Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 7, pp 987–994, 1996

PII: S0968-0896(96)00097-1
0968-0896/96 $15.00 + 0.00

Kinamycin Acetyltransferase I from *Streptomyces murayamaensis*, an Apparently Large, Membrane-Associated Enzyme†

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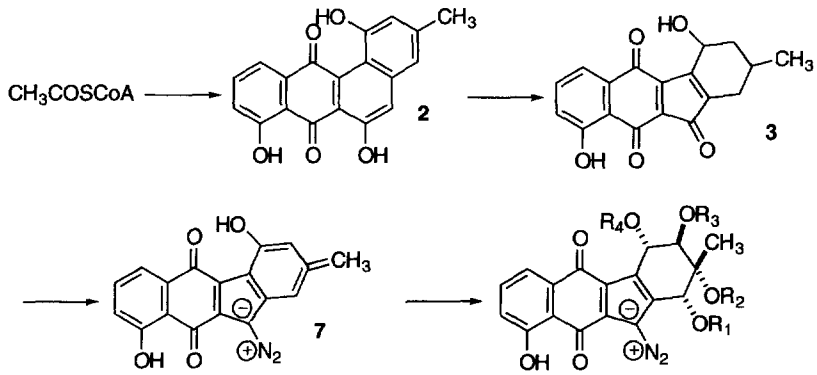
Abstract—Identification and initial characterization of an apparently large, membrane-associated multifunctional enzyme, kinamycin acetyltransferase I (KAT I), is described. KAT I activity was enriched 29-fold over the level in cell-free extracts of *Streptomyces murayamaensis*. Two acetyltransferase activities catalyzing acetyl coenzyme A dependent conversion of kinamycin F and E to kinamycin E and D, respectively, were inseparable in the course of the partial purification. Partial purification involved separation of KAT I from cytosolic proteins by differential ultracentrifugation, solubilization with 0.5% CHAPS zwitterionic detergent followed by ultracentrifugation, and Sephacryl S400 gel filtration chromatography of the resulting supernatant.

Introduction

The kinamycin antibiotics, diazo-substituted benz[*b*]fluorene quinones (e.g., kinamycin D, **1**),[1,2] contain a carbon skeleton that has been shown to be derived from 10 equiv of acetyl coenzyme A (AcCoA) via the polyketide benz[*a*]anthraquinones dehydrorabelomycin (**2**)[3] and kinobscurinone (Scheme 1, **3**).[4] Four kinamycins, A–D (corrected structures[5,6] are shown: **4–6**, **1**), were initially reported from *S. murayamaensis*.[1,2] The simplest member of this class, prekinamycin (**7**),[5,7] and the unacetylated kinamycin F (**8**)[7] have also been isolated from this organism. Additional kinamycin structural variants in which D-ring oxygens are modified with propionyl and isobutyryl substituents have been isolated from a different species of *Streptomyces*,[8] and *O*-isobutyryl-substituted kinamycin derivatives have also been identified in two other actinomycetes.[9,10]



1, $R_1 = R_3 = Ac$, $R_2 = R_4 = H$
4, $R_1 = R_2 = R_3 = Ac$, $R_4 = H$
5, $R_3 = Ac$, $R_1 = R_3 = R_4 = H$
6, $R_1 = R_3 = R_4 = Ac$, $R_2 = H$
8, $R_1 = R_2 = R_3 = R_4 = H$
9, $R_1 = Ac$, $R_2 = R_3 = R_4 = H$

Scheme 1.

The final kinamycin framework is in place at the level of **8**. Regiospecific *O*-acetylations of the highly oxygenated D-ring then lead to a set of acetylated kinamycins

†This paper is dedicated to Professor Sir Alan R. Battersby and Professor A. Ian Scott, recipients of the 1995 Tetrahedron Prize.
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(Scheme 1). Five of the 15 possible D-ring acetylation patterns, kinamycins A–E, have been isolated and characterized so far. Kinamycin E (**9**) had been proposed to be the monoacetate intermediate linking **8** and the diacetate **1**,[7] but had not been demonstrated biochemically up to this point; neither had any of the enzymology underlying the acylation chemistry of kinamycin biosynthesis been explored.

In order to use reverse genetics as an aid to isolating the genes for kinamycin biosynthesis, we attempted to detect and purify the acetyltransferase(s) from *S. murayamaensis* that presumably would convert **8** to **1**. Numerous soluble, modest molecular-weight acetyltransferases have been isolated from antibiotic-producing actinomycetes, including streptothricin acetyltransferase,[11] puromycin acetyltransferase,[12,13] chloramphenicol acetyltransferase,[14,15] blasticidin acetyltransferase,[16] and phosphinothricin acetyltransferase.[17,18] However, in this communication we report the detection, solubilization, and partial purification of enzymatic activities responsible for the sequential AcCoA-dependent conversion of **8** to **9**, and of **9** to **1**, KFAT and KEAT, respectively, that appear to reside on a single large protein or multienzyme complex, KAT I. The activities were inseparable and were found in a high molecular-weight, membrane-associated fraction of a cell-free extract (CFE).

