Structure of Antimycin A1, a Specific Electron Transfer Inhibitor of Ubiquinol−Cytochrome *c* Oxidoreductase

Hoeon Kim,[†] Lothar Esser,[†] M. Bilayet Hossain,[‡] Di Xia,[†] Chang-An Yu,[§] Josep Rizo,[||] Dick van der Helm,[‡] and Johann Deisenhofer*[,†]

Department of Biochemistry and Howard Hughes Medical Institute The University of Texas Southwestern Medical Center Dallas, Texas 75235
Department of Chemistry and Biochemistry University of Oklahoma, Norman, Oklahoma 73019
Departments of Biochemistry and Pharmacology The University of Texas Southwestern Medical Center Dallas, Texas 75235
Department of Biochemistry and Molecular Biology University of Oklahoma, Stillwater, Oklahoma 74078

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Antimycin A, a natural antibiotic from *Streptomyces*, inhibits specifically the electron transfer activity of ubiquinol−cytochrome *c* oxidoreductase (collectively called the bc1 complex) in most respiratory and photosynthetic organisms. Because of high specificity and strong affinity, antimycin A has been widely used in functional studies of the enzyme.[1] With this inhibitor bound to the bc1 complex, the phenomenon of "oxidant-induced reduction of cytochrome *b*" can be observed,[2] which provides strong experimental support for the protonmotive Q cycle hypothesis.[3] The specific interaction of antimycin A with the bc1 complex has been studied by structure−activity analysis using either synthetic analogues of the inhibitor [4−6] or resistant mutants of the protein.[7]

Recent progress in the determination of the three-dimensional structure of the eukaryotic bc1 complex revealed two separate cavities in the cytochrome *b* subunit, one of which binds inhibitors of quinone oxidation and the other inhibitors of quinone reduction, for example antimycin A.[8] We believe that these cavities are the binding sites for the substrate (ubiquinone).[9] On the basis of the diffraction data at the currently available resolution of about 3 Å, the specific interactions between functional groups of the inhibitor and the protein are difficult to analyze without knowing the exact geometry of the molecules involved. To facilitate the structural analysis, we have determined the three-dimensional structure of isolated antimycin A1 by X-ray crystallography.[10] Furthermore, we have employed two-dimensional nuclear magnetic resonance (NMR) spectroscopy to resolve a discrepancy between the X-ray structure and the chemical structure.[11]

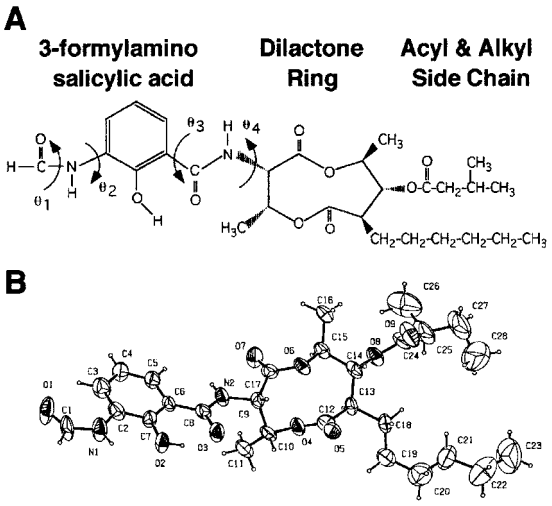

Figure 1. (A) Chemical formula. The heterogeneity in the alkyl and acyl chain is responsible for the diversity of the natural product.[14] (B) Conformation of antimycin A determined from the crystal structure analysis. The absolute configuration was determined from synthetic studies.[12]

The crystal structure of antimycin A1 revealed two ring moieties, a planar 3-formylamino salicylic acid (FASA) and a puckered dilactone, which are connected through an (N2−C9) amide bond bridge (Figure 1). The carbonyl oxygen atom O3 of the benzamide group is the acceptor for an intramolecular hydrogen bond, donated by the phenolic OH group of the 3-FASA ring, with an O2···O3 distance of 2.53 Å. This hydrogen bond was previously proposed from ^{1}H NMR spectra,[5] and its functional significance was also indicated by activity assays with inhibitor analogues.[4] The θ_1 and θ_2 dihedral angles of the 3-formylamino group are −2 and 185°, respectively. These values are similar to those deduced by conformational energy calculations.[6] The absolute configuration of the dilactone ring is [C9(S), C10(S), C13(S), C14(R), C15(R)], which had been deduced by NMR and CD spectroscopy.[12] Among these chiral centers, the configuration of C9(S) has been reported to be essential for inhibitory activity.[13] A search of the Cambridge structural database indicated that the

* To whom correspondence should be addressed.
[†] Department of Chemistry and Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center.
[‡] Department of Chemistry and Biochemistry, University of Oklahoma.
[||] Departments of Biochemistry and Pharmacology, The University of Texas Southwestern Medical Center.
[§] Department of Biochemistry and Molecular Biology, University of Oklahoma.

