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Crystallization and preliminary X-ray characterization of the *Bacillus amyloliquefaciens* YwrO enzyme

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CB1954 is an anticancer prodrug that is currently in clinical trials coupled with the *Escherichia coli* flavoenzyme nitroreductase (NTR) for use in directed-enzyme prodrug therapy (DEPT). The NTR enzyme is responsible for the conversion of the prodrug into a cytotoxic agent. The bifunctional alkylating agent produced by this bioactivation process leads to DNA damage and death of cancer cells. Recently, a novel flavoenzyme from *Bacillus amyloliquefaciens*, YwrO (Bam YwrO), was reported to be able to reduce CB1954 from its noncytotoxic form into its active form. The crystallization and preliminary X-ray diffraction analysis of two crystal forms of Bam YwrO are reported. The first crystal form is orthorhombic, with space group $P22_12_1$, and diffracts X-rays to 2.18 Å resolution. The second crystal form is tetragonal, with space group $P4_1$, and diffracts X-rays to 3.4 Å. Determination of the Bam YwrO crystal structure will provide an understanding of the molecular recognition between this enzyme and the anticancer prodrug CB1954.

1. Introduction

CB1954 (2,4-dinitro-5-aziridinylbenzamide) is a nitroaromatic compound that is presently in clinical trials as a prodrug in anticancer therapy. It was originally synthesized over 30 years ago at the Chester Beatty Research Institute, UK as part of a series of tumour-inhibitory nitrophenylaziridines (Khan & Ross, 1970). This low-molecular-weight compound was reported to eradicate rat Walker tumour 256 upon bioactivation by the NADPH-quinone oxidoreductase 1 enzyme (NQO1), which is overexpressed in this type of tumour cell line. Since then, CB1954 has been of interest for its potential use in the treatment of human cancer (Workman *et al.*, 1986) and has been described as 'a drug in search of a human tumour to treat' (Knox *et al.*, 2003).

Bioactivation of CB1954 from its relatively noncytotoxic form into the powerful anticancer agent involves the enzymatic reduction of one of the two nitro groups (Knox *et al.*, 2000, 2003). The nitro group at position 4 of the aromatic ring becomes enzymatically reduced to the hydroxylamine derivative, as shown in Fig. 1. This chemical modification yields a new alkylating functional group additional to the aziridine group. The resulting compound is therefore a bifunctional alkylating agent which eventually forms DNA interstrand cross-links.

There are two ways in which the prodrug CB1954 can be bioactivated: endogenously or by the direct-enzyme prodrug therapy (DEPT) approach, which relies on enzymes from an external source (Knox & Chen, 2004; Knox *et al.*, 2003; Denny, 2003). Endogenous bioactivation requires the overexpression of an enzyme able to activate CB1954 in the tumour tissue. For instance, in rat Walker tumour 256 the previously mentioned NQO1 enzyme is overexpressed and efficiently activates the prodrug. However, the homologous human NQO1 (86% sequence identity with the rat enzyme) showed poor catalytic activity towards CB1954, which explains the diminished cytotoxic effects of this compound in some human tissues compared with rat (Chen *et al.*, 1995). The low CB1954-reduction activity of human NQO1 was rationalized in terms of the change of Tyr104 (in the rat enzyme), which is located next to

Table 1
Oligonucleotide sequences of the primers used in the PCR steps for cloning.

Oligonucleotide	Sequence, 5′–3′
BamYwrO-F	CAGAAGGATTCAGCATATGAAAGTATTGGTAT-TAGCGGTTC
BamYwrO-R	AGCTGCAGAGTTAAAATGATTTCTTTATGTAAGCAGCC
YwrO-F-h6-Xa	CATATGCATCATCATCATCATCATATTGAAGGCCGCAT-GAAAGTATTGGTATTAGCGG
BamYwrO-R	AGCTGCAGAGTTAAAATGATTTCTTTATGTAAGCAGCC
YwrO-CB1	TAAGGCATGGGCAGAAGAATTAA
YwrO-CA	TTAATTCTTCTGCCCATGCCTTA
YwrO-CC	AAAGAACGTCAGCTGTGTGAACA
YwrO-CB2	CTGTTCACACAGCTGACGTTCTT

the FAD cofactor (Chen *et al.*, 1997), to Gln. Interestingly, the human enzyme NQO2, an isoform of NQO1 (47% sequence identity), has a tyrosine at position 104 and is capable of comparable catalytic activity to rat NQO1 (Chen *et al.*, 2000).

