Structure of the Heme Biosynthetic *Pseudomonas aeruginosa* Porphobilinogen Synthase in Complex with the Antibiotic Alaremycin

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The recently discovered antibacterial compound alaremycin, produced by *Streptomyces* sp. A012304, structurally closely resembles 5-aminolevulinic acid, the substrate of porphobilinogen synthase. During the initial steps of heme biosynthesis, two molecules of 5-aminolevulinic acid are asymmetrically condensed to porphobilinogen. Alaremycin was found to efficiently inhibit the growth of both Gram-negative and Gram-positive bacteria. Using the newly created heme-permeable strain *Escherichia coli* CSA1, we are able to uncouple heme biosynthesis from bacterial growth and demonstrate that alaremycin targets the heme biosynthetic pathway. Further studies focused on the activity of alaremycin against the opportunistic pathogenic bacterium *Pseudomonas aeruginosa*. The MIC of alaremycin was determined to be 12 mM. Alaremycin was identified as a direct inhibitor of recombinant purified *P. aeruginosa* porphobilinogen synthase and had a *K*ₐ of 1.33 mM. To understand the molecular basis of alaremycin’s antibiotic activity at the atomic level, the *P. aeruginosa* porphobilinogen synthase was co-crystallized with the alaremycin. At 1.75-Å resolution, the crystal structure reveals that the antibiotic efficiently blocks the active site of porphobilinogen synthase. The antibiotic binds as a reduced derivative of 5-acetamido-4-oxo-5-hexenoic acid. The corresponding methyl group is, however, not coordinated by any amino acid residues of the active site, excluding its functional relevance for alaremycin inhibition. Alaremycin is covalently bound by the catalytically important active-site lysine residue 260 and is tightly coordinated by several active-site amino acids. Our data provide a solid structural basis to further improve the activity of alaremycin for rational drug design. Potential approaches are discussed.

Modified tetrapyrroles are complex macromolecules and the most abundant pigments found in nature. Tetrapyrroles such as hemes and chlorophyll are essential prosthetic groups involved in numerous electron transport chains for energy recovery in essentially all forms of life. The biosynthetic pathways of tetrapyrroles are correspondingly highly conserved (16, 30), making heme biosynthesis an attractive target for antibacterial drug discovery and application (24). In fact, the tetrapyrrole biosynthetic pathway serves both as a source for the production of antibiotics such as asukamycin (25) and as a target for antibiotics, as in the case of gabaculin (1, 19). Recently, the antibiotic alaremycin was isolated from the culture broth of the actinomycete *Streptomyces* sp. A012304. Its structure, determined to be 5-acetamido-4-oxo-5-hexenoic acid (2) (Fig. 1A), is related to that of 5-aminolevulinic acid (ALA), the first common precursor molecule of all tetrapyrroles. The enzyme porphobilinogen (PBG) synthase (PBGS; EC 4.2.1.24) asymmetrically condenses two such molecules of ALA to generate porphobilinogen (26, 27) (Fig. 1B). The activity of alaremycin against *Escherichia coli* is enhanced by the presence of ALA, implying that its antimicrobial activity derives from the inhibition of PBGS (2). During tetrapyrrole biosynthesis, four molecules of porphobilinogen are fused to generate the first tetrapyrrole, uroporphyrinogen III. Thereafter, the biosynthetic pathways of heme and chlorophyll separate from those of vitamin *B*₁₂, siroheme, and factor F₄₃₀ due to distinct modifications of the initial tetrapyrrole skeleton (30).

PBGSs from a range of organisms have been investigated biochemically and structurally, providing in-depth insights into the catalysis of PBGS (4, 7–10). The two ALA substrate molecules occupy two distinct binding sites in PBGS, referred to as the A and the P sites, to indicate that they contribute to porphobilinogen an acetic acid and a propanoic acid side chain, respectively. The ALA A and P sites are covalently bound through a stable Schiff base with lysine residues 205 and 260, respectively (12). Although PBGSs from different organisms differ with respect to the metal ion requirements and localization, the amino acid sequences are highly conserved between bacteria, archaea, and eukaryotes. Whereas human PBGS is Zn²⁺ dependent (17), catalysis by PBGS from the opportunistic human pathogen *Pseudomonas aeruginosa* is metal independent but retains a structural Mg²⁺ site near the active site (9, 13).

