

Purification and Crystallization of *Pseudomonas aeruginosa* Chloramphenicol Acetyltransferase

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ABSTRACT A chloramphenicol acetyltransferase from *Pseudomonas aeruginosa* genomic DNA has been overexpressed, refolded, purified, and crystallized. Crystals suitable for a three-dimensional x-ray structure determination were obtained from solutions of polyethyleneglycol methyl ether 2000 containing NiCl₂ at pH 8.5. These crystals belong to the cubic space group P4₁/32 (a = 154.8 Å) and diffract x-rays to ~3.2 Å resolution. **Proteins 28:298–300, 1997.** © 1997 Wiley-Liss, Inc.

Key words: chloramphenicol acetyltransferase; protein structure; x-ray crystallography; antibiotic resistance

INTRODUCTION

Although bacterial chloramphenicol (Cm) resistance is mediated in a variety of ways, inactivation of this drug by enzymatic acetylation is the best understood mechanism.¹ The chloramphenicol acetyltransferases (CATs) that catalyze this reaction use acetyl-CoA as the acyl donor to create the inactive *O*-acetoxy derivative of Cm that neither binds to bacterial ribosomes or inhibits protein elongation. The CAT_{III} purified from *Escherichia coli* strains harboring the R factor R387 has been the subject of intense study, and both a proposed mechanism of action and a three-dimensional structure for this enzyme are available.^{1–3}

It is apparent, however, that a very different CAT exists in certain bacterial strains. The sequence of the CAT from *Pseudomonas aeruginosa* PA103 is dissimilar to the “classic” CAT_{III} enzyme⁴ but instead shows similarity to enzymes of the *cysE/nodL/lacA* family of acetyltransferases.⁵ Several other antibiotic-inactivating acetyltransferases of this family have also been identified. These include the CATs from *Agrobacterium tumefaciens* C58⁶ and *E. coli* transposon Tn2424⁴ and enzymes that acetylate the group A compounds of the Virginiamycin class of antibiotics, such as the gene products of *vat* from *Staphylococcus aureus* pIP680,⁷ *vatB* from *S. aureus* pIP1156,⁸ and *satA* from *Enterococcus faecium* BM4145.⁹ We are interested in the structure and function of these acetyltransferases and the means by which Cm is inactivated. We report here the overexpression, refolding, purification, and crystallization of one member of this family, the genomic chloramphenicol

acetyltransferase from *Pseudomonas aeruginosa* PA103.

RESULTS AND DISCUSSION

Overexpression of *Pseudomonas aeruginosa* CAT (Pacat)

A lyophilized sample of *Pseudomonas aeruginosa* PA103 was obtained from the American Type Culture Collection (ATCC 29260, Rockville, MD) and rehydrated in 6 ml Trypticase Soy Broth (TSB, BBL 11768; 30.0 g/l). Approximately 400 µl of this mixture was diluted to 4 ml with TSB, incubated overnight at 37°C, and 1.5 ml was used to prepare genomic DNA. The *cat* gene was amplified by the polymerase chain reaction using Vent polymerase (New England Biolabs, Beverly, MA) and the genomic DNA as the template. The upstream primer sequence contained an *Nde*I restriction site and was designed from the published nucleotide sequence of *regB*,¹⁰ a gene that contains an overlapping open reading frame with the *cat* gene, thus providing partial DNA sequence information. It had the sequence 5'-ACGCATATGGC-AACTATTTTCGAGAGC-3'.

The downstream primer contained a *Bam*HI restriction site and was designed on the basis of the published amino acid sequence of the *P. aeruginosa* CAT,⁴ taking into account the codon usage pattern of this bacterium. It had the degenerate sequence CGGGATCCGCTTA(G/C)GC(G/C)GT(G/C)GCCTG-(A/G/C)CGCTGCTTCCA(A/G)TG-3'. The PCR product obtained (0.6 kb) was digested with *Nde*I and *Bam*HI prior to ligation into a similarly digested pET3a overexpression vector (Novagen, Madison, WI). The DNA sequence of the *cat* gene contained in the overproduction plasmid (pYT1) was determined with an Applied Biosystems ABI 377 sequencer (Applied Biosystems, Foster City, CA) and primers that directed the complete sequence determination