The DEPT approach is used to introduce a prodrug-activating enzyme into tumour cells and in general this can be achieved in a number of ways: virus/gene DEPT (Melton *et al.*, 1999), antibody DEPT (Syrigos & Epenetos, 1999) and clostridial DEPT (Minton, 2003). In the case of CB1954, the virus/gene DEPT method is used to deliver a nitroreductase (NTR) gene from *Escherichia coli* to tumour cells using retroviral and adenoviral vectors so that the enzyme is expressed in the cells. The introduction of an appropriate activating enzyme such as *E. coli* NTR in the target tumour cells allows the conversion of the prodrug CB1954 into its cytotoxic form, thus achieving a high therapeutic effect. This *E. coli* NTR-CB1954 system using virus/gene DEPT proved to be successful and has reached phase III clinical trials (Dachs *et al.*, 2005; Palmer *et al.*, 2004).

The human NQO2 and *E. coli* NTR enzymes can both effectively bioactivate the prodrug CB1954, but with different ratios of the cytotoxic 4-hydroxylamine product. NQO2 reduces the nitro group at position 4, giving only the 4-hydroxylamine derivative (Fig. 1). On the other hand, *E. coli* NTR generates equimolar amounts of the 2-hydroxylamine and 4-hydroxylamine products. The 2-hydroxylamine derivative is not as cytotoxic as the 4-hydroxylamine form (Knox *et al.*, 1992, 2003).

A novel nitroreductase enzyme (Bam YwrO) of 174 amino acids in length with a molecular weight of 19 656 Da isolated from *Bacillus amyloliquefaciens* and overexpressed from the *ywrO* gene has been reported to have catalytic activity towards the prodrug CB1954, producing only the cytotoxic 4-hydroxylamine derivative (Anlezark *et al.*, 2002). The study showed that the enzyme has comparable kinetic properties to those of NQO2 and *E. coli* NTR. The catalytic efficiency of the novel enzyme Bam YwrO in reducing CB1954 is determined by the k_{cat}/K_m ratio, which is 0.013 μM^{-1} s^{-1} and lies between the value for NQO2 and that for *E. coli* NTR, which are 0.02 and 0.007 μM^{-1} s^{-1}, respectively. Moreover, despite being from a bacterial source, the Bam YwrO amino-acid sequence shares some striking homology with that of NQO2 (27% sequence identity), but has no similarity to that of *E. coli* NTR. While NQO2 is a dimer, YwrO was found to be a tetramer in solution (Anlezark *et al.*, 2002).

Investigation of the Bam YwrO crystal structure, both in its apo form and in complex with CB1954, will reveal details of the molecular interactions governing the binding of the prodrug. In addition, comparisons with the CB1954-complexed structures of NQO2 (AbuKhader *et al.*, 2005) and *E. coli* NTR (Johansson *et al.*, 2003) will help to explain the catalytic properties of this novel enzyme.