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Many organisms, including bacteria, produce dedicated antimicrobial compounds to ward off infections or competing microbes. Streptomyces sp. A012304 hence produces the compound alaremycin, thereby preventing growth in neighboring microorganisms. Strategies to counteract the effect of alaremycin on Streptomyces sp. A012304 have so far not been described. We have investigated the molecular basis for the antimicrobial activity of alaremycin by analyzing its activity against Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus megaterium, Streptomyces coelicolor, and Streptomyces avermitilis. As PBGS is the molecular target of alaremycin, we studied the impact of the antibiotic on recombinant Mg<sup>2+</sup>-stimulated P. aeruginosa PBGS and Zn<sup>2+</sup>-dependent Methanosarcina Barkeri PBGS. Furthermore, alaremycin was cocrySTALLized with the PBGS from P. aeruginosa, and its structure solved at a resolution of 1.75 Å to describe its mode of PBGS inactivation at the atomic level.

**MATERIALS AND METHODS**

**Materials.** ALA was purchased from Merck (Darmstadt, Germany), porphobilinogen was purchased from Porphyrin Products (Logan, UT), and other chemicals were purchased from Sigma-Aldrich (Hamburg, Germany). Oligonucleotides were purchased from Metabion (Planegg-Martinsried, Germany), Protino Ni-IDA resin was purchased from Machery-Nagel (Düren, Germany), and ethyl methanesulfonate was purchased from ABCR (Karlsruhe, Germany).

**Bacterial strains and growth conditions.** To quantify the activity of PBGS in cell extracts, MOPSO [3-(N-morpholino)-2-hydroxypropanesulfonic acid] minimal medium containing 20 mM hemin (20). B. megaterium (DSM 319) was grown in MOPS0 [3-(N-morpholino)-2-hydroxypropanesulfonic acid] minimal medium. To quantify the activity of PBGS in cell extracts, S. avermitilis was cultured in 2% dextrose, pH 7.2, at 30°C and 200 rpm for 4 days. S. coelicolor was grown in 0.4% glucose–0.4% yeast extract–1% malt extract, pH 7.2, at 30°C and 200 rpm for 4 days. S. avermitilis was cultured in 2% oat meal, pH 7.2, at 30°C and 200 rpm for 4 days. E. coli C647 was grown in LB medium at 37°C and 200 rpm.

**Isolation of alaremycin.** For alaremycin production, Streptomyces sp. A012304 was cultivated in production medium containing 6% dextrin, 2% yeast extract, 0.3% NaCl, 0.3% CaCO<sub>3</sub>, 0.1% dry bouillon, 0.3% soybean meal, 0.3% CaCO<sub>3</sub>, pH 7.0) at 30°C and 200 rpm for 4 days. All other bacterial strains were grown in LB medium at 37°C for 20 h.

**Protein production and purification.** Recombinant P. aeruginosa PBGS was produced and purified as described previously (10). Protein integrity was analyzed by mass spectrometry and Western blot analysis. Recombinant M. Barkeri PBGS was produced by using E. coli strain BL21(DE3)RIL (Stratagene, Heidelberg, Germany) in LB medium at 37°C and 180 rpm. At an optical density at 578 nm of 0.7, protein production was induced with 150 µM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were cultivated overnight at 17°C and 150 rpm, harvested, washed with buffer A (50 mM Tris HCl, pH 8.5, 300 mM NaCl, 10 mM ZnCl<sub>2</sub>) and resuspended in a minimal volume of buffer A. After cell disruption by sonication (HD 2070, Bandelin) and centrifugation (10,000 × g for 45 min), the soluble fraction was applied to Protino Ni-IDA agarose (Machery-Nagel, Düren, Germany) and washed and the PBGS was eluted with 300 mM imidazole in buffer A. Further purification steps involved anion-exchange chromatography on a DEAE-Sepharose column (PBGS was eluted with 200 mM NaCl in buffer A) and gel permeation chromatography on a 30-ml Superdex 200 HR 10/30 column (0.5 ml/min; GE Healthcare) in buffer A. The overall yield was ~9 mg of M. Barkeri PBGS per liter of culture.

