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Crystallization and preliminary X-ray characterization of VanA from *Enterococcus faecium* BM4147: towards the molecular basis of bacterial resistance to the glycopeptide antibiotic vancomycin

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A recombinant form of *Enterococcus facium* BM4147 D-alanine-D-lactate ligase (VanA) has been prepared and crystallized. VanA was found to crystallize only in the presence of a phosphinate inhibitor analogue of D-alanine-D-alanine. The crystals grow in 40–45% ammonium sulfate, 0.1 *M* 3-(*N*-morpholino)-propanesulfonic acid pH 6.0 and reach dimensions of 0.4 × 0.2 × 0.1 mm. The crystals diffract to at least 2.5 Å and are in the centred orthorhombic space group $C222_1$, with unit-cell dimensions $a = 123.2$, $b = 225.4$, $c = 72.4$ Å.

1. Introduction

The peptidoglycan layer of bacterial cell walls is a macromolecular assembly of unusual chemical composition which provides the cell wall with its strength and rigidity and which is essential for cell viability and osmotic integrity. Peptidoglycan consists of a long alternating matrix of polysaccharide chains of *N*-acetylmuramic acid and *N*-acetylglucosamine repeats, cross-linked by short pentapeptide chains. The enzymatic cross-linking of peptidoglycan is the target for a number of important glycopeptide antibiotics such as vancomycin, the use of which is now becoming limited owing to the ever-increasing resistance mechanisms employed by various clinically important bacteria such as the *Enterococci*. The search for novel antibiotics and novel antibiotic targets is therefore of increasing importance, as is a greater understanding of how resistant mechanisms arise.

The structure of the pentapeptide is unusual in that it contains a number of D-amino acids. The penultimate amino acid in the peptidoglycan chain (D-alanine) crosslinks with the central amino acid in an adjacent chain. In Gram-positive bacteria, this central amino acid is L-lysine, whereas in Gram-negative bacteria this position is occupied by *meso*-diaminopimelate. The glycopeptide antibiotic vancomycin acts by binding to the D-alanine-D-alanine termini of peptidoglycan chains, preventing crosslinking and therefore weakening the cell wall. Vancomycin-resistant organisms such as *Enteroccus facieum* BM4147 have become resistant by the production of peptidoglycan termini ending in a variety of alternate ester linked D-hydroxy acids such as D-lactate. A D-alanine-D-lactate depsipeptide is produced by the action of VanA from *E. facieum* BM4147, which has an altered affinity from D-alanine to D-lactate at the second binding site. The resultant peptidoglycan chain has a 1000-fold decrease in vancomycin-binding affinity which, in conjunction with the toxicity to humans of vancomycin, renders the drug unusable in patients with enterococcal infections. The gene for VanA is encoded by a transposon Tn1546 (Arthur *et al.*, 1993) and acts in conjunction with two other genes, *VanH* and *VanX*, to produce high-level vancomycin resistance. The recently crystallized VanH (Stoll *et al.*, 1998) is related to bacterial D-lactate dehydrogenase enzymes and is responsible for the production of D-hydroxy acids. VanX is a metallodipeptidase (Wu *et al.*, 1995) and reduces the cellular pool of D-alanine-D-alanine, which would otherwise be incorporated into peptidoglycan rendering the cell susceptible to vancomycin.

The X-ray crystal structure of D-alanine-D-alanine ligase (ddlB) from *Escherichia coli* has been solved at 2.3 Å resolution (Fan *et al.*, 1994) and mutational analysis has revealed some of the key residues involved in substrate binding (Shi & Walsh, 1995; Fan *et al.*, 1997). Using this information, inferences have been drawn concerning the key residues in VanA thought to mediate the altered substrate specificity crucial to the clinical importance of this enzyme. However, given that there is only 28% sequence identity between the two enzymes and the fact that VanA is some 6 kDa larger, only a full structural inspection of VanA will provide a clear description of how the substrate specificity of this clinically important enzyme is defined.