2. Materials and methods

2.1. Cloning

The *ywrO* gene was PCR-amplified from *B. amyloliquefaciens* ATCC23842 genomic DNA using the primers BamYwrO-F and BamYwrO-R (see Table 1) and cloned into the vector pCR2.1 (Invitrogen) and the nucleotide sequence was determined. The gene from this strain encoded a protein with two amino-acid differences compared with the previously characterized YwrO protein (Anlezark *et al.*, 2002). To ensure consistency with the previous study, the appropriate two codons were altered by site-directed mutagenesis: one portion of the gene was PCR-amplified using the primers YwrO-F-h6-Xa and YwrO-CA, a second portion using the primers YwrO-CB1 and YwrO-CB2 and a third portion using the primers YwrO-CC and BamYwrO-R (see Table 1 for primer sequences). The three PCR products were gel-purified and used as templates in a three-way overlap extension PCR using the outer primers YwrO-F-h6-Xa and BamYwrO-R. The full-length PCR product was cloned into pCR2.1 and its nucleotide sequence was verified. The resulting gene encodes a protein identical to that previously described, with the addition of an N-terminal six-histidine tag (MHHHHHHIEGR-YwrO). The gene was excised as an *Nde*I/*Pst*I fragment and inserted into the equivalent sites of the expression plasmid pMTL1015. This plasmid is a derivative of the vector pMTL1013 (Brehm *et al.*, 1991), in which the *trp* promoter has been replaced by the *E. coli mdh* promoter (Nichols *et al.*, 1989).

2.2. Expression and purification

The YwrO protein was overexpressed by inoculation of 500 ml LB medium containing 10 μg ml^{-1} tetracycline with 5 ml of an overnight culture. The culture was then incubated at 310 K and shaken at 200 rev min^{-1} for 24 h. Cells were harvested by centrifugation at 1828*g* (4000 rev min^{-1}) for 30 min, resuspended in 20 ml lysis buffer

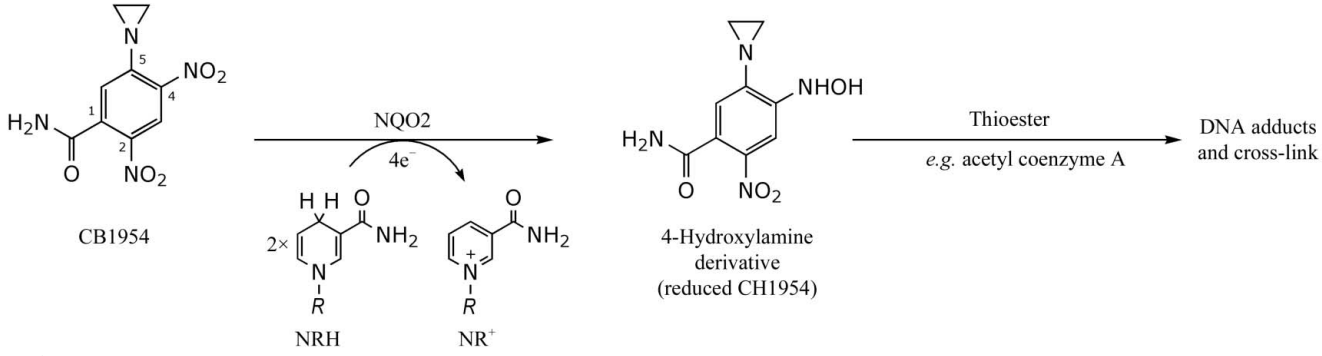


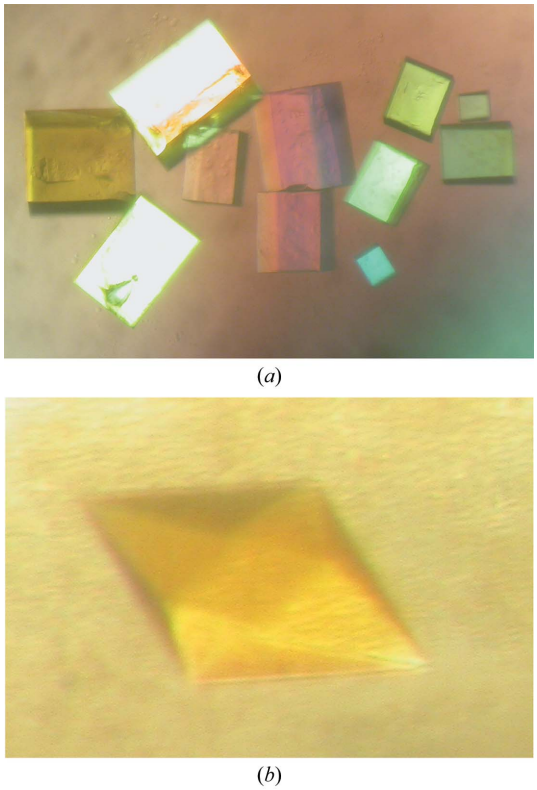
Figure 1
The four-electron bioactivation mechanism of CB1954 by NQO2 (Knox & Chen, 2004).

