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Crystallization and preliminary X-ray crystallographic analysis of a vancomycin–*N*-acetyl-D-Ala-D-Ala complex

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A vancomycin–*N*-acetyl-D-Ala-D-Ala complex has been crystallized by the sitting-drop vapour-diffusion method using imidazole maleic buffer at pH 7.6. The novel crystals obtained belong to the space group $P6_322$ with unit-cell parameters $a = b = 73.43\,(1)$, $c = 277.17\,(4)$ Å, $\gamma = 120°$. The crystal density was determined as 1.106 g cm^{-3} which gives a supercell of 24 molecules (12 dimers) per asymmetric unit for an acceptable Matthews number and an estimated solvent content of 42%. Data were collected at room temperature to 2.8 Å.

1. Introduction

The vancomycin family of antibiotics represents an effective method of control against staphylococcal and streptococcal infections and is effectively the last line of defence against the virulent methicillin-resistant *Staphylococcus aureus* (MRSA). Such bacteria are resistant to most other antibiotics such as penicillin, ampicillin, erythromycin, tetracycline and sulfonimides (Neu, 1992). The vancomycins are therefore of great clinical importance and the mechanism of their activity needs to be understood at a structural level. The vancomycins exert their bactericidal effect by complexing with C-terminal D-Ala-D-Ala precursor peptide units on the surface of Gram-positive bacterial cell walls, thus inhibiting the cross-linking of the polymeric lipid–PP-disaccharide–pentapeptide chains in the growing peptidoglycan cell wall (Barna & Williams, 1984; Reynolds, 1989). The action of transglycosylases and transpeptidases is impeded (Billot-Klein *et al.*, 1994) and the resulting cell wall becomes susceptible to lysis by osmotic shock (Wright & Walsh, 1992). NMR studies suggest that the vancomycin molecule binds with high affinity *via* its concave pocket to the *N*-acetyl-D-Ala-D-Ala termini by way of five hydrogen bonds and other non-covalent interactions between substrate and antibiotic (Walsh *et al.*, 1996; Barna & Williams, 1984).

At high concentrations, vancomycin and many of its analogues are known to aggregate in aqueous solution. This aggregation is assumed to be dimerization and has been shown from microcalorimetric (Cooper & McAuley-Hecht, 1993) and NMR (Waltho & Williams, 1989) studies to enhance binding affinity. Recent studies (McPhail & Cooper, 1997) have shown that ligand binding also promotes dimerization.

The first vancomycin crystal structure was the degradation product CDP-I which is in the form of a monomer (Sheldrick *et al.*, 1978), as was the structure of the first naturally occurring vancomycin, ureido-balhimycin (Sheldrick *et al.*, 1995). The first structure of the vancomycin dimer was determined by Schäfer *et al.* (1996) and also showed the presence of two chloride ions, an acetate and 105 water molecules. Recently the crystal structure of vancomycin in complex with *N*-acetyl-D-alanine has been determined (Loll *et al.*, 1998) which shows not only the back-to-back dimer conformation, but also a novel face-to-face oligomeric interaction. Interestingly, from this new structural motif it is possible to build longer naturally occurring peptide ligands by extension of the *N*-acetyl-D-alanine.

During microcalorimetric studies of the interaction of vancomycin with the *N*-acetyl-D-Ala-D-Ala ligand (McPhail & Cooper, 1997) precipitation was noticed at particular stoichiometric concentrations of the antibiotic and ligand. This appeared to offer a possible route to crystallizing the complex.

2. Materials and methods

2.1. Crystallization of the vancomycin–*N*-acetyl-D-Ala-D-Ala complex

Since initial crystallization trials using standard evaporation procedures were unsuccessful, we adopted a protein grid-screen approach. Measured aliquots of vancomycin and *N*-acetyl-D-Ala-D-Ala (both from Sigma Chemical Co.) were prepared and set up in 24-well Cryschem trays using the sitting-drop method. Both the pH and the stoichiometric concentrations were varied. Initially most of the wells showed precipitation. Several buffers were screened before optimum conditions for crystal growth were found. Eventually, two

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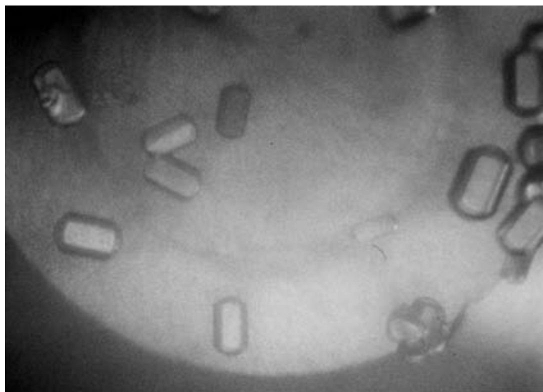

