The Active Conformation of Avilamycin A Is Conferred by AviX12, a Radical AdoMet Enzyme*

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The antibiotic avilamycin A is produced by Streptomyces viridochromogenes Tu57. Avilamycin belongs to the family of orthosomycines with a linear heptasaccharide chain linked to a terminal dichloroisoeverninic acid as aglycone. The gene cluster for avilamycin biosynthesis contains 54 open reading frames. Inactivation of one of these genes, namely aviX12, led to the formation of a novel avilamycin derivative named gavibamycin N1. The structure of the new metabolite was confirmed by mass spectrometry (MS) and NMR analysis. It harbors glucose as a component of the heptasaccharide chain instead of a mannose moiety in avilamycin A. Antibacterial activity tests against a spectrum of Gram-positive organisms showed that the new derivative possesses drastically decreased biological activity in comparison to avilamycin A. Thus, AviX12 seems to be implicated in converting avilamycin to its bioactive conformation by catalyzing an unusual epimerization reaction. Sequence comparisons grouped AviX12 in the radical S-adenosylmethionine protein family. AviX12 engineered with a His tag was overexpressed in Escherichia coli and purified by affinity chromatography. The iron sulfur cluster [Fe-S] present in radical AdoMet enzymes was detected in purified AviX12 by means of electron paramagnetic resonance spectroscopy.

Avilamycins are oligosaccharide antibiotics isolated from Streptomyces viridochromogenes Tu57. Along with everninomycins, curamycins, and flambamycins, they belong to the orthosomycin group of antibiotics (1). Structural features of the avilamycins are a terminal dichloroisoeverninic acid moiety (Fig. 1, residue A) and a heptasaccharide chain consisting of d-olivose (residues B and C), 2-deoxy-d-epsilonose (residue D), 4-O-methyl-d-fucose (residue E), 2,6-di-O-methyl-d-mannose (residue F), l-lyxose (residue G), and eurekanate (residue H). Avilamycin A, the main compound produced by S. viridochromogenes Tu57, was shown to be active against many Gram-positive bacteria, including emerging problem organisms, such as vancomycin-resistant enterococci, methicillin-resistant staphylococci, and penicillin-resistant pneumococci (2). Everninomycin (Ziracin), which is structurally very similar to avilamycin, was under investigation for approval by Schering-Plough.

Due to side effects and its poor water solubility, further development was stopped in 2000 (2). Both avilamycin and everninomycin were shown to inhibit protein biosynthesis by binding to the 50 S ribosomal subunit of the bacterial ribosomes (4–6). Recently, we reported that methylation of G2535 and U2479 in domain V of the 23 S rRNA confers resistance to avilamycin by preventing the antibiotic from binding to the ribosome (7). This was in accordance with results obtained by footprinting avilamycin on Escherichia coli ribosomal subunits (8). Based on these data, it is suggested that avilamycin interacts with the ribosomal A site and interferes with initiation factor IF2 and tRNA binding.

The complete avilamycin biosynthetic gene cluster containing 54 open reading frames was sequenced (9). The corresponding genes were named avi. Based upon sequence similarities of the deduced proteins to enzymes of known function in the data base, a putative biosynthetic pathway for avilamycin has been proposed (9). Gene disruption experiments with putative methyltransferase genes have led to new avilamycin derivatives with enhanced water solubility, named gavibamycins (2). After deletion of aviB1 and aviO2, components of an incomplete pyruvate-dehydrogenase complex, an avilamycin derivative was obtained lacking the terminal acetyl residue at position C-4 of the eurekanate moiety of avilamycin A (10).