Results and Discussion

As a prelude to a search for acetyltransferase activity, the positions of **8** and **9** in the pathway to **1** were first established in vivo. Addition of **8** to growing cultures of *S. murayamaensis* in a glycerol–asparagine (GA) medium resulted in greater accumulations of **9** than the traces detected in unsupplemented fermentations. Thus, when 5 mg portions of **8** were fed to each of three 200 mL broths 18 and 20 h after inoculation with a seed culture (30 mg total fed), work up[7] 24 h later yielded 4.4 mg of pure **9** (13% conversion).

[^{14}C]Kinamcyin F (**8a**) was then prepared by base hydrolysis[7] of **1a**, produced biosynthetically from sodium [1-^{14}C]acetate, and a portion of this [5.5 mg, 4.8×10^5 disintegrations/min (dpm)] was fed to 200 mL of an 18 h old culture. In this case, the fermentation was stopped 1 h later, and work up yielded **9a** (2 mg; 1.4×10^5 dpm) and **1a** (18 mg; 7.4×10^4 dpm) containing 29.2 and 15.5%, respectively, of the total radioactivity fed. These results conclusively established for the first time that **8** is converted to **9.** The similar specific radioactivities of **9a** and **8a** indicated that a relatively small pool of **9** existed in vivo at the time of the feeding. In contrast, the significant difference in the specific radioactivities of **9a** and **1a** implied that a substantial pool of unlabeled **1** existed in vivo at the time of the feeding. The alternative explanation, that **9** is not the direct precursor of **1**, was excluded by the identification of an enzyme activity (KEAT) for conversion of **9** to **1** in the course of this work.

The anticipated presence of at least two kinamycin acetyltransferase activities in the CFE (KFAT and KEAT) necessitated development of an assay method, which facilitated direct monitoring of consumption of **8** as well as production of **9** and **1**. An HPLC protocol yielding baseline separations of **8**, **9**, and **1** was developed for routine monitoring of acetyltransferase activity throughout the purification process. For cases in which small quantities of **1** were also detected, KFAT activity calculations were based upon the sum of the contributions of **9** and **1**. A convenient colorimetric assay based on the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent)[19] with liberated CoASH could not be used for routine screening of column fractions due to the strong absorbance of the kinamycins at 412 nm.

KFAT and KEAT activities were first demonstrated in a CFE. Cells from 18 to 24 h cultures were collected, washed to remove surface proteases,[20] and sonicated. Centrifugation gave a crude *S. murayamaensis* CFE that rapidly converted **8** to **9** at the expense of AcCoA. A second AcCoA-dependent reaction in which **9** was slowly converted to **1** was also observed during this experiment. When **9** served as the initial substrate, conversion to **1** was rapid. No other acetylated kinamycin products were detected and none of the putative alternative substrates (**5**, **1**, and kinamycin 3′-*O*-acetate) were altered by the CFE when tested under the same conditions. Also, butyryl coenzyme A could not replace AcCoA as the cofactor. Background levels of colored metabolites were significantly decreased by inclusion of polyvinylpolypyrrolidone (PVPP) during sonication of the mycelia and even TLC analysis of incubations showed a simpler mixture of compounds.

Assay mixtures were also subjected to HPLC under a standard set of conditions capable of separating all of the kinamycin metabolites for which authentic standards are available.[21] The identity of each product was confirmed by matching the HPLC retention time and photodiode array UV–vis spectrum to an authentic standard in an *S. murayamaensis* spectral library (Fig. 1). This analysis also served to rule out the presence of other acetylated kinamycin products.

Simultaneous efforts to purify the acetyltransferase activities catalyzing conversion of **8** to **9** and conversion of **9** to **1** were undertaken with the assumption that these activities were attributable to distinct, soluble enzymes. Reducing agents such as dithiothreitol (DTT, 1 mM), which frequently stabilize enzyme preparations, had an adverse effect, reducing the kinamycin quinone in the assay mixture. Initial efforts to purify the KFAT and KEAT activities yielded an 18-fold enrichment of both activities by treating the CFE with streptomycin sulfate to remove nucleic acids, followed by ammonium sulfate (AS) precipitation, gel filtration chromatography, and then DEAE chromatography at pH 6.5 with elution at 200 mM NaCl. Surprisingly, the two

acetylating activities co-eluted from the Sephacryl S200 gel filtration and from the DEAE DE-52 anion exchange columns. Both activities eluted in the void volume during gel filtration chromatography, indicating an $M_r > 200{,}000$. Further efforts to extend this purification were unsuccessful. However, Lineweaver–Burk analysis was used to estimate K_m values for **8** (85 μM) and AcCoA (13 μM) in the KFAT reaction and for **9** (46 μM) in the KEAT reaction (data not shown). The highest specific activity (SA) KAT I fraction from the anion exchange chromatography step served as the enzyme source.

