Purification, Characterization, and Cloning of an S-Adenosylmethionine-dependent 3-Amino-3-carboxypropyltransferase in Nocardicin Biosynthesis*

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S-Adenosylmethionine: nocardicin 3-amino-3-carboxypropyltransferase catalyzes the biosynthetically rare transfer of the 3-amino-3-carboxypropyl moiety from S-adenosylmethionine to a phenolic site in the β-lactam substrates nocardicin E, F, and G, a late step of the biosynthesis of the monocyclic β-lactam antibiotic nocardicin A. Whereas a number of conventional methods were ineffective in purifying the transferase, it was successfully obtained by two complementary affinity chromatography steps that took advantage of the two substrate-two product reaction scheme. S-Adenosylhomocysteine-agarose selected enzymes that utilize S-adenosylmethionine, and a second column, nocardicin A-agarose, specifically bound the desired transferase to yield the enzyme as a single band of 38 kDa on a silver-stained SDS-polyacrylamide gel. The transferase is active as a monomer and exhibits sequential kinetics. Further kinetic characterization of this protein is described and its role in the biosynthesis of nocardicin A discussed. The gene encoding this transferase was cloned from a sublibrary of Nocardia uniformis DNA. Translation gave a protein of deduced mass 32,386 Da which showed weak homology to small molecule methyltransferases. However, three correctly disposed signature motifs characteristic of these enzymes were observed.

Nocardicin A (see 1 in Fig. 1) is the most biologically active of a series of monocyclic β-lactam antibiotics isolated from the fermentation broth of the actinomycete Nocardia uniformis subsp. tsuyamanesis (ATCC 21806) (1–3). Co-occurring with the major metabolite 1 are the structurally related monocyclic β-lactams nocardicin B–G (Fig. 1, 2–7) (2, 4–6). Whole-cell incorporation experiments have shown 1 to be derived from the L-isomers of methionine, serine, and p-hydroxyphenylglycine (PHPG) (8), a degradation product of L-tyrosine. These are the most direct primary precursors of the homoserine, β-lactam, and aryl portions of the antibiotic, respectively (7–11). In an important result, it was demonstrated that the simplest member of the nocardicin family, nocardicin G (7), is a direct biosynthetic precursor to nocardicin A (12).

With nocardicin G (7) positioned as the central intermediate in the pathway leading to nocardicin A (1), the skeleton of the antibiotic can be completed, in unspecified order, by amine oxidation and 3-amino-3-carboxypropyl side chain attachment (Fig. 2). The stereochemical course of side chain attachment was shown to be inversion through the incorporation of methionine stereospecifically deuterated at C-4 (13). This configurational outcome parallels the stereochemistry of addition of the 3-aminopropyl moiety derived from decarboxylated S-adenosylmethionine (AdoMet)3 operative in the biosynthesis of the polyamines (14–16), as well as more conventional methyltransferases (17, 18), suggesting a role for AdoMet in nocardicin A biosynthesis in vivo. This postulate was borne out in preliminary cell-free studies in which an efficient, time-dependent conversion from nocardicin E (5) to nocardicin A in the presence of AdoMet was demonstrated (19). Assuming the epimerization of the amino acid terminus (C-9′) of nocardicin A is a late step in the biosynthesis, the product of this transferase enzyme was expected to be isonocardicin A (L at C-9′). Subsequently, it was demonstrated that an epimerase capable of equilibrating the stereochemistry at C-9′ of nocardicin A was also present in the cell-free extract (19).

The bioconversion of nocardicin E (5) to isonocardicin A in cell-free extracts lends support for pathway A (Fig. 2) to nocardicin A (1) in which the amine of nocardicin G (7) is oxidized to the oxime to form nocardicin E (5) and, in the penultimate step, the 3-amino-3-carboxypropyl side chain is attached to the phenolic hydroxy group. The final step, in analogy to penicillin biosynthesis (20) and demonstrated experimentally (see below), is then the epimerization of the amino acid terminus to the β-configuration.

Although rare, the transfer of the 3-amino-3-carboxypropyl group from AdoMet to a nucleophilic acceptor is not unprecedented. It has been demonstrated in the biosynthesis of the X-base from Escherichia coli tRNA59me (21), the Y-base from yeast tRNA59me (22), the germination inhibitor discadene from the cellular slime mold Dictyostelium discoideum (23), and plant siderophores of the mugineic acid family (24, 25). AdoMet has also been shown (26) to be the donor of the 3-amino-3-carboxypropyl unit in diphthamide, the post-translationally modified histidine of elongation factor 2. The only one of the enzymes responsible for this group transfer that has been purified (27) is nicotianamine synthase, but this enzyme cata-

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1 The abbreviations used are: AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; dd H2O, distilled, deionized water; GITC, 2,3,4,6-tetra-O-acetyl-b-D-glucopyranosyl isothiocyanate; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair; NAT, S-adenosylmethionine: nocardicin 3-amino-3-carboxypropyltransferase; TOP, transferase oligonucleotide probe; PCR, polymerase chain reaction; nt, nucleotides.

