, but no acetyltransferases have been characterized. In this work, we report an amino acid-regulated protein acetyltransferase (MaKat, Micau_1670) for acetylation of acetate scavenging acetyl-CoA synthetase (MaAcs, Micau_0428) in *M. aurantiaca*. Our research reveals the first biochemical characterization of a protein acetyltransferase that contains a fusion of a GNAT domain with an ACT domain and provides a novel signaling pathway for regulating cellular protein acetylation in actinomycetales, in which all of the proteins containing the domain organization of ACT-GNAT have been discovered.

It was previously known that the activities of protein acetyltransferases and deacetylases are carefully regulated in
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response to the change of intracellular signals (such as level of acetyl-CoA and NAD) that control the acetylation of specific proteins, which in turn affects the metabolic network. Acetyl-CoA and NAD are key indicators of cellular energy status and demonstrate that protein lysine acetylation serves as a link that connects cellular energy levels with protein acetylation/deacetylation activity. In mycobacteria, cAMP directly activates MsKat and MrKat by binding of cAMP to the cyclic nucleotide-binding domain of two protein acetyltransferases, indicating that the levels of protein acetylation can also be modulated by response to intracellular cAMP levels (27). This study demonstrated that ACT domains are linked to GNAT acetyltransferases, which confirms amino acid-induced allosteric regulation of these enzymes. Protein acetyltransferases with ACT-GNAT domain organization could be a novel mechanism for connecting cellular metabolic status (amino acid metabolism) with levels of protein acetylation in actinomycetes.

The allosteric stimulation of MaKat acetyltransferase activity by Cys and Arg exhibited mixed activation kinetics in which \( k_{cat} \) values increased; \( K_m \) values for MaAcs decreased. Our FRET and kinetic analyses indicate that IC\(_{50}\) values of MaKat for Cys and Arg are in the submicromolar ranges (Cys, 80 \( \mu \)M; Arg, 210 \( \mu \)M). This is comparable with the intracellular levels reported for Cys and Arg, which can reach cytoplasmic concentrations of 100–200 \( \mu \)M in growing E. coli cultures (no data for M. aurantiaca) (28, 29). The normal intracellular level of two amino acids is evidently sufficient to effectively contribute to allosteric regulation of MaKat acetyltransferase.

Recently, advancements in mass spectrometry and high affinity purification of acetylated peptides allow identification of thousands of lysine acetylation sites (acytyleprotome) in prokaryotic and eukaryotic cells. It was found that many enzymes involved in the amino acid metabolism were lysine-acetylated in E. coli (30, 31), S. enterica (32), B. subtilis (33), Geobacillus kaustophilus (34), Thermus thermophilus (35), Saccharopolyspora erythraea (36), and Saccharomyces cerevisiae (37). On the other hand, most of the ACT-domain-containing proteins appear to interact with amino acids and are involved in some aspect of regulation of amino acid metabolism in E. coli, including serine binding to 3-phosphoglycerate dehydrogenase, which catalyzes the first step in the biosynthesis of serine; phenylalanine binding to chorismate mutase, which catalyzes the first two steps in the biosynthesis of phenylalanine; valine binding to acetyl-CoA synthetase, or OAS-sulfhydrylase is inhibited by Cys is the metabolic source of sulfur for all thiol-containing compounds in cells and has a central role in sulfur metabolism for assimilation and utilization of sulfur. CysB or CymR controls the genes involved in cysteine synthesis and transport in response to N-acetylselenine or O-acetylselenine, precursor of cysteine in E. coli and B. subtilis. Meanwhile, the first or second enzyme of the pathway of cysteine biosynthesis from serine, serine acetyltransferase, or OAS-sulphydrylase is inhibited by Cys. The challenge ahead is to identify all substrates of MaKat and the structure and mechanism of MaKat (38) and to elucidate the signaling link that connects intracellular levels of Cys and Arg with the regulation of metabolic enzyme activity at the post-translational modification level.

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