In view of the unexpectedly large apparent size of KAT I relative to other *Streptomyces* acetyltransferases ($M_r < 80$ kDa), the presence of residual cell membrane was examined by high-speed centrifugation. Indeed, after centrifugation at $39{,}200 \times g$ for 3 h, the pellet contained 43% of the original activity with a 10-fold increase in SA, while the supernatant contained only 18% of the original activity. Only KFAT was assayed. Similarly, ultracentrifugation (1 h, $105{,}000 \times g$) of the CFE caused a dramatic drop in the total and specific activities of both KAT I activities in the supernatant. In contrast, the resuspended pellet fraction contained both activities and the specific activities of both were enhanced by a factor of 8. A greater recovery of total activity was obtained with these conditions; some heat denaturation may have occurred in the long centrifugation at the lower speed. A large portion of the total protein mass (>75%) remained in the post-ultracentrifugation supernatant, which typically contained less than 15% of the total KAT I activity.

A panel of detergents was next surveyed in order to identify an effective solubilizing agent for the acetyltransferase activities in the ultracentrifugation pellet. Measurement of the distribution of KAT I (monitored by assaying KFAT activity) among the detergent-treated ultracentrifugation supernatant, the redissolved ultracentrifugation pellet, and an uncentrifuged control sample containing no detergent revealed that most of the detergent treatments (deoxycholate, TWEEN 80, *n*-octyl-β-glucopyranoside, urea, NP-40, Triton X-100, SDS) resulted in substantial losses of activity at all concentrations tested, but that the neutral digitonin and the zwitterionic 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS)[22] were effective in solubilizing KAT I. Of the pelleted activity 56% was solubilized at 0.3% CHAPS, while all activity

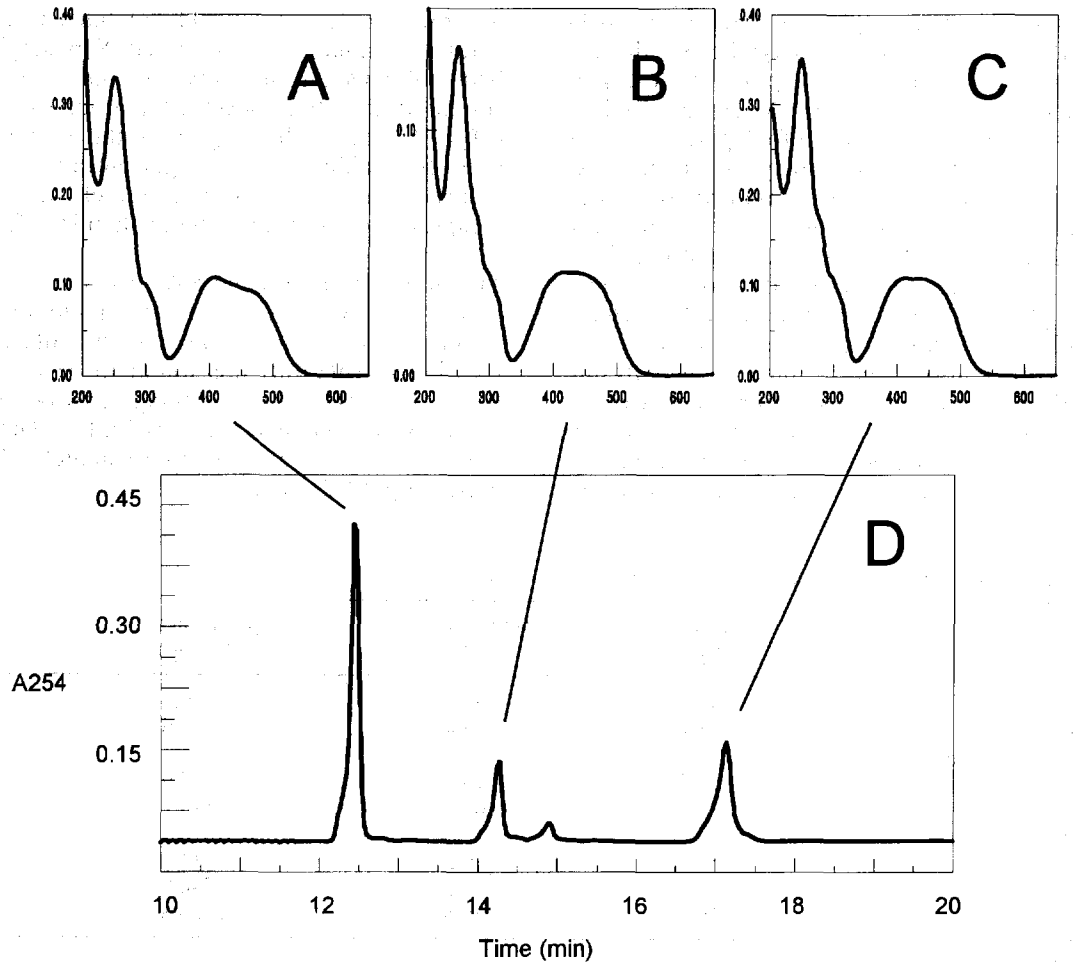

Figure 1. (A) UV–vis spectrum of kinamycin F (**8**) from photodiode array HPLC detector; (B) UV–vis spectrum of kinamycin E (**9**); (C) UV–vis spectrum of kinamycin D (**1**); (D) HPLC[21] (C_{18}) profile of a KAT I incubation with kinamycin F as substrate.

