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Crystallization of type I chloramphenicol acetyltransferase: an approach based on the concept of ionic strength reducers

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Chloramphenicol acetyltransferase (CAT) is responsible for bacterial resistance to chloramphenicol. It catalyzes inactivation of the antibiotic by acetyl-group transfer from acetyl CoA to one or both hydroxyl groups of chloramphenicol. Type I CAT possesses some unique properties which are not observed in other CAT variants. Type I CAT overexpressed in *Escherichia coli* was purified and crystals with a resolution limit of 2.22 Å have been obtained using a novel procedure which is based on the concept of 'ionic strength reducers'. The crystals have the symmetry of space group *P1* and unit-cell parameters $a = 96.46$, $b = 113.86$, $c = 114.21$ Å, $\alpha = 119.9$, $\beta = 94.1$, $\gamma = 98.6^\circ$. These dimensions are consistent with four to six trimers per unit cell, corresponding to a solvent fraction ranging from 65 to 47%.

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1. Introduction

Bacterial resistance to antibiotics is frequently achieved by enzymatically catalyzed covalent modification of the drug. For chloramphenicol, enzymatic inactivation is achieved by acetylation. Chloramphenicol bears two hydroxyl groups, one or both of which may be acetylated by acetyl transfer from acetyl CoA. The enzyme responsible for this acetylation is chloramphenicol acetyltransferase (CAT; Shaw, 1983).

Three different classes of CAT have been found in Gram-negative bacteria; they have been classified as types I, II and III (Foster & Shaw, 1973). All natural CAT variants are homotrimers (Shaw & Leslie, 1991). The crystal structure of type III CAT has been determined at 1.75 Å resolution (Leslie, 1990). Based on chemical modification studies, a mechanism has been proposed in which the N^α atom of residue His195 of the enzyme acts as a general base, extracting a proton from the 3-hydroxyl group of chloramphenicol and thereby promoting a nucleophilic attack on the carbonyl of the thioester of acetyl CoA (Kleanthous & Shaw, 1984). This mechanism, which requires the formation of a tetrahedral intermediate and its subsequent collapse to a 3-acetoxy ester of chloramphenicol and coenzyme A, is consistent with the three-dimensional structure of CAT III (Leslie, 1990).

Type I CAT, which is encoded by the transposon Tn9, possesses some unique properties which are not observed in other known CAT variants. Besides chloramphenicol, it is also capable of inactivating fusidic acid, a steroidal inhibitor of bacterial protein synthesis acting at the elongation phase (Proctor *et al.*, 1983). This mechanism of resistance does not involve a

covalent modification of the antibiotic (Bennett & Shaw, 1983). Fusidate competes with chloramphenicol in CAT binding despite the lack of a structural similarity between the two drugs (Proctor *et al.*, 1983). On the other hand, type I CAT binds not only fusidic acid but also bile salts, as well as totally different compounds such as triphenylmethane dyes (Proctor & Rownd, 1982). Type I CAT is inhibited by triphenylmethane dyes in the following order: ethyl violet, crystal violet, methyl violet and parafuchsin. It has been shown that all these inhibitors are competitors of chloramphenicol (Tanaka *et al.*, 1974). The two variants of CAT (type I and type III) share 46% sequence homology. Eight of the 17 residues involved in the chloramphenicol binding site are different (Shaw & Leslie, 1991).

To understand the structural basis of the unique enzymatic properties of type I CAT, we have initiated crystallographic studies of the enzyme.

2. Experimental procedures

2.1. Expression of type I CAT gene in *E. coli*

The type I CAT gene is derived from Tn9 (Kirby & Vapnek, 1979) and modified by PCR in order to introduce a *Hind*III site immediately before the initiation codon and a *Bam*HI site after the stop codon. This gene is cloned in a pBR322-based expression vector containing a strong constitutive promoter (*P*₁) and a consensus Shine–Delgarno sequence (*P*₉) used previously for a high level of expression of various structural genes in *E. coli* (Ivanov, Tam *et al.*, 1987; Ivanov, Gigova *et al.*, 1987; Ivanov *et al.*, 1989).