Figure 1
Crystals of the vancomycin complex. The size of the largest crystal shown is ~0.2 × 0.1 × 0.05 mm

morphologies of crystals were obtained by varying the buffer used. Small torpedo-shaped crystals formed in 0.1 *M* sodium phosphate at pH 7. These crystals proved to be extremely fragile. A second morphology (rectangular-shaped crystals) was derived from a drop comprising 11 µl of 5.41 m*M* *N*-acetyl-D-Ala-D-Ala and 17 µl of 4.63 m*M* vancomycin in 0.1 *M* imidazole maleic buffer pH 7.6. These crystals (Fig. 1) were used in the analysis. Crystallizations were carried out at 290 K and crystal growth took several months. The crystals were fairly robust but quickly became opaque in the absence of mother liquor.

2.2. Determination of crystal density

The experimental determination of crystal density was based on the density-gradient method described by Westbrook (1985) using a series of Ficoll solutions [from 30 to 60%(*w*/*w*)]. The gradient was prepared in a glass test-tube with an inner diameter of 4 mm and centrifuged for 1 h at 3000*g*. The gradient was calibrated with drops of a mixture of carbon tetrachloride and toluene, the densities of which were determined using a PAAR Digital density meter, DMA 35. The crystal was introduced along with a small volume of mother liquor at the top of the gradient, on the tip of a needle, and further centrifuged for 55 min at 1000*g*. From the equilibrium position the density of the vancomycin crystal is estimated as 1.106 (±0.01) g cm^{-3}. (The experiment was repeated twice.)

2.3. Molecular weight of the complex using electrospray mass spectrometry

To verify the nature of the crystals (in the light of the surprisingly large unit-cell dimensions) and to eliminate possible contamination or chemical degradation effects, the individual vancomycin and *N*-acetyl-D-Ala-D-Ala solutions, as well as a few crystals (which were washed thoroughly and dissolved in water), were characterized by mass spectrometry using a VG Platform quadrupole mass spectrometer fitted with a pneumatically assisted electrospray source and controlled *via* VG Mass-Lynx software. The instrument was calibrated immediately before use with horse heart myoglobin supplied by Sigma Chemical Co. Samples for analysis were diluted with an equal volume of 4%(*v*/*v*) formic acid in acetonitrile and 10–20 ml aliquots were injected directly into the carrier system. The results showed no contaminating protein or other high molecular weight species and showed that the crystals contained only vancomycin and the ligand.

3. Results and discussion

Three crystals of approximate dimensions 0.1 × 0.1 × 0.2 mm were used for data collection on a Siemens Xentronics area detector mounted on a rotating anode with Cu $K\alpha$ radiation. The crystals were sealed in quartz capillaries with some mother liquor. 93% of the data were collected to 2.8 Å from seven data sets collected at different resolution shells with these three crystals. The data were collected using 0.1° oscillation frames of 400 s exposure for a total of 2391 frames and were routinely processed using *XDS* (Kabsch, 1988), resulting in 48375 observations with $I \geq 3.0\sigma(I)$ being merged into 10380 unique reflections using *XSCALE* (Kabsch, 1988), with an $R_{\rm merge}$ of 7.2%. These crystals belong to the hexagonal space group $P6_322$ (No. 182) with unit-cell dimensions $a = b = 73.43$ (1), $c = 277.17$ (4) Å, $\gamma = 120°$. A vancomycin–peptide complex with $M_r = 1688$ Da and an experimental density measurement of 1.106 g cm^{-3} requires 24 molecules (or 12 dimers) per asymmetric unit to give a volume per unit molecular weight, V_m, of 2.6 Å^3 Da^{-1} which is an acceptable Matthews value (Matthews, 1968). Subsequently, this gives an estimated solvent content of 42% which is roughly in line with that found for the recent vancomycin dimer (Schäfer *et al.*, 1996).

Interestingly, had the crystal been mounted on our CAD-4 instead of the Siemens instrument the large cell dimensions would not have been resolved and the crystals assigned to be non-diffractors.

Initial self-rotation function calculations indicate (not surprisingly) stacking along the long *c* axis, possibly by hydrogen-bond networking between the complex and water which may also involve the buffer. With the accurate model available for the vancomycin dimer complexed to *N*-acetyl-D-alanine (Loll *et al.*, 1998), molecular-replacement techniques would seem to offer a possible route to structure determination. However, there is a superstructure motif which may deny solution by this technique, especially in such a high-symmetry space group.

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