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lyzes a transformation distinct from the enzyme in the present work and has not been characterized kinetically or mechanistically. A 420-fold partial purification has been reported for discadenine synthase, but the final preparation contained many contaminating proteins (28).

In this work, the first purification to apparent homogeneity, kinetic characterization, and cloning of a 3-amino-3-carboxypropyltransferase from a bacterial source is reported. In the presence of AdoMet this enzyme catalyzes the transformation of the substrates nocardicin E, F, and G to the products isonocardicin A, B, and C, respectively. By using reverse genetic techniques, the gene encoding the transferase was cloned from N. uniformis DNA. Translation revealed a 32,389-Da protein containing three modestly conserved motifs characteristic of S-adenosylmethionine-binding sites (29).

**EXPERIMENTAL PROCEDURES**

**Materials**—Except where noted, all chromatographic matrices, AdoMet (chloride salt) and its derivatives, and reagents for protein purification and affinity column synthesis were products of Sigma. Superose 6 HR 10/30 desalting columns (10 ml) and dye reagent concentrate for the Superose 6 HR 10/30 was a product of Amersham Pharmacia Biotech. All other chemicals were of reagent grade. Nocardicin substrates and peptides were purchased as a lyophilized pellet from the ATCC (Rockville, MD). All other chemicals were of reagent grade. Peptide sequence analyses were conducted on an Applied Biosystems 470A gas-phase sequencer (Protein/Peptide/DNA Facility, Department of Biological Chemistry, The Johns Hopkins Medical School). HPLC separations were carried out on a Waters 600 HPLC and 490 Programmable Multi-wavelength Detector equipped with a Vydac C4 reversed phase column (250 × 10 mm; Heperia, CA).

**Purification and Characterization of S-Adenosylmethionine:nocardicin A 3-Amino-3-carboxypropyltransferase (NAT)**

**Purification of NAT—**N. uniformis subsp. tsuyamanensis (ATCC 21806), 1 liter of total fermentation volume, was harvested by centrifugation (9,200 × g, 10 min, 4 °C). The cell paste was washed with sterile 0.9% NaCl, centrifuged as above, and suspended in 0.05 m sodium phosphate, pH 7.4, containing 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 25% glycerol (buffer P) in a 250-ml steel beaker (2 ml of buffer per g wet weight of cells). Cells were ruptured at 0 °C by ultrasonication (Bransonic, model W-225R) at 50% duty cycle, power level 7, for 5 min and centrifuged (16,300 × g, 30 min, 4 °C). The supernatant was used directly as the cell-free extract. Nucleic acids were precipitated with ethanol and centrifuged at 10,000 × g for 5 min at 4 °C. The resulting solution was adjusted to 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA and dialyzed overnight versus 2 liters of the same.

**S-Adenosylhomocysteine (AdoHcy):Agarose—**The dialyzed solution from above was diluted to 300 ml with dialysis buffer and loaded at a flow rate of 1 ml/min onto a 5-ml column of AdoHcy:agarose. The column was washed with 25 ml of the same buffer and an additional 25 ml of 50 mM buffer containing 1 mM AdoMet at the same flow rate. Subsequent chromatographic steps required the inclusion of 1 mM AdoMet in the buffer for activity to be retained.

**Nocardicin A:Agarose—**A 5-ml aliquot of the eluate from the AdoHcy column was loaded directly onto a 0.8-ml column of nocardicin A-equilibrated buffer P containing 1 mM AdoMet. After wash-
the column with 2 ml of the same buffer, it was eluted with 5 ml of buffer P containing 1 mM AdoMet, 1 mM nocardicin A, and 1 mM NaCl. This solution of homogeneous protein was concentrated using two Centricon-30™ concentrators to a final volume of about 1 ml. The combined concentrate was desalted on a prepacked column of Bio-Gel P6DG (10 mzl mL^-1) and used as buffer P containing 1 mM AdoMet.

Assay of Enzyme Activity—A Varian 5020 microprocessor-controlled liquid chromatograph equipped with a 250 × 4.6 mm Spherex (Phenomenex) 5-μm C-18 reversed phase column was used for HPLC assays. Column effluent was monitored by UV spectroscopy at 270 nm on an ABI 10005 Diode Array detector interfaced with a Waters 745 Data Module recorder in buffer P containing 1 mM AdoMet, 1 mM nocardicin A, and 0.1 mM sodium phosphate, pH 7.5, to yield a total volume of 15 ml. After standing for 1 h at 4 °C, the gel was washed with 1 liter of 0.1 mM NaCl, also at 4 °C. The washed gel was then equilibrated with 200 ml of 0.1 mM NaHCO₃, pH 9.0 (NaOH).