was lost at 1.0% CHAPS. However, essentially all of the activity was solubilized at 1.0–3.0% digitonin. Digitonin was initially chosen for further work and was included (1.0%) in the buffer for chromatography on Sepharose 6B. Recovery of KAT I activity was low and was detectable only in the first protein-containing fractions. Difficulties were encountered with digitonin solubility, CHAPS was reinvestigated, and a slight improvement was found at 0.5%, with 64% solubilization obtained. More consistent results were obtained with CHAPS than with digitonin, and CHAPS was used for all further work. Although KFAT activity was monitored during the detergent trials, the final CHAPS-solubilized ultracentrifugation pellet was also subjected to KEAT activity assays, which confirmed that both activities had been solubilized to the same extent and supported the hypothesis that both activities are attributable to KAT I. The designation KAT I distinguishes the enzyme or complex responsible for the KFAT- and KEAT-catalyzed acetylations from other as yet uncharacterized kinamycin acylating activities.

Based on these findings, a new protocol was developed that consisted of AS precipitation at 75% saturation, re-solution and desalting by gel filtration, ultracentrifugation at $105{,}000 \times g$, CHAPS treatment (0.5%) of the pellet followed by a second ultracentrifugation at $105{,}000 \times g$, and chromatography on a Sephacryl S400 column. Both KAT I activities emerged from the gel filtration matrix in a wide peak just prior to the thyroglobulin standard ($M_r = 669$ kDa). As summarized in Table 1, this procedure gave a 29-fold purification of KAT I (with respect to KFAT activity). Unfortunately, all efforts to purify the acetyltransferase activity further by a variety of chromatographies were uniformly disappointing. Cibacron Blue affinity chromatography,[23] which has been used in the purification of some bacterial acetyltransferases,[11] was tried first, but KAT I did not bind to this support under any of the tested conditions. KAT I also failed to bind to two freshly prepared affinity columns: a thiopropyl-coenzyme A column and a kinamycin F-diazo column. Attempts to use cation exchange, anion exchange, hydrophobic interaction, and hydroxylapatite chromatographies to enhance the purity of CHAPS-solubilized enzyme preparations were also unsuccessful. Indeed, while KAT I could be stored at −20 °C as a 20% glycerol suspension at this point, all of the tested chromatographic procedures resulted in significant losses of total activity and failed to improve the SA of KAT I. Although it had been intended to use a kinamycin acetyltransferase and reverse genetics as an aid to locate the kinamycin biosynthetic gene cluster, further efforts to purify KAT I from *S. murayamaensis* do not appear to be worthwhile. However, most of the gene cluster has recently been cloned using a different approach.[24] Thus, it may be possible to determine the size of the KAT I protein(s) by detecting the gene(s) in the regions flanking the cloned DNA, and to then obtain pure enzyme via genetic engineering.

The SA of the KAT I preparation was enhanced only modestly during the purification procedure, possibly due to the lability of KAT I or the removal of an essential component. A frequent problem accompanying attempts to solubilize membrane-associated proteins is loss of activity in a large percentage of the solubilized enzyme.[25] The highest SA KAT I fractions obtained in the course of this investigation contained approximately 15 bands when examined by SDS-PAGE on a 7.5% polyacrylamide gel (silver stain).

Several samples of CHAPS-solubilized KAT I were chromatographed on calibrated Sephacryl S300 and S400 columns, and in every case both KAT I activities co-eluted after blue dextran but prior to thyroglobulin ($M_r = 669$ kDa), the largest of the standard proteins used for calibration. The gel filtration data clearly indicate that KAT I is an extremely large enzyme or complex, even in its detergent solubilized form.

The optimum pH for the KAT I reaction was found to be 6.2. However, the long-term stability of the enzyme appeared to be compromised in this pH range. Tris–HCl buffer (pH 7.0) was found to be the best of the tested buffers for maintaining activity, and routine assays were conducted in this buffer. The activity of KAT I at pH 7.0 was approximately 70% of the maximal pH 6.2 activity.