It is most likely that the biosynthesis of avilamycin starts with the formation of the unusual pentose L-lyxose. The next plausible steps toward formation of the heptasaccharide chain might be the unusual C1-C1 linkage between lyxose and mannose and subsequently the attachment of the eurekanate to the l-lyxose moiety. A knock-out mutation in aviE2, the gene encoding a UDP-glucuronic acid decarboxylase, led to a breakdown of avilamycin biosynthesis, confirming the presumption of the start of avilamycin biosynthesis in the coupling of lyxose and mannose (11). Inactivation of aviG74, a putative glycosyltransferase, led to the formation of a new avilamycin derivative lacking the terminal eurekanate residue (11).

There still are genes with unknown function in the avilamycin biosynthetic gene cluster. One of these genes is aviX12, positioned in the center of the avilamycin biosynthetic gene cluster in proximity to methyltransferase and sugar biosynthetic genes (9). AviX12 shows no significant similarities to proteins of known function, but it contains a sequence motif typical for the radical S-adenosylmethionine (AdoMet)2 protein family. Members of this family are among others involved in oxidative processes and making AviX12 a good candidate for oxidative

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‡ The abbreviations used are: AdoMet, S-adenosylmethionine; DTT, dithiothreitol; MS, mass spectrometry; HPLC, high performance liquid chromatography; mT, millitesla(s).
reactions in the avilamycin biosynthesis, such as the building of the methylene bridge at the terminal eurakeanate moiety or the orthoester linkages. To gain insight into the function of AviX12, aviX12 was inactivated, and the structure of a new avilamycin derivative that accumulated in this mutant was determined. Our results indicated the involvement of AviX12 in the formation of the biologically active conformation of avilamycin A by catalyzing an unusual epimerization reaction. Furthermore, aviX12 was modified with a His tag and overexpressed in E. coli. The protein was purified by affinity chromatography, and the bound [Fe-S] cluster was characterized by means of EPR spectroscopy.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Conditions**—DNA manipulation was carried out using E. coli XL-1 Blue MRF (Stratagene) as the host strain. Before transforming S. viridochromogenes strains, plasmids were propagated in E. coli ET 12567 (dam–, dcm–, hsdS, CmR) (12) to obtain unmethylated DNA. E. coli BL21 (DE3) pLysS (Stratagene) was used for heterologous protein expression experiment. E. coli strains were grown on Luria-Bertani (LB) agar or liquid medium containing the appropriate antibiotic. S. viridochromogenes GW4 is a derivative of S. viridochromogenes Tu57, deficient in aviG4, a methytransferase gene, leading to the production of gabivamycin A1 with a free phenolic hydroxy group (9) (Fig. 1). Plasmids pBluescript SK– (pBSK–) (Stratagene) and pUC18 (New England Biolabs) were used for cloning. Plasmid pSP1 (13), conferring erythromycin resistance, was a kind gift of Dr. S. Pelzer (Combinature-Biopharm AG, Berlin, Germany), and pSET152 (14), conferring apramycin resistance, was obtained from Eli Lilly & Co. (Indianapolis, IN). The construction of pSET-1,erm has been described elsewhere (15).

**General Genetic Manipulation, PCR, and Sequence Analysis**—Routine methods were performed as described previously (16). Isolation of E. coli plasmid DNA, DNA restriction, DNA modification, and Southern hybridization were performed following the manufacturer’s directions (Amersham Biosciences, Roche Diagnostics, Promega, and Stratagene). Streptomyces protoplast formation, transformation, and protoplast regeneration were performed as described previously (17). PCR was carried out using a GeneAmp PCR System 9700 (Applied Biosystems). Oligonucleotide primers were purchased at Qiagen (Hilden, Germany). Computer-aided sequence analysis was done with the DNAsis software package (version 2.1, 1995; Hitachi Software Engineering). Data base searches were performed with the BLAST 2.0 program (18) on the server of the National Center for Biotechnology Information, Bethesda, MD.