Cloning and Characterization of Gene Encoding NAT

N-terminal Sequence of Native NAT—NAT from the nocardicin A-agarose column was purified further by reversed phase HPLC on a Vydac C₁₈ column. After pre-equilibration of the column with 0.1% aqueous trifluoroacetic acid, peptide fragments were eluted with an aqueous buffer gradient (10–60%, each component containing 0.1% trifluoroacetic acid) over 30 min.

Purification and Sequence Determination of Lys-C-generated NAT Proteolytic Fragments—NAT purified by nocardicin A-agarose column chromatography was treated with Lys-C endoprotease as described by the supplier (Sigma). The resulting peptide fragments were separated by reversed phase HPLC on a Vydac C₁₈ column. After pre-equilibration with 0.1% aqueous trifluoroacetic acid, peptide fragments were eluted with an aqueonitrile:water gradient (0–60%, each component containing 0.1% trifluoroacetic acid) over 30 min. NAT from the nocardicin A-agarose column was purified further by reversed phase HPLC on a Vydac C₁₈ column. After pre-equilibrium with 0.1% aqueous trifluoroacetic acid, peptide fragments were eluted with an aqueonitrile:water gradient (0–90%, each component containing 0.1% trifluoroacetic acid) over 30 min. Peptide 1-23-22 was further purified by a second gradient (5–35%) over 60 min, and similarly peptide 1-30-40 was further purified with a gradient (10–40%) over 60 min. The purified peptides were sequenced.

Genomic DNA Preparation from N. uniformis—High molecular mass (50 kb) N. uniformis gDNA was prepared using the cetyltrimethylammonium bromide, large scale bacterial genomic DNA procedure described by Ausubel et al. (39). RNA was removed by an overnight incubation at 37 °C with DNase-free RNase A (final concentration 40 μg/ml).

PCR Amplification of N. uniformis DNA Encoding a Portion of NAT—Amplification of the 5’ end of the NAT gene utilized one primer designed from the N-terminal sequence (1-30-40NQ) and two primers designed from the internal Lys-C peptide sequence (1-23-22CE and 1-23-22CD). Degeneracy of all three primers was minimized by using the reported Nocardia codon preferences (40). A typical 100-μl reaction in 1× cloned Pfu buffer contained 200 ng of N. uniformis gDNA, 250 mM of each primer, 200 μM of each dNTP, and 5% glycerol. The reaction mixture was overlaid with mineral oil, denatured for 5 min at 95 °C, and, upon the addition of 2.5 units of cloned Pfu polymerase, was subjected to 30 cycles (94 °C for 3 min and 72 °C for 1 min) and one cycle of (94 °C for 1 min and 72 °C for 10 min) in an Eppendorf Microcycler (Fremont, CA). PCR products were separated on a non-denaturing polyacrylamide gel. The desired band was excised, eluted from the gel using Smal-digested pBluescript II SK−, and transformed into E. coli XL1-Blue MR/F cells. Recombinant pBluescript II SK− was purified and sequenced.

Preparation of an N. uniformis Genomic Sublibrary—A non-degenerate oligonucleotide derived from the PCR-amplified 5’ end of the gene encoding NAT (transferrase oligonucleotide probe (TOP), TAC GAC CTG TTC TTC CTC) hybridized to a single 6.3-kb PstI fragment in 3× tetramethylammonium chloride, 50 mM NaPO₄, pH 6.8, 0.2% SDS, and
5× Denhardt's solution at 52 °C on a dried agarose gel (41–44). According to many, 30 μg of N. uniformis gDNA was digested with 500 units of PstI at 37 °C for 12 h followed by a 1-h incubation at 37 °C with an additional 100 units of enzyme. Using standard procedures, the digested DNA was precipitated, washed with 70% ethanol, and dissolved in 45 μl of dd H2O (39). The digested DNA was resolved on a 0.6% agarose gel and the DNA between 6 and 9 kb was excised and isolated.

The isolated PstI fragments were ligated into PstI-digested shrimp alkaline phosphatase-treated pBluescript II SK(–). The ligation product mixture was transformed into E. coli XL 1 BlueMRF(–) cells and grown on LB agar containing 90 μg/ml ampicillin, overlaid with isopropanol-96% galactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside at 37 °C. Recombinant colonies (200) were regrown on nitrocellulose filters and screened by hybridization with the end-labeled [32P]-TOP, as described above for the genomic digest. A single colony was isolated that contained a 6.3-kb insert, pSDB 05-152.

**DNA Sequencing and Analysis—** Sequencing of PCR products was accomplished using Sequenase-2.0 DNA polymerase as described by U. S. Biochemical Corp. using either the appropriate PCR primer or universal primers.

The DNA sequence of pSDB 05-152 was obtained by primer walking and using automated fluorescent sequencing methods. Sequence data were edited and compiled in Seqencher™ (Gene Codes Corp., Ann Arbor, MI) and analyzed with the GCG software package (Version 8, Genetics Computer Group, Madison, WI). Data base searches were performed online with BLAST 2 at the National Center for Biotechnology Information (45–47), and sequence alignments were made using PIMA through the Baylor College of Medicine.