Table 1. Partial purification of kinamycin acetyltransferase I from *S. murayamaensis*

Step	Protein (mg)[a]	Activity × 10^3 (U)[b,c]	Specific activity (U/mg)	Purification
CFE	552.0	54.8	99.3	1.0
AS treatment	525.1	75.4	143.6	1.5
Differential ultracentrifugation:				
Supernatant	424.2	16.1	37.9	0.4
Pellet	98.3	109.7	1119.7	11.3
0.5% CHAPS:				
Supernatant	55.3	100.2	1811.8	18.3
Pellet	35.2	26.3	746.0	7.5
Sephacryl S400	16.1	46.8	2906.8	29.3

[a]Starting with 15 g of wet cells.
[b]1U = 1 nmol of **9** formed per hour under conditions described in the Experimental.
[c]A pronounced increase in KAT I total activity was observed following solubilizaton with CHAPS detergent.

A number of antibiotic-producing streptomycetes rely upon antibiotic-modifying enzymes for protection against their own toxic secondary metabolites. In at least five cases, acetylation has been identified as a mechanism of antibiotic resistance in antibiotic-producing actinomycetes.[11–18] However, the kinamycin producer *S. murayamaensis* is capable of elaborating a much more extensive array of acetylation patterns than any other streptomycete reported to date. Results of tests with various Gram-positive and Gram-negative bacteria have previously revealed that kinamycin F is only slightly more active than any of the acetylated derivatives.[26] Thus, the purpose of the various *O*-acetylations is not yet clear. One possibility is that increasing the hydrophobicity of kinamycin promotes sequestering of antibiotic in membranes, where it can be exported/used to ward off predators.

The enzyme(s) described in this report account for two of the five D-ring acetylation patterns observed in naturally occurring kinamycins (Scheme 1). Throughout the purification process, KFAT and KEAT activities were copurified. The membrane association and large apparent size of KAT I represent unusual features relative to other acetyltransferases,[27] as does the apparent multifunctional nature of the enzyme. Although both 1-acetylchloramphenicol and 3-acetylchloramphenicol have been isolated from the same fermentation, chloramphenicol acetyltransferase (CAT) catalyzes *O*-acetylation of the C-3 hydroxyl moiety of chloramphenicol only. This intermediate can then undergo a nonenzymatic, intramolecular transfer of the C-3 acetyl group to the C-1 position. Both chloramphenicol and 1-acetylchloramphenicol are substrates for CAT, yielding 3-acetylchloramphenicol and 1,3-diacetylchloramphenicol, respectively.[14,28]

The minimum pathway which accounts for all reported acetylated kinamycins is illustrated in the top branch of Figure 2. In addition to the KFAT and KEAT activities of KAT I, at least two additional kinamycin acetyltransferases, KAT II and KAT III, would be required to assemble **4** and **6**; **5** could be a dead-end product, or may undergo further acetylations leading to kinamycin A through either of two hypothetical diacetates. With **8** as substrate, we have not observed production of any kinamycin monoacetate other than **9**, and the only detected diacetate was **1** when **9** served as substrate. No triacetates or tetraacetates were observed under any conditions. We have previously shown that **8** and **9** are produced by *S. murayamaensis*. The current study establishes the relationship between **8**, **9**, and **1**.

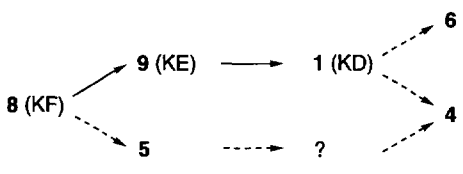

Figure 2. Possible acetylation pathways in kinamycin biosynthesis; solid arrows denote demonstrated enzymatic activity; dashed arrows denote hypothetical enzymatic steps.

Experimental

General

All chemicals, protease inhibitors, and gel filtration protein standards were purchased from Sigma. Sephacryl S300 HR and S400 HR gel filtration media were purchased from Pharmacia, and DEAE DE-52 anion exchange medium from Whatman. UV–vis absorption measurements were made on a Hewlett Packard 8452A photodiode array spectrophotometer. HPLC analyses were performed on an IBM HPLC instrument with a Kratos Spectroflow 757 UV detector (set at 408 nm) and a Hewlett Packard 3396A integrator. A Whatman EQC C18 column (10 μm, 4.6 × 216 mm) was employed. The mobile phase was 35% acetonitrile:0.1% trifluoroacetic acid (TFA) in H_2O and the flow rate was 1.4 mL/min.

Refrigerated centrifugations were performed in an IEC B-20A centrifuge. Refrigerated ultracentrifugations were done in a Beckman L-8 ultracentrifuge equipped with Ti 42.1 rotor. Cell disruption was performed with a Model W-225R sonicator (Heat Systems-Ultrasonic, Inc.). For open columns, the flow rate was controlled by peristaltic pump P-3 (Pharmacia), and fractions were monitored with a UV-2 dual path optical control unit (Pharmacia). Water used for fermentations and biochemical preparations was purified by a Milli-Q water system (Millipore Corp.). All steps in the enzyme purification procedure were conducted at 4 °C.