**Gene Inactivation of aviX12**—AviX12 was PCR-amplified using oligonucleotides 5′-CTACCTGGAATTCTGCTGACC-3′ (aviX12F) and 5′-CAGGCCGCTACAGAATTCCAG-3′ (aviX12R) containing engineered EcoRI and XbaI restriction sites (underlined), respectively. The resulting fragment was isolated with the Nucleospin Prep kit (Macherey Nagel), digested with EcoRI and XbaI, and ligated into pUC18 previously digested with the same enzymes, generating plasmid pUC-aviX12. The product was confirmed by DNA sequencing (4base lab). pSET-1,erm was transferred to plasmid pSP1 to generate IKX12. The inactivation construct was used to transform protoplasts of S. viridochromogenes GW4. Transformants were propagated in HA medium without erythromycin selection for 16 generations. Selection of erythromycin-sensitive colonies gave the double crossover mutant S. viridochromogenes GW4-X12. The deletion within the gene was confirmed by PCR. PCR fragments obtained from mutant S. viridochromogenes GW4-X12 using primers 5′-GGCCGGACCCGGAGAAAGCCGGAG-3′ (aviX12P-F) and 5′-TCTTGGCAGCCCTTGCGCAACCA-3′ (aviX12P-R) could not be cleaved by SacII (Fig. 2a), whereas the PCR fragment obtained from S. viridochromogenes GW4 could be digested by SacII.

**Southern Hybridization**—For Southern hybridization, genomic DNA from mutant and wild-type strains was completely digested with SacII, fractionated by agarose gel electrophoresis, and transferred to a positively charged nylon membrane (Hybond N+; Amersham Biosciences). The probe was a 1302-bp EcoRI/XbaI fragment from IKX12 labeled with digoxigenin-dUTP by the random priming method. Hybridization of the probe with DNA fragments on the nylon membrane was detected by the chromogenic method using procedures described by Roche Diagnostics.

**Complementation of Mutant S. viridochromogenes GW4-X12**—To determine clearly that the mutation event affected only the desired gene and not other genes, aviX12 was ligated behind the ermE+ promoter of pSET-1,erm, and the resulting complementation construct pSETerm-aviX12 was introduced by protoplast transformation into the ΔaviX12 mutant. The complementation led to restored gavibacamycin production.

**Construction of pRSET-X12 Expressing AviX12**—The AviX12 gene was PCR-amplified using primers 5′-AGGGCCGGACCCCGAGC-3′ (X12ProtF) and 5′-CGTCTCAATTGG-3′ (X12ProtR) containing engineered PstI and EcoRI restriction sites (underlined), respectively. The product was isolated with the Nucleosep® Extract kit (Machery Nagel), digested with PstI and EcoRI, and ligated into the T7 expression vector pSETb (Invitrogen) previously digested with the same enzymes. The product pRSET-X12 was confirmed by DNA sequencing (4base lab).

**Expression and Purification of AviX12 (N-terminal His6-tagged)**—For purification of expressed protein, E. coli strain BL21 (DE3)pLysS cells (Stratagene), carrying either pRSET-X12 with aviX12 or the pRSETb vector alone, were grown in LB broth containing 50 μg/ml carbenicillin and 30 μg/ml chloramphenicol to an A600 of ~0.6. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and growth was continued at 37 °C for 4 h. Cells were harvested by centrifugation and stored at −20 °C. The cell pellet from the 100-ml culture was resuspended in 4 ml of lysis buffer consisting of 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, and 5 mM diethiothreitol (DTT). The cells were broken by a single pass through a French pressure cell (Thermo Spectronic) at 700 pounds/square inch. After centrifugation, the supernatant fraction was used for the purification procedure. Protein was bound to nickel nitrotriacetic acid-agarose (Qiagen) and loaded onto a column. The column was washed twice with washing buffer containing 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, and 5 mM DTT. The protein was recovered with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, and 5 mM DTT). The imidazole was removed by passing the protein over a PD-10 (Sephadex™ G-25; Amersham Biosciences) desalting column equilibrated in 10 mM Tris, pH 8.0, and 5 mM DTT. Expression and purification of AviX12 was monitored by SDS-PAGE (4% stacking gel and 12% resolving gel) followed by Coomassie Blue staining. The concentration of purified protein was estimated by the Bradford dye binding method (19). The molecular mass of the purified protein was determined by means of liquid chromatography/electrospray ionization-MS.