**RESULTS**

The purification of AdoMet:nocardicin 3-amino-3-carboxypropyltransferase (hereafter referred to as the "transferase" or NAT) was begun by preparing a cell-free extract of N. uniformis in phosphate buffer by sonication. Nucleic acids and a significant fraction of cellular protein were removed using polyethyleneimine. A single ammonium sulfate cut at 60% saturation precipitated remaining proteins. The transferase activity in the pellet was stable for 2 weeks at 4 °C in the presence of protease inhibitors and 25% glycerol. The transferase was inactivated by high ionic strength (~0.2 or greater), but activity was partially regained by removing the salts by dialysis or gel filtration.

The effectiveness of chromatographic techniques was assessed by specific activity determinations. Anion exchange was plagued by nearly complete loss of activity using DEAE-Sepharose, DEAE-Sepharose, or DEAE-Trisacryl. Hydroxypatite and hydrophobic interaction chromatography were also unsatisfactory, suffering from poor resolution and low recovery, especially in the case of hydrophobic interaction, suggesting NAT is significantly hydrophobic in character.

Dye-ligand chromatography was attempted in an effort to solve the recovery problem. Adsorption of NAT to dye gels was anticipated as the adenosyl moiety of AdoMet resembles the adenosyl moiety of AdoMet as a pseudoaffinity step conducted under hydrophobic interaction conditions (48). In approaches to the purification of the transferase, agarose columns modified with blue, red, yellow, green, or brown triazine dyes (Sigma) were tested. NAT was, in all cases, either not bound or bound irreversibly.

Affinity chromatography was turned to in order to circumvent the problems of lack of specificity and low recovery of activity encountered with conventional solid supports. Many enzymes requiring adenosyl sulfur compounds (AdoMet, S-bromobutylated AdoMet, S-adenosyl homocysteine, AdoHcy, methylthioadenosine) use substrates that have been purified by affinity chromatography using substrate, product, or an analog of either as ligands (37, 49–64). For example, S-formycinylhomocysteine, a substrate analog modified in the purine ring, coupled to α-aminohexyl-Sepharose 4B was utilized to achieve a 25-fold purification of S-adenosylhomocysteine/5’-methylthioadenosine nucleosidase (53). Spermidine synthase from bovine brain was purified 570-fold utilizing S-adenosyl-(5’)-3-thiopropylamine (decarboxylated AdoHcy)-Sepharose as the adsorbent. In this gel, the ligand forms a sulfonium linkage to the solid support by displacing the bromide of bromoacetyl-Sepharose (59). A similar coupling strategy was employed in the synthesis of AdoHcy-Sepharose (37). A 360-fold purification of AdoMet:protein-carboxy O-methyltransferase was achieved from an ammonium sulfate pellet using 20 μM AdoMet in dilute phosphate buffer to release the enzyme (37).

Ligands proposed for affinity chromatography were screened by testing for inhibition of the 3-amino-3-carboxypropyl transferase in a partially purified cell-free extract processed through ammonium sulfate precipitation and dialysis. If inhibition was observed, it was assumed that the potential ligand bound to the enzyme. After a number of largely unsuccessful trials with ligands that were positively charged at C-5’, neutral AdoMet analog ligands were examined in order to eliminate ion exchange effects on the affinity column. Peptide coupling of AdoHcy 9 (Fig. 3) to 6-aminoxenoxylic acid-agarose provided a gel with residual carboxylic acid functionalities (versus amines), precluding anion exchange interactions with the residual unmodified groups on the spacer arm. The identity of the solid support itself was also found to be important; much milder conditions were required for desorption of NAT from agarose versus Sepharose.

In buffer P, the protein solution processed through ammonium sulfate precipitation and dialysis was introduced at a flow rate of <1 ml/min onto AdoHcy-agarose. Under these conditions, >90% of the total protein was not adsorbed, whereas the transferase was quantitatively retained. A stepwise elution using 1 mM AdoMet was found to be as effective as a gradient in the final purification of the transferase, producing a protein solution increased in specific activity by 20-fold. In favor of the stepwise elution, salt did not have to be removed, and the enzyme was found to be greatly stabilized by the high concentration of AdoMet. This procedure is particularly appealing as it is a true affinity process; the enzyme binds to an immobilized inhibitor and is released by the natural substrate. This purification step is shown in the SDS-polyacrylamide gel depicted in Fig. 4. NAT is the major protein band at this point (lane 4), contaminated by about 15 other bands of lower intensity.

After partial purification on the AdoHcy column, a second affinity column, nocardicin A-agarose, was designed to take advantage of the co-substrate specificity of NAT compared with other AdoMet-requiring enzymes. Nocardicin A was coupled by the carbodiimide method first to aminohexyl-agarose (Fig. 3), forming a peptide bond with carboxylic acid functionalities at C-10’ and C-10 of 1, and second to 6-aminoxenoxoic acid agarose, forming a peptide bond with the amino group of C-9’. Neither matrix was effective as a purification tool for the transferase owing to poor recovery of activity. In contrast, when nocardicin A was attached by the bromoacetyl method, the resulting gel was successful in the purification of the transferase. In this preparation, attachment of 1 to the gel is postulated to occur at the C-9 phenol versus the α-amino acid. This alternative position of coupling could provide the sterically necessary for optimized binding.