Abbreviations: Cm, D-threo chloramphenicol; CAT, chloramphenicol acetyltransferase; Pacat, *Pseudomonas aeruginosa* chloramphenicol acetyltransferase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TEA, triethanolamine; CoA, coenzyme A; DTNB 5-5'-Dithiobis(2-nitrobenzoic acid); IPTG, isopropyl β-D thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Received 26 November 1996; Accepted 5 December 1996

of both strands. The deduced amino acid sequence of Pacat differs from one published report at positions 97 (Val → Ala) and 203 (Arg → Gln).⁴

Ampicillin-resistant colonies of *E. coli* BL21(DE3) harboring the pYT1 overexpression plasmid were used to inoculate tube cultures and grown to OD₆₀₀ of 0.2–0.8. These cultures were induced with 1 mM IPTG and shaken for 2 h prior to preparation of soluble and pellet fractions for SDS-PAGE analysis. Although a greatly overexpressed band of the correct molecular weight (~23 kDa) was visible on these gels, the great majority of protein was found in the insoluble fraction, presumably because of the formation of inclusion bodies.

Large Scale Preparation of Pacat

Single colonies of *E. coli* BL21(DE3) harboring the overproduction plasmid pYT1 were used to inoculate four tube cultures of Luria-Bertani medium containing 50 µg/mL ampicillin (LB-Amp) and allowed to incubate overnight at ambient temperature. Each of the four tubes was used to inoculate 2-liter flasks containing 1 liter of LB-Amp media, which were shaken at 37°C until an A₆₀₀ of 0.6 was reached. The cultures were induced with 0.5 mM IPTG and continued for 1.5 h. The cell suspension was centrifuged at 7280 × *g* for 30 min, and the resulting 16-g pellet resuspended with 16 ml 50 mM TEA, pH 7.8, 1 mM EDTA and frozen in aliquots at –70°C for future use.

Because adjustment of incubation temperature and induction protocols did not markedly improve the fraction of protein present in the soluble fraction, a purification strategy designed to refold the insoluble protein was adopted.¹¹ Six frozen cell suspension aliquots (each 2-g cells in 5 ml) were thawed, and to each tube 250 µl 1 M Tris, pH 8.5, 1 ml 500 mM NaCl, 10 µl 500 mM EDTA, and 80 µl 10 mg/ml lysozyme were added. All subsequent steps were conducted at 0–4°C. The samples were set on ice for 20 min, disrupted with a probe sonicator, and pelleted by centrifugation at 12,000 × *g*. The pellet was washed in 30 ml H₂O and then in 30 ml wash solution (2 M urea, 5 mM EDTA, 0.02% [w/v] sodium azide, 100 mM Tris, pH 8.5, 10 mM DTT, and 1.3 mM PMSF). The pellet was extracted twice with 30 ml denaturation buffer (wash solution containing 5 M urea), and the pooled supernatant fractions was diluted to 200 ml in this buffer. The 200-ml Pacat solution was transferred to a dialysis bag (molecular weight cutoff 12–14 kDa) and dialyzed for 3-h time periods against 2-liter volumes of refolding buffer (5 mM EDTA, 0.02% sodium azide, and 10 mM DTT) containing successively lower urea and glycerol concentrations according to the following schedule: i) 4 M urea/40% glycerol, ii) 2 M urea/40% glycerol, iii) 0.5 M urea/40% glycerol, iv) 0.1 M urea/20% glycerol, v) 5% glycerol, and vi) refolding buffer.