Buffers

The following buffers were routinely used: buffer I [Tris–HCl (50 mM):NaCl (50 mM), pH 7.5 at 4 °C], buffer II [buffer I + 1 mM EDTA], buffer III [buffer II + 20% (v/v) glycerol], and buffer IV [buffer III + 0.5% CHAPS detergent].

Bacterial strain and culture conditions

Streptomyces murayamaensis spp. nov. Hata et Ohtani was maintained as previously described.[29] Small-scale cultures (6 × 200 mL) were grown aerobically at 26 °C, 285 rpm for 24 h (Lab-line incubator-shaker) in GA medium.[29] At the end of the fermentation, the broths were combined, subjected to sequential washes with 400 mL each of 1 M KCl, 0.8 M NaCl, and 50 mM Tris buffer (pH 7.2), with centrifugation (10,000 × *g*, 10 min) after each wash. The mycelia were frozen at −20 °C, then stored at −80 °C (yield: 8–9 g wet cell mass/1.2 L). Large-scale fermentations (7.5 L) were conducted in a New Brunswick Microferm fermentation console fitted with a 14 L fermentation vessel. A two-stage Kikano soybean medium seed culture[29] was used to inoculate the GA production medium and the organism was grown for 22–26 h (300 rpm, 27–28 °C, 10 L/min aeration), then harvested by centrifugation (Sharples T-1 continuous flow centrifuge). Sequential washes were carried out as described above using 1 L of each solution. The mycelia were frozen at −20 °C,

then stored at −80 °C in 15 g portions (yield: 57–60 g wet cell mass/7.5 L).

In vivo feeding studies

Pure **8** (5 mg per flask at each time point) was added to each of three small-scale fermentations (200 mL in 1 L flasks) 18 and 20 h after inoculation. Following the second administration of **8**, the fermentations were continued for an additional 24 h. The cultures were combined, centrifuged (10,000 × *g*, 10 min), and the supernatant was reserved. The mycelia were resuspended in H_2O (60 mL), sonicated at full power (4 °C, 5 min, 90% duty), centrifuged (10,000 × *g*, 10 min), and the supernatant was recombined with the broth. Purified **9** (4.4 mg) was obtained by silica gel column chromatography as previously described.[7]

A supply of **1a** isolated following administration of sodium [1-^{14}C]acetate to *S. murayamaensis* was hydrolyzed as previously described,[7] and **8a** (11 mg, 9.6×10^5 dpm) was isolated by silica gel chromatography. A portion of the isolated **8a** (5.5 mg, 4.8×10^5 dpm) was administered to a growing culture (18 h) of *S. murayamaensis* (200 mL, 1 L flask) under the conditions described above. The fermentation was continued for 1 h, then worked up as described above. **9a** (2 mg, 1.4×10^5 dpm) and **1a** (18 mg, 7.4×10^4 dpm) were isolated and purified to constant specific radioactivity.

Measurement of KAT I activity

One unit of KFAT activity is defined as the amount of enzyme required to convert 1 nmol of **8** to **9** in 1 h, at 30 °C, under the specified assay conditions. One unit of KEAT activity is required to convert 1 nmol of **9** to **1** in 1 h, at 30 °C, under the specified assay conditions. Each assay mixture (115 μL) contained enzyme solution, **8** or **9** (250 μM, added in 5 μL of DMSO), and AcCoA (500 μM, added in 10 μL of buffer I). Assays were initiated by the addition of the AcCoA and were carried out at 30 °C for 20 min in an IBM 9950 heating/cooling fluid circulator (±0.5 °C). All experiments included a control assay in which enzyme solution was replaced with heat-inactivated enzyme solution (95 °C, 15 min). Reactions were terminated by the addition of an equal volume of 70% acetonitrile:0.2% (v/v) TFA in H_2O, and protein was removed by centrifugation. A portion of the quenched assay mixture (20 μL) was analyzed by HPLC (retention times: **8**, 4.5 min; **9**, 7.8 min; **1**, 22.7 min). Protein content was measured using the Bradford method[30] with bovine serum albumin as the standard.

Preparation of CFE for initial KAT I purification procedure

Thawed cells (9 g) were suspended in buffer I and treated with PVPP (2 g). The mixture was placed in an ice bath, sonicated at maximum power, 90% duty for 30 s, cooled for 5 min, the sonication was repeated, and the mixture was centrifuged (10,000 × *g*, 10 min). The pellet was suspended in buffer I (75 mL), sonicated under the same conditions and centrifuged (10,000 × *g*, 10 min). The combined supernatants (170 mL) comprised the CFE.

Cell-free *O*-acetylation of 8 and 9

A mixture of the CFE (1.0 mL), AcCoA (500 μM, added in 20 μL of buffer I), and **8** or **9** (250 μM, added in 10 μL of DMSO) was incubated at 30 °C for 1 h. Aliquots (200 μL) were removed every 15 min, treated with an equal volume of 70% acetonitrile:0.2% TFA in H_2O, centrifuged, and analyzed by TLC and HPLC.