**Isolation of Avilamycin Derivatives**—Strains were grown in SG medium containing 2% glucose, 1% soy peptone, 0.1% CaCO3, 20 mM L-valine, and 1 ml of 0.1% CoCl2 solution for 72 h at 28 °C. Cultures were filtered, and the filtrate was extracted twice by ethyl acetate and evapo-

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rated to dryness. The mycelium was broken with acetone and filtered again. After evaporation of the acetone, the mycelium was also extracted with ethyl acetate and evaporated to dryness. Crude extracts from the filtrate and mycelium were combined and applied to a solid-phase extraction cartridge (SepPak C18; Waters Associates). The cartridge was eluted with 50 and 80% methanol. The 80% fraction contained ~90% of the avilamycin derivatives. The evaporated and lyophilized fractions were redissolved in acetonitrile and water. Further isolation was performed on an Agilent 1100 system using a semipreparative column (Zorbax SB-C18; 5 μm, 9.4 μm × 150 mm). For elution, the following gradient profile was used: solvent A, 5 mM ammonium acetate in water; solvent B, acetonitrile, nonlinear gradient, 30–50% of B within 20 min at a flow rate of 3.5 ml/min. A mass-based fraction collector was used to isolate the avilamycin derivatives. The final isolation step was performed using a gel permeation column (PLgel, 5 μm, 4.6 × 150 mm) at a flow rate of 3.5 ml/min. As a result, the acetonitrile at a flow rate of 1 ml/min was used. Again, a mass-based fraction collector was used to isolate the avilamycin derivatives.

Biological Properties—The antimicrobial activity of the new derivative was determined by agar plate diffusion assay using Bacillus subtilis as the test strain. Susceptibility of staphylococci, streptococci, and enterococci to different derivatives was determined by the microdilution test according to National Committee for Clinical Laboratory Standards guidelines. Vancomycin was used as a standard to ensure the reliability of the determined minimum inhibitory concentrations.

Structural Characterization of the New Gavibamycin Derivative Accumulated by S. viridochromogenes GW4-X12—High performance liquid chromatography (HPLC)/electrospray ionization-MS was performed on an Agilent 1100 Series system with an electrospray chamber and a quadrupole detector. HPLC analysis was carried out on a Zorbax SB-C18 column (5 μm, 4.6 × 150 mm; Agilent) with a Zorbax SB-C18 precolumn (5 μm, 4.6 × 12.5 mm; Agilent). A nonlinear gradient from 20 to 70% acetonitrile in 0.5% acetic acid over 22 min at a flow rate of 0.5 ml/min was used. The column temperature was 23 °C, and the UV detection wavelengths were 254 and 300 nm. The chamber settings were drying gas flow, 12 liters/min; nebulization pressure, 50 pounds/square inch on the gauge; and drying gas temperature, 300 °C. The samples were analyzed in positive and negative scan mode with a mass range of 700–1500 Da. NMR analysis spectra were recorded on samples dissolved in 330 μl of Me2SO-d6 in SHIGEMI® NMR (Tokyo, Japan) tubes at a temperature of 295 K on a Bruker DMX 750-MHz NMR spectrometer of the Bavaria NMR Center in Garching, Germany. The following spectra have been used for the structure elucidation: 13C-1D, 13C-filtered nuclear Overhauser enhancement spectroscopy (20), correlated spectroscopy (21), heteronuclear single quantum coherence (22), and heteronuclear multiple bond coherence (23). The spectra were analyzed using XWINNMR version 3.6 (Bruker) and SPARKY version 3.3.3.