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(10) X-ray crystallographic analysis: Antimycin A1 were purchased from Sigma (catalogue number A0149). X-ray quality crystals were obtained by vapor diffusion from an ethyl acetate solution using hexane as the nonsolvent; 2407 reflections with $2\theta = 120°$ were measured, of which 1521 were considered observed ($I = 2\sigma$). The structure was solved by direct methods and refined by full-matrix least-squares (SHELXL93) of $F2$ using anisotropic thermal parameters for the non-hydrogen atoms. Hydrogen atoms were placed in their calculated positions and were refined using isotropic thermal parameters. Distance restraints were applied to the long side chains. The structure refined to a final $R = 0.082$ for the observed, and $R = 0.121$ for all data, with $S = 1$. The FASA moiety exhibits unusual variations in bond distances; similar variations were found in related trisubstituted aminophenols.[17]

(11) NMR experiments were performed on a Varian Unity 500 spectrometer at 25 °C using a 70 mM sample of antimycin A1 dissolved in perdeuterated methanol. The spectral width in all proton dimensions was 4200 Hz. The DQF-COSY spectrum consisted of 400 complex increments (4096 complex points each), with 16 scans per increment. For HSQC and HMBC spectra, the spectral width in the 13C dimension was 10 000 Hz, and 180 complex increments of 2048 complex points each were acquired (32 and 64 scans per increment for HSQC and HMC, respectively).

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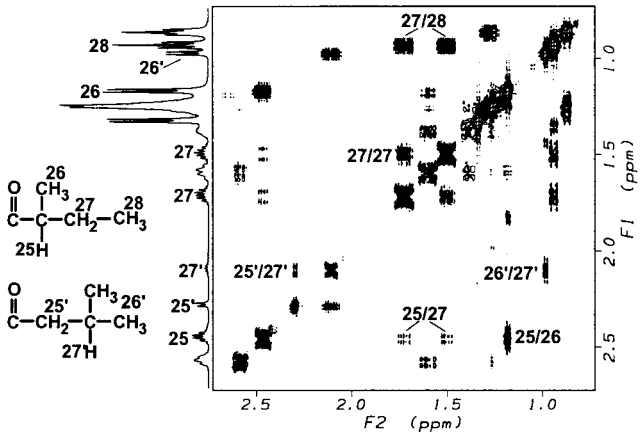

Figure 2. Contour plot of the region of the DQF-COSY spectrum of antimycin A that contains the cross-peaks corresponding to the acyl side chain. An expansion of a 1D NMR spectrum of antimyicin A is also shown. Signals and cross-peaks corresponding to the acyl chain protons have been labeled in the 1D spectrum and the contour plot, respectively, using the numbering scheme indicated in the chemical formulas shown at the upper left corner of the figure.

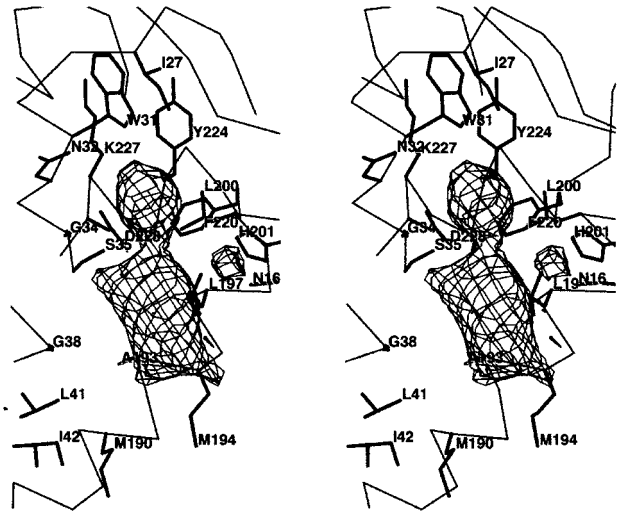

Figure 3. Stereoview of the density of antimycin in the difference Fourier map and the neighboring residues of cytochrome *b* in the bc1 complex. The difference map was calculated in the 20−3.5 Å resolution range. The map was contoured at the 5σ level.