2.2. Purification of type I CAT

Type I CAT was purified from an overnight grown culture of *E. coli* LE 392 cells transformed with the expression plasmid. The cells were harvested by centrifugation at 277 K, washed with 10 mM Tris buffer pH 8.0, 0.1 mM EDTA, centrifuged and re-suspended in 50 mM Tris buffer pH 7.8 containing 50 μ M β -mercaptoethanol (TM buffer). The cells were disrupted at 273 K by pulsed sonication for 5 min. The insoluble material was removed by centrifugation at 277 K. The supernatant was dialyzed overnight against TM buffer and loaded onto a chloramphenicol-Sepharose 4B affinity column pre-equilibrated with TM buffer. The column was washed with TM buffer containing 1 M NaCl and the protein was eluted with TM buffer containing 1.5 M NaCl and 5 mM chloramphenicol. CAT-containing fractions were pooled, dialyzed

overnight against 50 mM Tris buffer pH 7.8 containing 50 mM NaCl, 1 mM mercaptoethanol, and were concentrated to \sim 16 mg ml⁻¹ using Amicon Centriprep 10 and Centricon 10 microconcentrators.

2.3. CAT assay

CAT activity was measured spectrophotometrically at 277 K according to Shaw (1975). The standard assay mixture contained 0.1 M Tris-HCl pH 7.8, 0.1 mM acetyl-CoA, 0.1 mM chloramphenicol and 0.4 mg ml⁻¹ 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB). One unit of enzyme activity is defined as the amount of CAT required to acetylate 1 μ mol of chloramphenicol per minute under standard assay conditions.

2.4. Crystallization

Crystallization trials were initially performed by conventional hanging-drop vapour-diffusion methods (Ducruix & Giegé, 1992) using tissue-culture plates and siliconized glass cover slips (McPherson, 1982). Crystals appeared in drops containing equal volumes of protein concentrate and a reservoir solution consisting of 0.1 M MES buffer (pH range 5.4–6.2) and 44–48% MPD. These crystals frequently exhibited poorly defined morphologies and showed high mosaicity. Furthermore, it has not been easy to reproduce the results of these crystallization experiments.

A more controlled strategy was thus developed which utilizes the concepts of the so-called ionic strength reducers (Papanikolaou & Kokkinidis, 1997). These are organic solvents or hydrophilic polymers (*e.g.* polyethylene glycol) which have drastic effects on the solubility of biological macromolecules in aqueous electrolytic solutions. After developing a generalized form of Green's equation (Riès-Kautt & Ducruix, 1992), Papanikolaou & Kokkinidis (1997) have shown that for a given concentration of electrolyte, ionic strength reducers should decrease macromolecular solubility under salting-in conditions and increase it under salting-out conditions. Addition of salt should have the opposite effects. These predictions have been verified experimentally and have been shown to have potentially wide applications in macromolecular crystallization (Papanikolaou & Kokkinidis, 1997). In the case of type I CAT solutions, we

have observed that MPD and NaCl have opposite effects on CAT solubility, showing typical ionic strength reducer behaviour: at a constant concentration of MPD, a decrease of the salt concentration (*e.g.* by dialysis) reduces the solubility of CAT.

This observation allowed us to fine-tune the solubility of type I CAT by carefully balancing the MPD and salt concentrations; it is the basis of a more controlled approach to crystallization.

The new crystallization procedure combines dialysis methods with seeding techniques; changes in the balance of salt and ionic strength reducer concentrations are used to change protein solubility. Each crystallization experiment consists of a microseeding step (Stura & Wilson, 1992) performed in microdialysis cells, where protein solubility can be conveniently manipulated by equilibrating the protein solution across the dialysis membrane against a reservoir containing suitable concentrations of salt and MPD. For crystallization experiments, a stock of microscopic crystal seeds (usually obtained by crushing crystals grown by conventional hanging-drop vapour diffusion) is added to a protein solution (8 mg ml⁻¹) in a microdialysis cell containing 30% MPD, 50 mM MES buffer pH 6.0, 75 mM NaCl. This balance of MPD and NaCl concentrations keeps the protein soluble. Subsequent reduction of the NaCl concentration in the protein solution across the dialysis membrane (this is achieved by immersing the cell into a 3 ml reservoir containing the same MPD concentration as the protein solution but lower NaCl concentration, *i.e.* 30% MPD, 50 mM MES buffer pH 6.0, 50 mM NaCl) leads to a gradual decrease of protein solubility and to favourable growth conditions for the microscopic seeds. These reach dimensions of up to 1.0 mm and their quality is sufficient for crystallographic analysis (Fig. 1).