Spectroscopic Procedures—For UV-visible spectroscopy, A TIDAS-UV1/1001–1 diode array spectrometer with 512 diodes (J & M Analytische Mess-μ. Regeltechnik, EmbH, Aalen, Germany) was used for UV-visible spectroscopy. Spectra were recorded in the range from 200 to 600 nm in a buffer of 10 mM Tris, pH 8.0, and 5 mM DTT. The spectrum of the 1-ml aliquot of the preparation, as isolated, corresponded to the spectrum of the oxidized protein. The sample was mixed in a stirred optical cell with a few grains of sodium dithionite at room temperature while spectra were continuously recorded every 2 s. When there was no more spectral shift, the spectrum corresponded to the spectrum of the reduced protein. Spectra were processed using the Spectrachrom software package (J & M, Aalen, Germany). For EPR spectroscopy, the EPR spectra of the air-oxidized and dithionite-reduced preparation were recorded with an X-band spectrometer EMX 6/1 (Bruker) equipped with a helium flow cryostat (Oxford). Spectra were recorded at a 9.46-GHz microwave frequency, a modulation amplitude of 0.6 mT, time constant of 0.164 s, and scan rate of 17.9 mT/min. The optimal signal was measured at a temperature of 10 K and 1 milliwatt of microwave power. Computer simulations of the spectra were performed using the program SimFonia (Bruker), assuming no hyperfine interaction and gaussian line shape.

Calculation of the Avilamycin Conformation—Possible conformers of avilamycin were identified in a two-step molecular modeling process. A low energy conformation was constructed and optimized within the ArgusLab program package (25, 26) using a classical unified force field (27). In the relaxed molecule (by visual inspection), five C–O single bonds were identified that permit rotations of major fragments of the molecule around in steps of 120 degrees. The corresponding S3 = 743 rotamers have been generated and checked for a significant atomic overlap using the repulsive part of the Lennard-Jones potential applying standard van der Waals radii. The geometry of each of the conformers has been optimized by a combination of Broyden-Fletcher-Goldfarb-Shanno and steepest descent techniques. The energy at the nine conformational minima has again been calculated using the unified force field referenced above.

GenBank™ Accession Number—The GenBank™ accession number for the DNA sequence reported in this paper is AAK83189.1.

RESULTS

Inactivation of aviX12—The inactivation of aviX12 was achieved by insertion of a frameshift mutation at a singular SacII restriction site central in aviX12 (Fig. 2a). As the wild-type strain for the inactivation experiment, S. viridochromogenes GW4 was chosen, a mutant carrying a deletion in the methyltransferase gene aviG4 leading to the production of gavibamycin A1 and A3 (Fig. 1). Protoplasts of S. viridochromogenes GW4 transformed with inactivation construct IKX12 were screened for a erythromycin-resistant phenotype. The strains selected were propagated without erythromycin selection. Two erythromycin-sensitive strains were examined by PCR; one of these proved the deletion of aviX12. This was verified by Southern hybridization (Fig. 2b). The mutant strain was designated S. viridochromogenes GW4-X12. HPLC analysis showed the production of new gavibamycin derivatives in comparison to the wild-type strain.

Identification of New Avilamycin Derivatives in S. viridochromogenes GW4-X12—For analysis of secondary metabolite formation, both wild type and the ΔaviX12 mutant were cultivated in production medium as described under “Experimental Procedures.” Ethyl acetate extracts of the culture supernatants were analyzed by HPLC-UV and HPLC/electrospray ionization-MS. Gavibamycin A1 (1388 atomic mass units) and gabibamycin A3 (1390 u) were detected in extract of S. viridochromogenes GW4. In contrast, the ΔaviX12 mutant accumulated four new compounds with atomic mass units of 1376, 1374 (main compound), 1332, and 1262, respectively. The mass of the main compounds indicate the loss of a methyl group in comparison to the main compounds of the wild-type gavibamycin A1 (1388 atomic mass units) and gabibamycin A3 (1390 atomic mass units). The new compounds were named gabibamycin N1 (1374 atomic mass units) and gabibamycin N3 (1376 atomic mass units). The other two compounds could be related to a derivative missing in addition to the methyl group, the acetate moiety at the eurekanate (1332 atomic mass units), and a derivative missing additionally.