(50 m*M* Tris–HCl pH 8, 300 m*M* NaCl) and lysed by sonication. The resulting lysate suspension was centrifuged at 24 696*g* (17 500 rev min^{-1}) for 30 min and the resulting supernatant solution was further centrifuged at 33 889*g* (20 500 rev min^{-1}) for 20 min. The supernatant, which has the characteristic yellow colour of the flavin cofactor, was then filtered using 0.22 µm filters, mixed with 2 ml Ni–NTA resin (4 ml 50% Ni–NTA resin slurry from Qiagen) and equilibrated by gentle mixing for 2 h at 277 K. Imidazole was added to the binding mixture to a final concentration of 10 m*M* to reduce nonspecific binding. The equilibrated mixture was loaded onto a 10 ml Bio-Rad gravity column and washed with three bed volumes of 50 m*M* Tris–HCl pH 8, 300 m*M* NaCl, 20 m*M* imidazole (followed by a further wash at 50 m*M* imadazole). Elution was carried out with two bed volumes of 50 m*M* Tris–HCl pH 8, 300 m*M* NaCl and 250 m*M* imidazole. The purified protein solution was dialysed against 50 m*M* Tris–HCl pH 8 and 300 m*M* NaCl overnight at 277 K and showed a high degree of homogeneity by SDS–PAGE. The dialysed protein solution was then concentrated using Centricon devices (VivaScience 10 000 Da cutoff). Four concentrations were prepared (4, 10, 15 and 21 mg ml^{-1}) and the Pre-Crystallization Test kit (Hampton Research) was used to determine the most appropriate protein concentration for crystallization screening. A protein concentration of 21 mg ml^{-1} was used for crystallization.

2.3. Crystallization

A Hydra II micro-dispensing system (Robbins Hydra, Matrix Technologies Ltd, Wilmslow, Engand) was used in the initial high-throughput crystallization and screening process. The sparse-matrix factorial search (Jancarik & Kim, 1991) method was used for the screening of initial crystallization conditions using the following five crystallization screens (Nextal Biotechnology/Qiagen): Classics, Anions and Cations, pH Clear, $AmSO_4$ and MPD. 96-Well crystallization plates (Intelliplates, Hampton Research) were used; equal volumes of protein and reservoir solution (2 µl each) were dispensed and combined into sitting drops. The plates were sealed tightly with clear tape and kept in an incubator at 293 K.

After two weeks, Bam YwrO crystals were obtained from two conditions. These hit conditions were (i) 65% MPD and 0.1 *M* bicine pH 9 and (ii) 2.4 *M* ammonium sulfate and 0.1 *M* citric acid pH 4. It was noticed that crystals grew at two different pH conditions (pH 4 and pH 9) with apparently differing crystal symmetry. The first hit condition was optimized using the sitting-drop technique in 24-well plates. Stock solutions of 100% MPD and 1 *M* bicine pH 9 were utilized. Improved crystals were obtained with 67.5% MPD and 0.1 *M* bicine pH 9 with a protein concentration of 21 mg ml^{-1}. The second hit condition is still being optimized.