Initial partial purification of KAT I

CFE (170 mL) was treated with 30 mL of streptomycin sulfate (10%, w/v) added over a 30 min period, then centrifuged (10,000 × *g*, 15 min). The supernatant (200 mL) was brought to 75% saturation by slow addition of solid AS (96.6 g). Following centrifugation (10,000 × *g*, 15 min), the pellet was suspended in buffer I (pH 6.8; 18 mL) and solids were removed by centrifugation. A portion of this solution (5 mL) was applied to a Sephacryl S200 column (2.5 × 50 cm) which had been pre-equilibrated with buffer I (pH 6.8) and eluted at a flow rate of 1.2 mL/min. Enzyme-active fractions (10.8 mL total) were combined and applied to a Whatman DEAE DE-52 cellulose column (1.5 × 10 cm), which had been pre-equilibrated with buffer I (pH 6.5). The column was washed with buffer I (pH 6.5) and KAT I was then eluted with 40 mL of buffer I (pH 6.5) containing NaCl (200 mM). Active fractions were combined and dialyzed against buffer I (pH 6.8).

High-speed centrifugation and determination of a suitable solubilizing agent

Desalted post-ammonium sulfate treated protein solution served as the enzyme source for detergent solubilization trials. Total and specific activities were first measured in the absence of detergent. Detergent/buffer stock solution (210 μL) was then mixed with enzyme solution (500 μL) at 4 °C, and the solution was vortexed gently every 10 min for 1 h. Following ultracentrifugation (105,000 × *g*, 1 h), the supernatant was collected and the pellet was resuspended in an equal volume of the same buffer/detergent solution. The solutions were then assayed for KFAT activity. Deoxycholate, TWEEN 80, *n*-octyl-β-glucopyranoside, NP-40, triton X-100, digitonin, and CHAPS were tested. All of the detergents were tested at 0.1 and 0.3% (w/v). Digitonin and CHAPS were further tested at 0.5, 1.0 and 3.0% (w/v).

Preparation of CFE for revised KAT I purification procedure

Washed cells (15 g) were suspended in 110 mL of buffer I, and PVPP (3.4 g) was slowly added. Pepstatin A (2 μM, added in methanol), leupeptin (2 μM, added in H_2O), and phenylmethylsulfonyl fluoride (PMSF,

200 μM, added in ethanol) were added. These protease inhibitors were replenished regularly throughout the purification. Sonication at maximum power, 90% duty was carried out four times in 30 s intervals, with 5 min cooling periods between treatments. Following centrifugation (10,000 × *g*, 15 min), the supernatant (92 mL) was saved and the pellet was redissolved in buffer I (50 mL), sonicated as above, and centrifuged. The two supernatants were combined, DNase I (3.5 mg) and 2.5 M $MgCl_2$ (300 μL) were added, and the solution was kept at 4 °C for 1 h. Centrifugation to remove solid materials yielded a CFE (142 mL).

Solubilization and partial purification of membrane-associated KAT I

The CFE was brought to 75% saturation by addition of solid AS (68.6 g). The suspension was stirred for 1 h and centrifuged (10,000 × *g*, 30 min) to collect the precipitate. The precipitate was dissolved in buffer II and desalted (Bio-Rad 10DG columns equilibrated with buffer II). The desalted solution (36 mL) was subjected to ultracentrifugation (105,000 × *g*, 1 h) and the supernatant was discarded. The pellet was dissolved in buffer III and CHAPS detergent was added (10% (w/v) solution in buffer III) to a final concentration of 0.5% (w/v). The solution was gently agitated overnight at 4 °C, then subjected to ultracentrifugation (105,000 × *g*, 1 h). The resulting supernatant was concentrated (<4 mL) by ultrafiltration (Centriprep-100, Amicon). The solubilized, concentrated KAT I sample was subjected to Sephacryl S400 column chromatography (1.5 × 71.5 cm) using buffer IV as the eluent (0.4 mL/min). Samples were loaded in a volume of 1 mL. The highest SA KAT I-containing fractions from the S400 step were pooled and either used immediately for testing new chromatographic steps or stored at −20 °C as 20% (v/v) glycerol suspensions.

Attempted estimation of the M_r of KAT I

Prior to chromatographing KAT I-containing samples, the S400 column was calibrated with a set of standard proteins [thyroglobulin (669 kDa), apoferritin (443 kDa), amylase (200 kDa), bovine serum albumin (66 kDa)] under conditions identical to those used when chromatographing KAT I.