2 T. D. Goddard and D. G. Kneller, personal communication.
the isobutyryl group at the lyxose moiety (1262 atomic mass units), respectively.

**Structure Elucidation of the Gavibamycin A1 and A3 Derivatives**

To elucidate the structure of the main compound of mutant *S. viridochromogenes* GW4-X12, this product was isolated as described under “Experimental Procedures.” The NMR analysis of the mutants in this study is based on the completed $^1$H and $^{13}$C resonance assignment of avilamycin A and avilamycin C (2). The samples under investigation were prepared by feeding the bacteria $^{13}$C-labeled L-methionine resulting in a partial $^{13}$C-labeling of the products. For gavibamycin N1, carbons C-27, C-34, C-43, and C-61 showed enhanced signal intensities because of the labeling (the nomenclature is given in Fig. 1). The unambiguous identification of these carbons was done by the analysis of the $^{13}$C-filtered nuclear Overhauser enhancement spectroscopy and the heteronuclear multiple bond coherence spectra. In Fig. 3, the resonances of atoms in the vicinity of C-37 (C-2 position in ring F) are shown in a section of the heteronuclear single quantum coherence spectra of gavibamycin N1 and avilamycin A. In comparison to avilamycin A, two methyl groups (C-7 and C-41) are missing in gavibamycin N1. Surprisingly, this is the same labeling pattern found in gavibamycin N1 (2). Because of the fact that it is very unlikely for an organism to have two enzymes with the same function, a further structural analysis was car-
rried out. Careful HPLC-UV analysis revealed that both compounds showed different retention times under the conditions described under “Experimental Procedures.” As a first step, all $^1$H and $^{13}$C chemical shifts of gavibamycins I1 and N1 were compared. No significant difference was observed, except for the atoms in the vicinity of C-37. In a second step, the $^{3}J_{HH}$ couplings, which are directly linked to the dihedral angle of two hydrogens by the Karplus equation, were analyzed at the site of C-37. For the pair H-36/H-37 (the hydrogens bound to carbons C-36 and C-37, respectively) a $^{3}J_{HH}$ coupling of 8 ± 0.5 Hz was found for gavibamycin N1. This value is characteristic for an axial/axial orientation of H-36 and H-37 with respect to the ring. In contrast, for avilamycin A, a value of 3.5 ± 0.5 Hz was found for the $^{3}J_{HH}$ coupling, corresponding to an axial/equatorial orientation. This result indicated that a glucose is incorporated in gavibamycin N1 instead of a mannose in wild-type gavibamycin A1. Similar studies were carried out on the X12 mutation derivative with a molecular weight of 1332 atomic mass units. The conformation of H-36/H-37 was found to be axial/axial as for gavibamycin N1. Compared with gavibamycin N1 and avilamycin A, this compound is lacking the acetyl moiety in ring H, which is replaced by a hydrogen. This result is analogous to the changes reported for the muta-

**FIGURE 3.** Section of the $^{13}$C-heteronuclear single quantum coherence spectra of avilamycin A (solid contour lines) and gavibamycin N1 (dashed contour lines). Resonances for C-37, C-38, C-39, C-40, and C-42 are lettered. Arrows indicate the changes in chemical shifts for the resonances of the two compounds. For C-36 (not shown), a change in chemical shift of 0.31 and 1.6 parts/million is observed for $^{1}$H and $^{13}$C, respectively.