The active fraction from AdoHcy-agarose was loaded directly onto the nocardicin-agarose column. The enzyme was completely retained. Again, elution was optimal using a stepwise procedure with buffer P containing 1 mM nocardicin A, 1 mM AdoMet, and 1 M NaCl. This protein solution was homogeneous by SDS-PAGE, showing a single band at about 38 kDa. For optimal recovery of activity, the enzyme sample was rapidly
concentrated using Centricon-30® microconcentrators followed by desalting on Bio-Gel P6DG. The optimized purification scheme is shown in Table I accompanied by the gel in Fig. 4. This preparation of the transferase is homogeneous not only by SDS-PAGE but also by gel filtration (Superose), reversed phase chromatography (Vydac Protein C4), and ion exchange chromatography (Mono-Q®). Several of these preparations were silver-stained, again yielding a single band, even on severely overloaded gels.

Properties—The product of NAT was expected to be isonocardicin A (t at C-9), resulting from direct S,2 displacement of the 3-amino-3-carboxypropyl group of L-AdoMet by the phenolic hydroxyl group of nocardicin E. An authentic sample of isonocardicin A was found to be indistinguishable from nocardicin A by the HPLC conditions of the standard assay as well as by UV and 1H NMR spectroscopy at 400 MHz (19). The presence of an epimerase capable of interconverting nocardicin A and isonocardicin A has been demonstrated in crude and partially purified cell-free extracts of N. uniformis (19); therefore, the actual stereochromic requirements at C-9 of the transferase reaction remained in doubt. Stated another way, it remained to be determined if the transferase and epimerase were separate enzymes. Additionally, it could be possible that the epimerase acts upon AdoMet and then v-AdoMet is used by the transferase. To address these questions, the AdoHcy-agarose-purified transferase was incubated with nocardicin E and AdoMet on a large scale. The resulting product was purified by preparative HPLC and derivatized with 2,3,4,6-tetra-O-acyl-β-D-glucopyranosylisothiocyanate (GITC) (36). An HPLC comparison with GITC-derivatized samples of both nocardicin A and isonocardicin A yielded the expected result; the product of the transferase was indeed isonocardicin A. Accordingly, it can be concluded that the epimerase is a separate enzymatic activity and that the transferase is specific for the natural stereoisomer of AdoMet.

A Superose® 6HR column calibrated with commercial gel filtration standards was used to determine the native molecular weight. The transferase activity eluted as a single peak at 40.5 kDa, showing that the transferase is active as a monomer. Many small molecule (65–68) and DNA methyltransferases (69, 70) are monomers, especially those isolated from non-mammalian sources. Spermine and spermidine synthase, on the other hand, appear to be dimers of identical subunits (59, 61, 62). The data are less conclusive in the case of nicotineamine synthase. SDS-PAGE indicated a molecular mass of about 30 kDa, whereas the estimate by gel filtration was 40–50 kDa (27).

Kinetics—Analyses were undertaken to characterize the kinetic mechanism of the 3-amino-3-carboxypropyl transfer, to determine the kinetic constants for the natural substrates, and to determine the inhibition type and magnitude for the affinity ligands used in the purification. The high specific activity fraction from the AdoHcy column was chosen for further study.

Prior to these studies, it was determined that no competing activities that consumed nocardicin E or AdoMet were present. The transferase is an example of a BiBi system in which two substrates combine with the enzyme and two products are released. Recognizing that inversion of stereochromic observed for the 3-amino-3-carboxypropyl addition in the present case and in the 3-aminopropyltransferase involved in polyanine biosynthesis suggested direct displacement (55, 56, 58, 71–75), the transferase was expected to operate by a sequential mechanism. In the large majority of cases, methyltransferases utilize a sequential mechanism, although significant variation has been observed with respect to ordered or random substrate binding (76). The steady state rate equation for a sequential mechanism in the absence of products can be expressed as shown in Equation 1 (77).

\[ V = \frac{V_{\text{max}}[A][B]}{K_{\text{m}A} + [A][B] + K_{\text{m}B} + [A][B] + K_{\text{m}A}K_{\text{m}B}} \]  

where \( V_{\text{max}} \) is the maximum initial velocity when both A and B are saturating; \( K_{\text{m}A} \) is the concentration of A which gives 0.5 \( V_{\text{max}} \) when B is saturating; \( K_{\text{m}B} \) is the concentration of B which gives 0.5 \( V_{\text{max}} \) when A is saturating; and \( K_{A} \) is the dissociation constant for \( E + A \) in equilibrium with \( EA \).