2. Materials and methods

VanA was purified from *E. coli* BL21(DE3) transformed with pAT214 (Dukta-Malen *et al.*,

1990) using a revised protocol derived from Bugg *et al.* (1991). Cells were freshly transformed and grown in Luria broth to an OD_{600} of 0.5 before induction with isopropyl β-D-thiogalactopyranoside to 0.5 m*M*. After a further 6 h of growth, ampicillin was added to 100 mg ml^{-1} to maintain plasmid selection prior to addition growth for 12 h. The cells were then harvested by centrifugation and stored at 193 K prior to purification of the enzyme. Crude extracts were produced by sonication in 25 m*M* HEPES pH 8.5, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA. After centrifugation at 30 000*g* for 30 min to remove cellular debris, the bacterial cell extract was brought to 25% saturation with ammonium sulfate and precipitated proteins were removed by centrifugation. The supernatant from this step was brought to 50% saturation with ammonium sulfate to precipitate the VanA protein. The ammonium sulfate pellet was dissolved in 25 m*M* HEPES pH 8.5, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA and dialysed against the same buffer overnight at 277 K. The resulting dialysed extract was applied to a 50 ml Q-Sepharose HP column and extensively washed in the same buffer. Proteins were eluted with a linearly increasing gradient of sodium chloride to 0.5 *M*, with VanA eluting at approximately 100 m*M* NaCl. Fractions containing VanA were further purified by gel filtration using a Superdex 75 HR26/60 column equilibrated in 50 m*M* HEPES pH 8.5, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA and 100 m*M* NaCl. The non-aggregated fractions were pooled and, after twofold dilution, applied to a 5 ml Q-Sepharose HP column. Proteins were eluted using a shallow gradient of NaCl to 0.3 *M* over 20 column volumes. Fractions containing VanA were pooled and concentrated prior to crystallization experiments. Following SDS–PAGE analysis, VanA-containing fractions were taken and concentrated prior to crystallization. Analysis of these fractions by both SDS–PAGE and native PAGE showed them to be free of any major or minor contaminating species and the molecular mass was confirmed by MALDI–TOF mass spectrometry.

2.1. Crystallization, data collection and processing

Crystals of VanA were grown by vapour diffusion using the hanging-drop method. Crystals were obtained in two sets of conditions from the commercially available Hampton 1 screening method (Hampton Research). Refinement of the ammonium sulfate conditions at pHs between 6.0 and 6.5 using 0.1 *M* 3-(*N*-morpholino)-propanesulfonic acid buffer gave crystals of sufficient quality for data collection. The protein concentration used for crystallization varied between 15 and 20 mg ml^{-1} in 10 m*M* HEPES pH 8.5, 5 m*M* ATP and 5 m*M* 1-(*S*)-aminoethyl [2-carboxy-2-(*R*)-methyl-1-ethyl] phosphinic acid phosphinate inhibitor (Ellsworth *et al.*, 1996; Parsons *et al.*, 1988), using a ratio of two volumes protein to one volume of precipitant. Crystallization experiments were carried out at 290 K. For data collection, single crystals of VanA were transferred using a rayon-fibre loop into mother liquor containing glycerol, with stepwise increases in glycerol concentration from 15 to 35%. Once at 35% glycerol concentration, the loop was placed in a stream of N_2 gas at 120 K to cryo-cool the crystal. Data were collected with an oscillation range of 1° per image using a Cu $K\alpha$ radiation source and a MAR Research MAR345 image-plate device. The data were processed and reduced using the *HKL* suite of programs (Otwinowski & Minor, 1997).