(*a*)

(*b*)

Figure 2
Bam YwrO crystals grown from (*a*) 67.5% MPD and 0.1 *M* bicine pH 9 (crystal form 1, orthorhombic) and (*b*) 2.4 *M* ammonium sulfate and 0.1 *M* citric acid pH 4 (crystal form 2, tetragonal). The crystals were grown using the vapour-diffusion technique with sitting drops in 24-well plates.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Crystal form 1	Crystal form 2
Space group	$P22_12_1$	$P4_1$
Unit-cell parameters (Å)		
a (Å)	55.5	152.7
b (Å)	77.4	152.7
c (Å)	93.7	86.3
α (°)	90	90
β (°)	90	90
γ (°)	90	90
Resolution range (Å)	26–2.18 (2.30–2.18)	35–3.4 (3.58–3.40)
No. of observations	93246 (14217)	112905 (16646)
No. of unique reflections	21823 (3131)	27459 (3946)
Multiplicity	4.3 (4.5)	4.1 (4.2)
Completeness	99.8 (99.9)	99.6 (98.8)
R_{sym}† (%)	4.7 (38.9)	14.8 (46.4)
Mean I/σ (I)	14.5 (2.8)	6.0 (2.3)

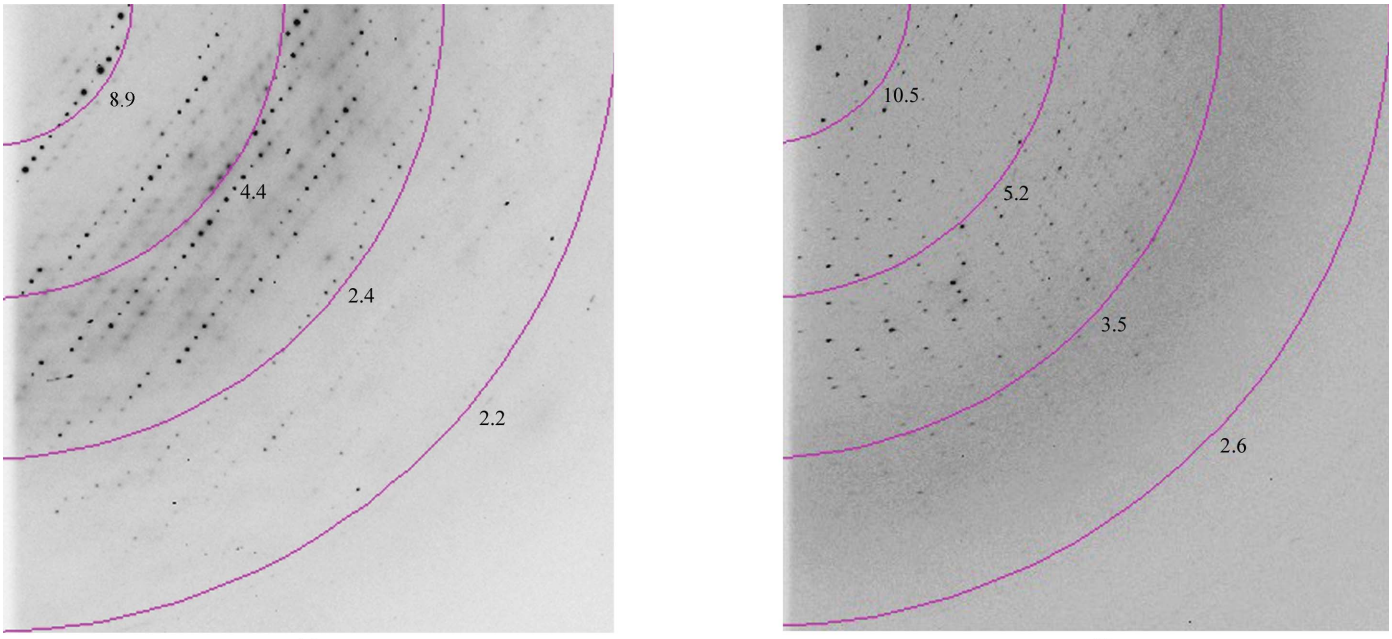
† $R_{sym} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i |I_{h,i}|$, where the outer summation is overall unique reflections with multiple observations and the inner summation is overall observations of each reflection.