If either substrate is saturating, Equation 1 reduces to the simple Michaelis-Menten equation. With these assumptions made, the \( K_{\text{m(app)}} \) and \( V_{\text{max(app)}} \) values for AdoMet, nocardicin E, nocardicin F, and nocardicin G were determined. The experimental data are shown in Table II. Numerical results were obtained by a computerized nonlinear regression fit of the experimental data to the direct linear plot of velocity versus substrate concentration (33, 34).

In the case of nocardicin G (7), it was impossible to conduct the reactions under Michaelis-Menten conditions (less than 10–15% of the substrate consumed). The enzyme was not diluted to slow the rate because it was desirable to use same sample of enzyme at the same concentration for the \( K_{m} \) determination for each of the nocardicin substrates to allow direct comparison of the \( K_{m} \) and \( V_{\text{max}} \) values. Furthermore, the required dilution would have produced inactivation.

Owing to the excess consumption of nocardicin G, it was no longer possible to assume a constant substrate concentration, making the use of the Michaelis-Menten equation invalid. Thus, the reduced substrate concentration (Equation 2) was used for the calculations. The reduced substrate approximation makes use of the close agreement between Equation 2 and the parenthetical term in the integrated rate equation (Equation 3), which is valid for all extents of reaction (77).

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**Fig. 3.** Ligands utilized for the affinity purification of the transferase.

**Fig. 4.** 10% SDS-PAGE analysis of the purification of the transferase. Lanes 1, 2, 6, and 7 are molecular weight markers. Lane 3 is the protein fraction from the 60% ammonium sulfate pellet. Lane 4 is the active protein fraction eluted from the AdoHcy-agarose affinity column. Lane 5 is the homogeneous enzyme recovered from the nocardicin A-agarose affinity column. Lane 9 shows the fraction of protein that was not adsorbed to the nocardicin A column (note that all bands from the AdoHcy column are present except for that of the transferase).
**AdoMet-dependent 3-Amino-3-carboxypropyltransferase**

**Table I**

Optimized purification protocol for AdoMet:nocardicin 3-amino-3-carboxypropyltransferase

| Sample                  | [protein] mg/ml | Volume ml | Total protein mg | Specific activity nmol/min/mg | Total units | Purification fold | Recovery % |
|-------------------------|-----------------|-----------|------------------|-----------------------------|-------------|-------------------|------------|
| 60% ammonium sulfate    | 3.42            | 300       | 1026             | 0.20                        | 29.5        | 1                 | 100        |
| AdoHcy-agarose          | 0.15            | 50        | 7.5              | 3.93                        | 20          | 2                 | 14         |
| Nocardicin A-agarose    | 0.06            | 2.4       | 0.14             | 11.7                        | 58          | 54                |            |

*The values are based on 5.0 ml of AdoHcy eluate introduced onto the nocardicin column (0.75 mg of protein, 2.95 units).*

where \([S_0]\) is the initial substrate concentration and \([S]\) is the final substrate concentration (\([S_0] - [\text{product}]\)).

\[
\frac{1}{V} = \frac{K_m}{V_{max}} \left( \ln \left( \frac{[S]}{[S]_0} \right) + 1 \right)
\]

With the kinetic constants in hand, an initial velocity plot (77) was constructed using the nocardicin F/nocardicin B conversion. The experiment was conducted by simultaneously varying the concentration of AdoMet and nocardicin F. Each data point was recorded in triplicate, and the averages were fit to an ordered BiBi mechanism using the computer programs developed by Cleland (34). The expected sequential mechanism predicts an intersecting initial velocity pattern (77), and this was the observed result, shown in Fig. 5. The intersecting plot eliminates a ping-pong mechanism.

From this plot, the following information can be extracted. First, the intersection point above the 1/[nocardicin F] axis for the family of lines in the double-reciprocal plot shows that the binding of one substrate increases the affinity of the transferase for the other. Second, the \(K_{m(app)}\) for the varied ligand decreases as the concentration of the fixed substrate increases. This is a result of increased saturation of the enzyme with the fixed substrate. The \(K_m\) values calculated from the computer fit of the data set are 158 ± 15 \(\mu\)M for AdoMet and 0.5 ± 0.4 \(\mu\)M for nocardicin F. These values represent the true \(K_m\) for each substrate at an infinite concentration of the other, and, as expected, are lower than the values generated under pseudo-first order conditions (77).

**Gene Cloning and Characterization of NAT**—To obtain the primary structure of NAT and compare it to other S-adenosylmethionine-dependent enzymes, amino acid sequence data were sought to identify its encoding gene by reverse genetic methods from *N. uniformis* DNA. A sample of homogeneous enzyme from the nocardicin A-agarose affinity chromatography step was isolated by HPLC and gave N-terminal sequence. Another portion of affinity purified NAT was digested with Lys-C endoproteinase and gave several fragments on reversed phase HPLC. Two well resolved peaks were collected and gave stoichiometrically credible sequence data based on the amount of NAT analyzed. The data obtained for each of these fragments and the N terminus are shown in Fig. 6. Fortuitously, one of these sequences, 1-30-40NQ, overlapped with the N-terminal amino acid sequence of NAT. Therefore, peptide 1-23-22 must reside toward the C-terminal end of the protein. The unambiguous relative position of the peptide fragments allowed the design of a PCR experiment which amplified that portion of the gene encoding these peptide fragments and the intervening DNA. The design of each PCR primer was based on the corresponding peptide fragment and took advantage of the reported Nocardia codon preferences (40) to minimize degeneracy. Primer 1-23-22CE was nested (21 nt) within primer 1-23-22CD, providing an internal control to reduce the chance of cloning spurious PCR products.