2.5. X-ray diffraction analysis

Data-collection work extending to the present resolution limit of 2.22 Å (Fig. 2) was performed at 100 K using cryoprotected crystals (the stabilizing solution contained 45% MPD as cryoprotectant agent) which were mounted in a stream of nitrogen gas using Hampton Research cryo-loops. Diffraction data were collected at the EMBL/DESY synchrotron-radiation beamline X11 using a MAR Research 345 imaging-plate detector. The rotation method (Arndt & Wonacott, 1977) was used. Oscillation frames of width 1.0° were recorded.

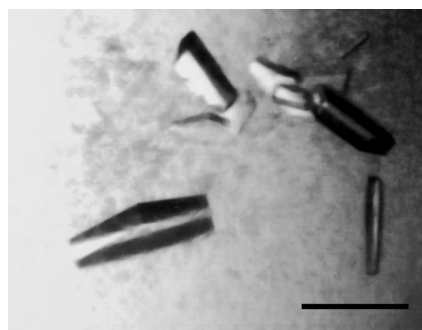


Figure 1
Crystals of type I chloramphenicol acetyltransferase. The scale bar corresponds to 0.5 mm.

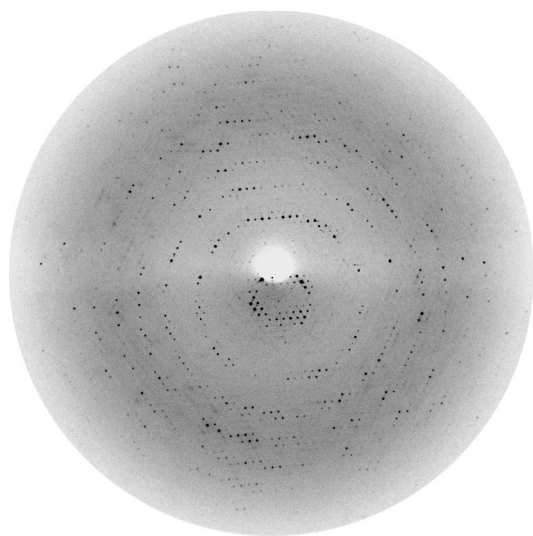


Figure 2
Diffraction pattern of a type I chloramphenicol acetyltransferase crystal obtained with a rotating-anode X-ray source and Cu K α radiation. The detector edge corresponds to 2.6 Å resolution.

Intensities were integrated with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The crystals have the symmetry of space group *P1* and unit-cell parameters $a = 96.46$, $b = 113.86$, $c = 114.21$ Å, $\alpha = 119.9$, $\beta = 94.1$, $\gamma = 98.6^\circ$. These dimensions can accommodate four to six trimers per unit cell, corresponding to a solvent content ranging from 65 to 47% (the corresponding Matthews coefficients, V_M , vary from 3.53 to 2.35 Å³ Da⁻¹; Matthews, 1968). A set of native diffraction data extending to the present resolution limit of 2.22 Å [357 527 observed reflections, 185 965 unique reflections with $\langle I/\sigma(I) \rangle = 12.6$ for all data and $\langle I/\sigma(I) \rangle = 3.0$ for the last resolution shell (2.3–2.2 Å), $R_{\text{merge}} = 5.3\%$, completeness of 91.7%] is used for the determination of the crystal structure. Because of the relatively high degree of amino-acid sequence homology (46%) between type I and type III CAT, molecular replacement using the structure of the type III variant will be attempted for the determination of the type I CAT structure.

The simple and efficient crystallization procedure described in this work, which is based on the concept of ionic strength reducers and combines dialysis with seeding techniques, was essential for the crystallization of the protein. Our results should warrant further investigation of the ionic strength reducer concept as potentially generally applicable in macromolecular crystallization.

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