**Enzyme activity assay.** PBGS activity was quantified as described previously (10). Protein, buffer, and alaremycin solutions were prepared in buffer K<sub>1</sub> (100 mM bis-Tris-propane, pH 8.5). ALA (40 mM) and alaremycin solutions were prepared in buffer K<sub>1</sub> (100 mM bis-Tris-propane, pH 8.5). E. coli (40 mM) and alaremycin solutions were prepared in buffer K<sub>1</sub> (100 mM bis-Tris-propane, pH 8.5). As a control, cell extracts were inactivated by heating to 95°C for 10 min, which resulted in negligible background activity. Reactions without additional ALA were used to determine the concentration of cellular PBGS in the cell extracts.

**Determination of inhibition constants.** P. aeruginosa PBGS at 2 µg/ml and M. Barkeri PBGS at 25 µg/ml were diluted to a final absorbance of 5 at λ<sub>max</sub> with potassium buffer K<sub>1</sub> (100 mM bis-Tris-propane, pH 8.5). ALA (40 mM) and alaremycin solutions were prepared in buffer K<sub>1</sub>. For P. aeruginosa PBGS, 10 mM MgCl<sub>2</sub> was added, and for M. Barkeri PBGS, 10 mM ZnCl<sub>2</sub> was added. Protein, buffer, and alaremycin (0 to 10 mM) were mixed and incubated at 37°C for 10 min. Longer incubation times did not affect enzyme inhibition. Substrate (5 mM) was added to start the reaction. The PBGS-catalyzed reaction was stopped at times of between 1 and 60 min by adding equivalent volumes of stop reagent (50% trichloroacetic acid, 100 mM HgCl<sub>2</sub>) to the reaction mixture. After centrifugation (at 5,000 × g for 3 min), the supernatant was treated with an equivalent amount of Ehrlich’s reagent (0.4 g 4-dimethylaminobenzaldehyde in 10 ml acetic acid and 10 ml HClO<sub>4</sub>). After 15 min of incubation at room temperature, the product was quantified by measurement of the absorbance at 555 nm (ε = 60,200 M<sup>-1</sup> cm<sup>-1</sup>). The alaremycin concentration that inhibited the enzyme...
Alaremycin inhibits the growth of Gram-negative and -positive bacteria. The MICs for all Pseudomonas aeruginosa strains were generally lower than those for Escherichia coli DH10b (Table 2). To determine the general applicability of alaremycin as an antibiotic, a range of bacterial species was exposed to the compound. Representatives of Gram-positive bacteria included B. megaterium and B. subtilis, and representatives of Gram-negative bacteria included P. aeruginosa and E. coli DH10b. Alaremycin-producing and non-alaremycin-producing Streptomyces strains were analyzed for their intrinsic immunity to the antibiotic. To identify the cellular target of alaremycin activity, heme-permeant E. coli strain CSA1 was created to test alaremycin independently of intrinsic heme biosynthesis. The alaremycin inhibition of PBGS was determined in cell extracts for the species listed above and of the enzymes purified from P. aeruginosa and M. barkeri. The PBGS from P. aeruginosa was also used for biochemical characterization as well as for cocrystalization with alaremycin and crystal structure determination.

Bacterial growth assays. E. coli laboratory strains have generally lost their ability to import heme from the surrounding medium (29). We have created an E. coli strain capable of heme uptake by treating hema-negative E. coli strain EV61 (28), which normally requires ALA for growth, with ethyl methanesulfonate to induce chemical mutagenesis (20, 21). Colonies were screened for their ability to grow in ALA-free, heme-containing medium. Our isolated heme-permeant hema-negative strain was designated E. coli CSA1. If heme biosynthesis is the target of alaremycin activity, the addition of heme to E. coli CSA1 should allow the strain to overcome the growth-inhibitory phenotype. The treatment of E. coli CSA1 with alaremycin (≤40 mg/ml) in the presence and the absence of ALA completely inhibited bacterial growth. The addition of additional heme rendered E. coli CSA1 alaremycin insensitive. This implicates heme biosynthesis as the target of alaremycin.