2.4. Data collection

X-ray diffraction experiments were performed at the European Synchrotron Radiation Facility (Grenoble, France). Data were collected on the ID14-2 beamline using an ADSC Q4R detector and an X-ray wavelength of 0.933 Å. Data collection was carried out under cryogenic conditions (100 K). Crystals from the first condition did not require special cryoprotection because they were grown from solutions containing 67.5% MPD. On the other hand, crystals from the second condition were soaked for a few seconds in a solution of artificial mother liquor containing 20% glycerol. Crystals were mounted in a nylon loop and flash-cooled in a liquid-nitrogen stream at 100 K. For each of the crystal forms, 120 frames with 1° oscillation and 15 s exposure (no attenuation) were collected. Diffraction data sets were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The crystallographic statistics for the processed data for both crystal forms are shown in Table 2.

3. Results

We have established conditions for the growth of diffraction-quality crystals of the Bam YwrO protein (Figs. 2 and 3). The best condition, which gave crystals of high diffraction quality (crystal form 1) with



(*a*) (*b*)

Figure 3
Diffraction patterns obtained from (*a*) crystal form 1 (orthorhombic) and (*b*) crystal form 2 (tetragonal). Resolution ranges are shown (values are in angstroms).

average dimensions of 0.2 × 0.2 × 0.1 mm, was 67.5% MPD and 0.1 *M* bicine pH 9. The other condition, consisting of 2.4 *M* ammonium sulfate and 0.1 *M* citric acid pH 4, gave small crystals with average dimensions of 0.08 × 0.08 × 0.05 mm and weak diffraction (crystal form 2). However, better results may be obtained with this crystal form after more extensive optimization.

From the diffraction data collected, the space group was determined to be $P22_12_1$ for crystal form 1 and $P4_1$ for crystal form 2 and the Matthews coefficients V_M were calculated to be 2.57 Å^3 Da^{-1} with a solvent content of 52.1% and 6.38 Å^3 Da^{-1} with a solvent content of 80.7%, respectively. These values are compatible with the presence of two molecules in the asymmetric unit of the orthorhombic crystal form, consistent with a homodimer, presumably similar to NQO2, which would then form through crystallographic symmetry the particular tetrameric assembly observed in solution for YwrO (Anlezark *et al.*, 2002). In crystal form 2, a tetrameric arrangement may be present in the asymmetric unit, *i.e.* with fourfold NCS, in line with a weak molecular-replacement solution obtained for the tetragonal data. However, based on the V_M coefficient we cannot yet rule out the presence of a second tetramer in the asymmetric unit.

While the proteins Bam YwrO and NQO2 are believed to have homologous structures, they only have 27% sequence identity and 36% sequence similarity. This level of homology may be sufficient to carry out molecular replacement with the program *Phaser* (Storoni *et al.*, 2004) using the crystal structure of NQO2 (PDB code 1qr2; Foster *et al.*, 1999) as a search model. However, molecular-replacement calculations with the NQO2 coordinates did not return any clear solutions. When the search model was modified, based on the differences identified through sequence alignments, by removing the last 10–12 residues, truncating many amino-acid side chains to alanine and including any conservative substitutions (*e.g.* isoleucine to leucine), a molecular-replacement solution could be identified with a *Z* score of 18.9 and a log-likelihood gain (LLG) value of 389. The solution found confirmed that two molecules are present in the asymmetric unit. This is a good starting point from which to solve the crystal structure of the Bam YwrO enzyme; the presence of the twofold NCS could further help the model building and refinement through electron-density averaging calculations.

Given that the Bam YwrO amino-acid sequence contains seven S atoms, in six methionine residues and one cysteine residue, it will also be possible to use the technique of sulfur-based single-wavelength anomalous diffraction (S-SAD; Dauter *et al.*, 2002; Debreczeni *et al.*, 2003). Phase information derived from the anomalous signal of S atoms may be combined with the molecular-replacement phases to help with the interpretation of the electron-density maps and the rebuilding of the model. Determination of the Bam YwrO crystal structure will provide an understanding of the molecular recognition between this enzyme and the anticancer prodrug CB1954.

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