The PCR reactions containing both primer pairs 1-30-40NQ with 1-23-22CE or 1-23-22CD, gave products of 122 and 143 nt in length, respectively. Both PCR products were sequenced directly. Additionally, the 143-base pair PCR product was cloned into the Smal site of pBluescript II SK(-), and the entire PCR product was sequenced. DNA sequence of both PCR products agreed with the amino acid sequence of the peptide fragments (Fig. 6). From these nucleotide sequences a nondegenerate oligonucleotide, TOP, was designed both to screen restriction digests of genomic DNA and to prime directly a sequencing reaction to confirm that the NAT-encoding gene had been found.

In dried agarose gel hybridizations in 3 \(\times\) tetramethylammo-
nium chloride (41–44), the $^{32}$P-TOP hybridized to discrete fragments of different restriction digests of *N. uniformis* genomic DNA, which include a 6.3-kb *Pst*I, a 15-kb *Apa*I, and a 20-kb *Kpn*I fragment. Based on these hybridizations, a *Pst*I genomic sublibrary was constructed by cloning digested and size-fractionated (6–9 kb) *N. uniformis* genomic DNA into the *Pst*I site of pBluescript II SK(+) and transforming into *E. coli* XL 1 Blue MRF9 cells. Colony hybridizations with labeled TOP led to the isolation of one clone containing the NAT gene, pSDB 05-152. Immediate sequencing with TOP secured the presence of *nat*, and primer-walking completed its double-stranded sequencing. The full sequence revealed that NAT is encoded by an open reading frame of 906 base pairs, *nat*, corresponding to 301 amino acids. The deduced protein has a molecular mass of 32,386 Da, which is in modest agreement with a molecular mass of 30 kDa for the native protein estimated by SDS-PAGE and gel filtration chromatography. The GC bias and codon preferences observed for *nat* were consistent with reported *Nocardia* genes (40) as well as those for the closely related, but better studied, actinomycete *Streptomyces* (78, 79).

Upstream 11 nt from the initiation codon lies a conserved *Streptomyces* ribosomal binding site (GGAGG), but obvious transcription terminator and promoter sequences were not identified (80). The deduced amino acid sequence of NAT was in good agreement with the experimentally determined 18 N-terminal amino acids and in complete accord with the amino acid sequence determined for the internal Lys-C peptide 1-23-22.

**Primary Structure Analysis of NAT**—By using the BLAST 2.0 algorithm, the translated amino acid sequence of NAT was compared against the Brookhaven protein, SWISS-PROT, PIR, and GenBank™ data bases (45–47). These analyses indicated quite weak similarity between NAT and a group of bacterial AdoMet-utilizing enzymes and even poorer homology to mammalian enzymes. The most significant scores were represented by two methyltransferases and two putative methyltransferases. However, a spermidine synthase identified in the *Mycobacterium tuberculosis* genome sequencing project exhibited weaker similarity. Despite the poor overall homology to AdoMet-utilizing enzymes, NAT possesses three conserved motifs proposed in AdoMet or AdoHcy binding (Figs. 7 and 8) (29). The homology that does exist between NAT and the methyltransferases is localized within the proposed AdoMet/AdoHcy binding motifs. Moreover, as predicted by Kagan and Clarke (29), a conserved acidic residue was observed 19 amino acids C-terminal to motif I (Asp-191) followed immediately by a hydrophobic residue (Leu-192) noted by Cheng et al. (81).
matrices was superior to Sepharose. The reason for this observation is unknown. Third, the two substrate-two product reaction mechanism of NAT afforded the opportunity to design complementary affinity columns that exploited each binding site in turn to give an extremely efficient purification. The successful affinity steps are true, bioisocpic affinity procedures. AdoHcy was demonstrated to be a competitive inhibitor of the transferase, the enzyme bound to the immobilized inhibitor, and was released by the natural substrate AdoMet. Even though nocardicin A is an epimer of the product of the reaction, the enzyme bound to the column containing it and was released by excess nocardicin A in the elution buffer.