dilactone structure in antimycin A1 provides the first atomic structure of this kind of nine-atom-ring lactone. The FASA and the dilactone ring are constant features in the naturally occurring antimycins and therefore likely to be necessary for the inhibitory action; in contrast, the alkyl and acyl side chains are structurally diverse.[14]

To our surprise, the electron density observed for the acyl side chain of antimycin A1 indicated that it corresponds to a 1-methyl butanoate, instead of the 2-methyl isomer described earlier.[14] To confirm this observation, we analyzed antimycin A1 in solution using two-dimensional nuclear magnetic resonance (NMR) spectroscopy. All carbon atoms and nonexchangeable protons of the molecule were assigned using double-quantum filtered homonuclear correlation spectroscopy (DQF-COSY), heteronuclear single quantum correlation spectroscopy (HSQC), and heteronuclear multiple bond correlation spectroscopy. The spectra were fully consistent with the chemical structure of antimycin A1 but revealed heterogeneity in the acyl side chain; about 80% of the acyl chain correspond to 1-methyl butanoate and 20% to 2-methyl butanoate. This can be clearly derived from the splitting patterns in the one-dimensional spectra and from the correlation cross-peaks in the COSY spectra shown in Figure 2. Therefore, we conclude that antimycin A1, as prepared from *Streptomyces* sp. by Sigma Co., consists of two isomers with the ratio of 4:1 and that the 1-methyl butanoate form crystallized preferentially over the 2-methyl butaonate isomer.

To determine the inhibitory mechanism of antimycin A1, cocrystals of the mitochondrial bc1 complex with the inhibitor were grown[15] and analyzed by X-ray crystallography. The difference Fourier map between data from antimycin A-bound and native crystals contains strong density features that indicate the inhibitor binding site near the high potential *b* heme, as shown in Figure 3. The eccentric shape of the density matches well the crystal structure of antimycin A (Figure 1). Mutational analysis had previously indicated that residues N32, G38, K227, and D228 in the vicinity of this density interact with the inhibitor.[7]

The density in the cocrystal provides insights into how antimycin A1 binds to protein. The polar residues W31, N32, Y224, K227, and D228 interact with side chains of the FASA ring moiety, whereas the nonpolar residues L41, I42, M190, A193, M194, L197, and F220 constitute the hydrophobic surroundings for the dilactone ring moiety. This uneven distribution of polarity around antimycin agrees well with structure−inhibitory activity relationships as the sensitivity to chemical modification is higher in the FASA ring moiety than in the dilactone moiety.[5−7] The two carbonyl groups in the dilactone ring moiety are capable of forming hydrogen bonds to neighboring residues. Residues N16 and S35 of cytochrome *b* might be involved in these hydrogen bonds since their polar side chains are most closely positioned to the dilactone ring. The intermolecular hydrogen bond of antimycin A1 (O2···O3) is likely to break down in order for them to interact with the protein when the inhibitor binds. On the basis of our best-fit model of antimycin, the carboxyl group of D228 is likely to be involved in the hydrogen bond formation with antimycin. D228 is one of the highly conserved residues in cytochrome *b*;[16] its potentially essential role in antimycin binding is consistent with the fact that mutation of D228 causes resistance to the inhibitor.[7] There are four protonic residues, W31, N32, Y224, and K227, near the tip of the antimycin A structure. Among those, two polar residues N32 and K227 seem to interact specifically with the 3-formylamino group. Point mutations in those residues also cause resistance to the inhibitor.[7] For these reasons it is likely that the FASA ring moiety and carbonyl groups of the dilactone ring provide the specificity for inhibitor binding, whereas the remaining structural elements provide the nonspecific hydrophobic interactions.

In our current understanding, antimycin A1 works as an electron transfer inhibitor by displacing ubiquinone from the Qi pocket of cytochrome *b*. Therefore, further structural refinement of the bc1 complex, using the accurate atomic model of the inhibitor, will provide valuable insights into the binding of both the inhibitor and ubiquinone in the bc1 complex. Furthermore, based on this structural knowledge, the design of more potent insecticides and herbicides that specifically and selectively inhibit the bc1 complex of certain species should be possible.

Supporting Information Available: Crystal data, atomic coordinates, bond lengths and angles, anisotropic displacement parameters, torsion angles, and ^{1}H and ^{13}C chemical shifts of antimycin (PDF) and an X-ray crystallographic file, in CIF format, for antimycin. This material is available free of charge via the Internet at http://pubs.acs.org.

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