The dialysate (186 ml) was removed, centrifuged, and applied in two runs to a 68-ml POROS 50 HQ

anion exchange column (Perseptive Biosystems, Framingham, MA) equilibrated in 20 mM TEA, pH 7.7, washed with two column volumes of this buffer, and eluted with a 1 M NaCl gradient over three column volumes. The fractions containing Pacat from each run were pooled on the basis of SDS-PAGE analysis and concentrated to 6 ml by using a stirred cell concentrator equipped with a YM10 membrane (Amicon, Beverly, MA). The concentrated Pacat sample of 86 mg (BioRad Protein Assay; BioRad, Hercules, CA) was applied to a 320-ml Superdex 200 prep grade column (Pharmacia, Piscataway, NJ) equilibrated in 20 mM TEA, pH 7.7, 50 mM NaCl and eluted isocratically at a flow rate of 1.5 ml/min. Fractions containing Pacat were pooled (61 mg), concentrated to 4 mg/ml, frozen in a dry-ice ethanol bath, and stored at –70°C.

The activity of the purified enzyme was measured by spectrophotometric quantitation using DTNB to quantify the reduced (unesterified) CoASH product of the reaction. A 0.8-ml reaction mixture containing 50 mM Tris, pH 8.5, 1 mM acetyl-CoA, 0.4 mg/ml DTNB, and enzyme was initiated with 0.2 ml 5 mM Cm at room temperature. Color development corresponding to production of the thionitrobenzoate ion produced on formation of the mixed disulfide between the product CoA and DTNB was followed spectrophotometrically at 412 nm, and the rate of color development in the absence of Cm was subtracted from this rate to assess Cm-dependent DTNB reacted. One unit of enzyme activity (U) is defined as the activity required to acetylate 1 µM of Cm per minute at ambient temperature. The specific activity of the final Pacat preparation was 262 U/mg.

The subunit mass of purified Pacat was determined by electrospray injection mass spectrometry to be 23,351 ± 3 d. This value is in good agreement with the subunit mass predicted from the DNA sequence determined here (also 23,351 d), presuming proteolytic cleavage of the leader methionine residue by the host *E. coli* methionine aminopeptidase.¹²

Crystallization of Pacat

Prior to crystallization, samples of Pacat were thawed and loaded onto a 7.8-ml POROS HQ 50 column at a flow rate of 10 ml/min. The column was washed with two columns of 20 mM TEA, pH 7.7, and eluted with a 1 M NaCl gradient in the same buffer over five column volumes. The enzyme eluted from the column as a single peak. The fractions were pooled, and a Centricon-30 concentration device (Amicon) was used to exchange buffer to 10 mM TEA, pH 7.7, 50 mM NaCl and to concentrate the enzyme to 16–21 mg/ml.

Crystals of Pacat were grown by the hanging drop vapor-diffusion method in Linbro tissue culture plates at room temperature. Either 4 or 5 µl of concentrated Pacat solution were mixed on silanized glass cover-

slips with a similar volume of the trial crystallization solutions contained in the Crystal Screen I and II crystallization kits (Hampton Research, Laguna Hills, CA) and inverted over reservoirs containing 0.75 ml of the crystallization solution. A parallel series of experiments was conducted with 2 mM Cm and 2 mM CoA added to the concentrated enzyme solution.

Although many hanging drops with and without the substrate-product pair displayed crystals of dodecahedral morphology, particularly from solutions containing high concentrations of phosphate and sulfate, all of these crystals failed to diffract x-rays beyond 5.0 Å resolution. However, crystals produced from solutions containing 20% polyethyleneglycol monomethyl ether 2000, 100 mM Tris, pH 8.5, and 10 mM NiCl₂ were of a distinct bipyramidal habit and attained a maximum size of $\sim 0.3 \times 0.3 \times 0.4$ mm. These crystals diffracted x-rays to 3.2 Å resolution at room temperature on a Rigaku RU200 rotating anode generator operating with fine focus at 50 kV and 80 mA and were assigned to space group as P4₁32 or enantiomorph ($a = 154.8$ Å) on the basis of the symmetry of x-ray reflections and pattern of systematic absences. These crystals of *Pseudomonas aeruginosa* CAT are suitable for a moderate resolution x-ray structure determination by the method of isomorphous replacement, which is in progress.

ACKNOWLEDGMENTS

We thank Drs. Steven C. Almo and John S. Blanchard, and Mr. Renjian Zheng for helpful discussions.

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