The relative values of the \( K_{\text{m(app)}} \) deserve comment. The value of 160 \( \mu M \) for AdoMet (from the initial velocity experiment) is within the widely ranging literature values for the \( K_{\text{m(app)}} \) for O-methyltransferases. For example, AdoMet:aminoacyl-6-O-methyltransferase operates with a \( K_{\text{m(app)}} \) for AdoMet of 260 \( \mu M \) (65). In contrast, catechol O-methyltransferase has a \( K_{\text{m(app)}} \) of 3.2 \( \mu M \) (82), identical to the value for emodin O-methyltransferase (48). The \( K_{\text{m(app)}} \) for AdoMet for discadenine synthase is reported to be 18.5 \( \mu M \) (28). The kinetic profile of nicotianamine synthase (27) has not been determined. As none of the other 3-amino-3-carboxypropyltransferases has been purified, it is impossible to compare the value reported herein to them. The \( K_{\text{m(app)}} \) for decarboxy-AdoMet in spermidine synthesis is less than 1 \( \mu M \) (59, 61), but it must be borne in mind that decarboxy-AdoMet is present in the cell at a much lower concentration than AdoMet (62).

It was somewhat surprising to observe that the \( K_{\text{m(app)}} \) obtained for nocardicin G was approximately five times lower than the value for nocardicin E. In parallel with these data was the observation that the \( V_{\text{max/Km}} \) value for nocardicin G was 12 times higher than for nocardicin E at the same enzyme concentration. This could suggest that nocardicin E is not the principal substrate in vivo. If this is true, then the order of amine oxidation and homoserine side chain attachment proposed in the introduction (Fig. 2) should be reversed. An experiment demonstrating conversion of either nocardicin G to nocardicin E or isonocardicin G (9' epimer of 3') to isonocardicin A (9' epimer of 1') would be required to unambiguously ascertain the true order of the biosynthetic reactions. To date, all attempts to observe amine oxidation (oxime formation) have been unsuccessful.

NAT is the first example of an enzyme that catalyzes the transfer of the 3-amino-3-carboxypropyl side chain from AdoMet to homoserine. Although rare, this transfer is not unprecedented. In this case, the nucleophile is a phenol, much like the substrate of catechol O-methyltransferase and many O-methyltransferases of secondary metabolism. However, as opposed to the more electrophilic, more accessible methyl group, the sterically hindered 3-amino-3-carboxypropyl moiety is transferred to the nucleophile acceptor. For nocardicin-producing organisms, perhaps the transferase evolved from an O-methyltransferase since the 3-amino-3-carboxypropyl side chain increases the biological activity of the antibiotic (4). Major structural changes in the protein would be required for such an evolution; the new catalytic site would need to shield the methyl group while exposing the 3-amino-3-carboxypropyl side chain to nucleophilic attack. Stereochemical inversion observed in both of these transferase reactions requires in-line geometry of the nucleophile, in these cases a phenol, and the electrophile AdoMet.

Catechol O-methyltransferase cloned from rat liver has been crystallized in the presence of a competitive inhibitor, Mg\(^{2+}\) ion and AdoMet (83). Unfortunately, primary sequence comparisons between catechol O-methyltransferase and NAT reveal very low similarity and afford no structural insight to account for reaction at one or the other electrophilic sites in AdoMet. Alignment of NAT to a group of spermidine synthases, which catalyze the chemically analogous 3-amino-3-propynol transfer with stereochemical inversion from decarboxy-AdoMet (14–16), also shows only very weak similarity. In fact, the sequence similarity among AdoMet-requiring enzymes, and even the more limited subset of methyltransferases, is widely recognized to be poor (84–86). Despite this common generalization, high resolution structural studies of methyltransferase enzymes acting on substrates as diverse as DNA (87–89), RNA (86, 90, 91), proteins (85), and small molecules (83, 92) all contain AdoMet binding domains of marked topological similarity.

Kagan and Clarke (29) identified three loosely conserved signature motifs in non-DNA methyltransferases and spermidine synthases. The significance of motif I is generally recognized for both small molecule/protein and DNA/RNA methyltransferases. Recent x-ray crystal structures of members of each of these groups has shown that this motif is involved in the creation of a binding pocket for the methionine and ribose moieties of AdoMet. An acidic residue found 17–19 amino acids C-terminal to motif I was identified by Kagan and Clarke (29). Structural correlations reveal hydrogen bonding between this conserved residue and the hydroxyl groups of the AdoMet ribose, whereas the side chain of the next adjacent amino acid packs against the face of the adenine ring. The central Asp, Glu, Asn, or Gln characteristic of motif II forms a hydrogen bond to the exocyclic amino group of this nucleoside base. Inspection of the alignment in Fig. 8 shows that NAT contains each of these critical amino acids. In addition, the typical separations of motifs I and II (57 ± 22) and motifs II and III (22 ± 5) reported by Kagan and Clarke (29) were found in NAT to be 47 and 22, respectively. This good agreement of motif composition and relative location suggests that NAT has an AdoMet-binding site quite similar to those of the methyltransferases. The structural basis for the reaction with AdoMet to transfer a 3-amino-3-carboxypropyl group rather than methyl will reside in the orientation of substrate binding relative to this domain to fulfill the geometric requirements of an \( S_n \) displacement reaction.
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