Alaremycin inhibits the growth of Gram-negative and -positive bacteria. The MICs of alaremycin for several Gram-positive and -negative bacteria ranged from 4 mM to over 20 mM (Table 2). For example, for E. coli, the MIC for alaremycin was 4 mM. Eight and 14 mM alaremycin inhibited the growth of S. coelicolor and S. avermitilis in complex medium, whereas the
TABLE 3. **Kinetic parameters of** *P. aeruginosa* **and** *M. barkeri* **PBGSs and IC₅₀ for alaremycin**

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>P. aeruginosa</em></th>
<th><em>M. barkeri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>General catalytic properties</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>0.32</td>
<td>0.07</td>
</tr>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>16.1</td>
<td>0.012</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (µM⁻¹s⁻¹)</td>
<td>50.3</td>
<td>0.17</td>
</tr>
<tr>
<td>Inhibition by alaremycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC₅₀ (mM)</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>$k_i$ (mM)</td>
<td>1.33</td>
<td>1.51</td>
</tr>
</tbody>
</table>

* The Michaelis-Menten constant ($K_m$) and the $k_{cat}$ and the $k_{cat}/K_m$ values for the PBGSs were determined from substrate velocity plots by measuring the constant velocity formation of porphobilinogen from ALA over a substrate concentration range of from 1 to 10 mM. Values were determined by the use of iterative, curve-fitting Lineweaver-Burk plots (SigmaPlot program, version 8.0; Enzyme Kinetics program, version 1.1). For inhibition studies, 0 to 40 mM alaremycin was added to the enzyme activity test. The standard errors of the results were between 5 and 10%.

*Quantifying PBGS inhibition by alaremycin in cell extracts.*

As alaremycin is structurally related to ALA (the substrate of PBGS), we analyzed the effects of 0, 5, and 10 mM alaremycin on PBGS activity in cell extracts of *S. coelicolor*, *S. avermitilis*, *Streptomyces* sp. A012304, *E. coli*, *B. megaterium*, *B. subtilis*, *P. aeruginosa*, *E. coli* DH10b, and *E. coli* CSA1. Surprisingly, the trend of alaremycin inhibition in cell extracts was distinct from the trend of alaremycin inhibition of the PBGS from the corresponding organisms (Table 2). Thus, 5 mM alaremycin essentially abolished the PBGS activity of *B. megaterium* cell extracts, whereas the *S. coelicolor* (22%), *P. aeruginosa* (25%), and *B. subtilis* (30%) cell extracts retained residual PBGS activity even in the presence of 10 mM alaremycin. The differences in the alaremycin inhibitory activities between intact organisms (MICs) and cell extracts (PBGSs) observed for *P. aeruginosa* were presumably due to the various permeabilities and uptake capacities of the individual organisms. Interestingly, *Streptomyces* sp. A012304 cell extracts retained 78% PBGS activity in the presence of 10 mM alaremycin, implying that the PBGS from this species possesses significant immunity against this antibiotic.

**Determination of inhibitory effect on recombinant PBGS.**

To unambiguously identify the molecular target of alaremycin, we tested its effect on recombinant purified PBGS from *P. aeruginosa* (metal independent) and *M. barkeri* (Zn²⁺ dependent). The kinetic parameters (Table 3) of *P. aeruginosa* PBGS were essentially as published previously (11). The specific activity of purified native *M. barkeri* PBGS is 0.39 µmol min⁻¹ mg⁻¹ (3), while the $K_m$ of 0.07 µM is quite low. To quantify the potency of the inhibitor, IC₅₀/ₐₖ₃ₖ₈₂ were determined as described previously (2) and are listed in Table 3. No time dependence of inhibition was observed. The $k_i$ for the *P. aeruginosa* PBGS was 1.33 mM, and that for the *M. barkeri* PBGS was 1.51 mM.