Determination of optimum pH

A 10-fold-diluted aliquot (20 μL) of KAT I from the S400 gel filtration chromatography step served as the enzyme source. The enzyme and **8** (250 μM, added in 5 μL of DMSO) were added to buffer I (total volume = 200 μL) and heated to 30 °C prior to AcCoA addition (500 μM, added in 20 μL of buffer I). At 0 and 20 min time points, a 50 μL aliquot was withdrawn and quenched with an equal volume of 70% acetonitrile:0.2% TFA in H_2O. Following removal of protein by centrifugation, samples were examined by HPLC. MES buffer (100 mM; pK_a 25 °C 6.1) was used for pH range 5–7, bis-Tris buffer (100 mM, pK_a 25 °C 6.8) was used for pH range 6.5–7.5, and Tris–HCl buffer (100 mM, pK_a 25 °C 8.3) was used for pH range 7.5–8.5.

Acknowledgments

This research was supported by U.S. Public Health Service Grant GM 31715 to S. J. G. Professor S. Omura of Kitasato University and Professor U. Hornemann of the University of Wisconsin are thanked for cultures of *S. murayamaensis*.

References

1. Ito, S.; Matsuya, T.; Omura, S.; Otani, M.; Nakagawa, A.; Takeshima, H.; Iwai, Y.; Ohtani, M.; Hata, T. *J. Antibiot.* **1970**, *23*, 315.

2. Hata, T.; Omura, S.; Iwai, Y.; Nakagawa, A.; Otani, M. *J. Antibiot.* **1971**, *24*, 353.

3. Seaton, P. J.; Gould, S. J. *J. Am. Chem. Soc.* **1987**, *109*, 5282.

4. Gould, S. J.; Melville, C. R. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 51.

5. Gould, S. J.; Tamayo, N.; Melville, C. R.; Cone, M. C. *J. Am. Chem. Soc.* **1994**, *116*, 2207.

6. Mithani, S.; Weeratunga, G.; Taylor, N. J.; Dmitrienko, G. I. *J. Am. Chem. Soc.* **1994**, *116*, 2209.

7. Seaton, P. J.; Gould, S. J. *J. Antibiot.* **1989**, *42*, 189.

8. Young, J.-J.; Ho, S.-N.; Ju, W.-M.; Chang, L.-R. *J. Antibiot.* **1994**, *47*, 681.

9. Isshiki, K.; Sawa, T.; Naganawa, H.; Matsuda, N.; Hattori, S.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1989**, *42*, 467.

10. Smitka, T. A.; Bonjouklian, R.; Perun, J. T. J.; Hunt, A. H.; Boeck, L. D.; Yao, R. C. *J. Antibiot.* **1992**, *45*, 433.

11. Kobayashi, T.; Horinuchi, S.; Uozumi, T.; Beppu, T. *J. Antibiot.* **1987**, *40*, 1016.

12. Perez-Gonzalez, J. A.; Vara, J.; Jiminez, A. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 772.

13. Paik, S.-Y.; Sugiyama, M.; Nomi, R. *J. Antibiot.* **1985**, *38*, 1761.

14. Shaw, V. W. *CRC Crit. Rev. Biochem.* **1983**, *14*, 1.

15. Nakano, H.; Matsuhashi, Y.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1977**, *30*, 76.

16. Sugiyama, M.; Takeda, A.; Paik, S.-Y.; Mini, O.; Nomi, R. *J. Antibiot.* **1986**, *39*, 827.

17. Thompson, C. J.; Movva, N. R.; Tizard, R.; Crameri, R.; Davies, J. E.; Lauwereys, M.; Botterman, J. *EMBO J.* **1987**, *6*, 2519.

18. Botterman, J.; Gossele, V.; Thoen, C.; Lauwereys, M. *Gene* **1991**, *102*, 33.

19. Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

20. Hutchinson, C. R. *Nat. Prod. Rep.* **1986**, *3*, 133.

21. Cone, M. C.; Melville, C. R.; Gore, M. P.; Gould, S. J. *J. Org. Chem.* **1993**, *58*, 1058.

22. Hjelmeland, L. M. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 6368.

23. Lowe, R. C.; Pearson, J. C. *Meth. Enzymol.* **1984**, *104*, 97.

24. Gould, S. J.; Hong, T.-S., unpublished results.

25. Hjelmeland, L. M. *Meth. Enzymol.* **1990**, *182*, 253.

26. Cone, M. C.; Gould, S. J., unpublished results.

27. Although rare, other membrane-associated secondary metabolic enzymes have been reported from *Streptomyces* spp.: Jones, G. H. *J. Bacteriol.* **1987**, *169*, 5575; Frenzel, T.; Zhou, P.; Floss, H. G. *Arch. Biochem. Biophys.* **1990**, *278*, 35.

28. Murray, I. A.; Derrick, J. P.; White, A. J.; Drabble, K.; Wharton, C. W.; Shaw, W. V. *Biochemistry* **1994**, *33*, 9826.

29. Cone, M. C.; Seaton, P. J.; Halley, K. A.; Gould, S. J. *J. Antibiot.* **1989**, *42*, 179.

30. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.

(*Received in U.S.A.* 24 *January* 1996; *accepted* 8 *March* 1996)