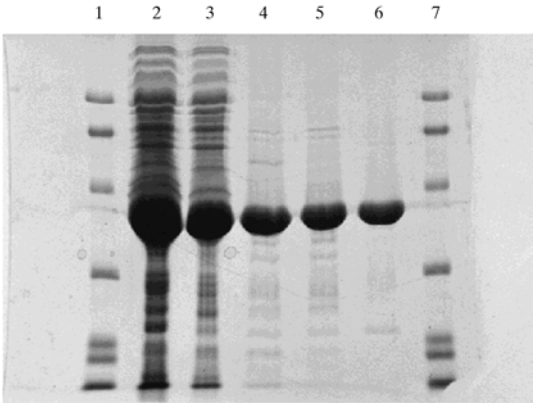

Figure 1
10% SDS–PAGE analysis of VanA purification. Lane 1, molecular-weight markers: (97.4, 66.2, 45.0, 31.0, 26.6, 21.5, 14.4 kDa); lane 2, crude extract; lane 3, 25–50% ammonium sulfate pellet; lane 4, pool of Q-Sepharose HP fractions; lane 5, pool of non-aggregated Superdex 75 fractions; lane 6, pool of Q-Sepharose fractions; lane 7, molecular-weight markers (as in lane 1).

3. Results and discussion

Attempts to overexpress the protein in a T7 promoter-based system using a polyhistidine tag for rapid affinity purification were successful, but for unknown reasons did not yield protein that would crystallize. Expression of VanA using a constitutive expression system achieved a yield of approximately 10–20% of total soluble protein after overnight induction. The recombinant protein was purified using a modified protocol to that published (Bugg *et al.*, 1991) to above 99% purity as judged by SDS. Purification of the enzyme is shown in Fig. 1. The protein was found to be unstable at neutral pH, in addition to low-salt conditions as previously described by Bugg *et al.* (1991). By maintaining the protein at an alkaline pH of 8.5, we were able to minimize aggregation, which had proved to be a serious problem in early preparations of VanA. The aggregation state of the protein was monitored throughout the purification using native gel electrophoresis. It was also our experience that VanA either precipitated or bound irreversibly to high-resolution ion-exchange media including Mono-Q, Resource-Q and Source-Q media, which hampered the production of protein samples of sufficient purity for crystallization. The subunit molecular weight of the protein was determined by MALDI–TOF MS as 37 499 Da, compared with a calculated molecular weight of 37 419 Da.

Crystals of VanA appear within 5–7 d at 290 K in the presence of ATP and D-alanine-D-alanine phosphinate inhibitor (see Fig. 2) and were of a size suitable for X-ray data collection. The crystals are of space group $C222_1$, with unit-cell dimensions a = 123.2, b = 225.36, c = 72.44 Å. Using the method of Matthews (1968), the solvent content of these crystals was calculated to be 63.13%, with a Matthews coefficient of 3.36 Å Da^{-1}, assuming two molecules in the asymmetric unit. X-ray diffraction data to 2.5 Å resolution (values for the outer resolution shell, 2.54–2.50 Å, are given in parentheses) are complete to 93.3% overall (96.6%) with an $R_{\rm merge}$ of 5.8% (21.7%), a multiplicity of 4.23 (3.85) observations per reflection and a mean $I/\sigma(I)$ of 29.9 (4.1).

A structure for VanA will further elucidate the molecular events leading to peptidoglycan biosynthesis in the clinically relevant *Enterococci* and shed further light

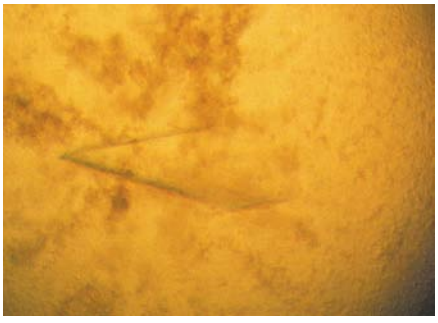

Figure 2
Photograph of VanA crystal of approximate dimensions 0.5 × 0.2 × 0.1 mm.

on possible mechanisms and targets for novel antibacterial agents.

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