**Crystal structure of the P. aeruginosa PBGS-alaremycin complex.** *P. aeruginosa* PBGS was cocrystallized with alaremycin to elucidate its mode of inhibition in detail. The structure was solved and refined to a resolution of 1.75 Å. The overall structure is unchanged from that presented in earlier reports (10, 13), despite a change in the space group from *P4₂₁₂* (one dimer per asymmetric unit) to *J4₂₂* (one monomer per asymmetric unit). Interestingly, the antibiotic is observed to bind as 5-acetamido-4-oxo-5-hexanoic acid in the active site of PBGS, a reduced derivative of 5-acetamido-4-oxo-5-hexenoic acid. We correspondingly refer to this compound as alarem (Fig. 1C). Alaremycin 2, which is located in the active site (Fig. 2) and whose electron density is clearly defined, is coordinated by the P-site Lys260 via a Schiff base to its C-4 atom. This resembles substrate ALA binding to the P site and has a binding mode comparable to that of the inhibitor 5-fluorolevulinic acid (Fig. 3) (9, 14). Inhibitor binding is stabilized by two hydrogen bonds from the antibiotic carboxylate to Ser286, as described for 5-hydroxylevulinic acid, a substrate analogue, in the P site of *P. aeruginosa* PBGS (12). Alaremycin 2 thus mimics the P-site substrate ALA rather than the product PBG. The Schiff base bond of a second A-site substrate ALA with Lys260 is not mirrored by alaremycin 2 coordination. Instead, the amino group of the A-site Lys260 hydrogen bonds to the C-7 keto group of alaremycin 2. Inhibitor binding is further stabilized by hydrogen bonds to water molecules that in turn form hydrogen bonds with Gln233, Asp127, and Ser175. An Mg²⁺ in the active site (12) is coordinated by Asp139, Asp131, and Asp 176. The part of the antibiotic that blocks the A site of the active site has a conformation similar to that previously observed for inhibitors mimicking the intermediate of the condensation reaction (18). The methyl group attached to the nonplanar C-5 carbon atom of alaremycin 2 is coordinated only by weak van der Waals interactions (≥3.9 Å) in the active site of PBGS, implying that the modification to alaremycin may be of limited relevance for its antibiotic function. The conversion of alaremycin (characterized by NMR [2]) to alaremycin 2 (observed in the active site of PBGS by X-ray crystallography) appears to occur during crystallization by an as yet unknown mechanism. However, the inhibitory features of the compound are not affected.

**Alaremycin as a lead compound for antibiotic development.**

Alignment of the amino acid sequences of PBGSs from a range of bacteria demonstrated a high degree of conservation at the catalytic site. Correspondingly, the amino acid residues in contact with alaremycin 2 are conserved in many bacteria, which explains the broad antibiotic activity of the compound. It also provides a structural basis for rational drug design to further improve its specificity and affinity. Clearly, the observed alare-
meflin 2 IC$_{50}$ of 2.1 mM for the $P$. aeruginosa PBGS catalytic site. The two FLA substrate molecules and the alaremycin 2 inhibitor are indicated in orange, the conserved lysine residues of the P and A sites are indicated in violet, amino acid residues involved in substrate recognition are indicated in black, and hydrogen bonding is indicated as dotted lines. The two FLA molecules are covalently bound to the natural inhibitor alaremycin 2 is bound by a Schiff base bond to Lys260 (P site) and Lys205 (A site) but additionally partly blocks the A site.

FIG. 3. Comparison of the binding modes of alaremycin 2 (A) and the two 5-fluorolevulinic acid (FLA) substrate analogue molecules (B) in the PBGS catalytic site. The two FLA substrate molecules and the alaremycin 2 inhibitor are indicated in orange, the conserved lysine residues of the P and A sites are indicated in violet, amino acid residues involved in substrate recognition are indicated in black, and hydrogen bonding is indicated as dotted lines. The two FLA molecules are covalently bound to Lys260 (P site) and Lys205 (A site) by Schiff bases linked by Na$^+$. The natural inhibitor alaremycin 2 is bound by a Schiff base bond to Lys260 (P site) but additionally partly blocks the A site.

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