**TABLE 1**

| Strain                | Vancomycin | Gavibamycin A1 | Gavibamycin N1 |
|-----------------------|------------|----------------|----------------|
| *Staphylococcus aureus* | 1          | 8              | 16             |
| ATCC 25923            |            |                |                |
| MRSA RV 5/98          | <0.5       | 4              | 16             |
| *Streptococcus epidermidis DSM 1798* | 1          | 4              | 32             |
| *Streptococcus pyogenes* |            |                |                |
| E12449/98             | <0.25      | 2              | 8              |
| *Streptococcus pneumoniae* E2919/94 | <0.25     | <0.5          | 4              |
| *Enterococcus faecalis* |            |                |                |
| ATCC 19212            | <0.25      | 2              | 16             |
| *Enterococcus faecalis* H10513/99 | >128      | 1              | 8              |
| *Enterococcus faecium* Vanco-H8914/00 | >128      | 8              | 16             |

**FIGURE 4.** U shape conformation of avilamycin A. Carbon atoms are shown in gray, hydrogen atoms are shown in white, oxygen atoms are shown in black, and chlorine atoms are shown in dark gray.
FIGURE 5. Spectra of the purified AviX12. A, UV-visible spectrum of the oxidized and reduced preparation. The inset shows the reduced minus-oxidized difference spectrum. B, EPR spectra of the oxidized preparation at 40 K (a), the oxidized preparation at 10 K (b), the reduced preparation at 10 K (c), and the simulated spectrum (d). mT, milliteslas.
The protein was overexpressed in *E. coli* BL21 (DE3)pLysS. A protein of the predicted molecular mass of AviX12 (48,995 Da) was observed by SDS-PAGE in extracts of *isopropl-β-d-thiogalactopyranoside*-induced cells. AviX12 was purified by nickel chelation chromatography. The preparation was desalted and subjected to liquid chromatography/electrospray ionization-MS. The molecular mass of the preparation was determined to 49,011 Da. The difference of 16 atomic mass units might be due to oxidation of methionine.

**Spectroscopic Characterization of the Iron-Sulfur Cluster**—The UV-visible spectrum of the AviX12 preparation, as isolated, showed (beside the peak of the aromatic amino acids at 280 nm) a broad structureless absorbance from 300 to 550 nm, indicating the presence of a non-protein cofactor (Fig. 5). This signal was bleached by the addition of dithionite leading to a broad negative peak ~450 nm in the reduced minus-oxidized difference spectrum (Fig. 5). This is a typical spectral feature of protein-bound [Fe-S] clusters (29). EPR spectra were recorded to determine the type of [Fe-S] cluster present in the AviX12 preparation. No EPR signals were detectable, with the sample reduced by dithionite in the temperature range from 5 to 100 K. However, the oxidized protein showed a signal at temperatures below 20 K, which was clearly seen at 10 K (Fig. 5). The signal exhibited an axial symmetry with a slight rhombic distortion and is typical for a [3Fe-4S] cluster. It was simulated with the following parameters: \( g_x = 2.007, g_y = 2.018, \) and \( g_z = 2.025, L_x = 1.65 \) mT; \( L_y = 1.1 \) mT, and \( L_z = 1.4 \) mT (Fig. 5). From spin quantitations, the presence of approximately one [3Fe-4S] cluster per AviX12 was calculated. Preliminary attempts to reconstitute an [4Fe-4S] cluster in the preparation under anaerobic conditions and in the presence of AdoMet were not successful, so far.

**DISCUSSION**

The biosynthesis of saccharide containing polyketides usually starts with the formation of the polyketide moiety. The sugar is attached to the hexasaccharide at the very end of the biosynthetic route. D-Mannose was discussed to be one component of the disaccharide with the formation of the polyketide moiety. The sugar is attached to the active conformation of Avilamycin A

The antibiotic activity of gavibamycin N1, the main product of *S. viridochromogenes* GW4-X12, is very low. This strongly reduced activity might be explained by a conformational change of the whole molecule leading to an inability to bind to the ribosome. AviX12 activity is believed to induce an important conformational change of the molecule, resulting in its active form.

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