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Crystallization and preliminary X-ray analysis of bacterial cytosine deaminase

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Cytosine deaminase (CD) is found in prokaryotes and fungi (but not higher eukaryotes) and catalyzes the deamination of cytosine and 5-fluorocytosine to uracil and 5-fluorouracil, respectively. The former activity is an important function within the pyrimidine-salvage pathway, while the latter activity allows the formation of a cytotoxic chemotherapeutic agent from a non-cytotoxic precursor. Recombinant bacterial CD from *Escherichia coli* has been subcloned, overexpressed, purified and crystallized for structural analysis. The crystals belong to space group *R*32, with unit-cell parameters $a = b = 109.1$, $c = 240$ Å and diffract to at least 1.5 Å resolution at a synchrotron X-ray source. There is one enzyme subunit per asymmetric unit and the Matthews coefficient V_M is 2.8 Å^3 Da^{-1}, corresponding to a solvent content of 56%. Selenomethionine-containing protein has been prepared and crystallized for MAD phasing.

1. Introduction

Cytosine deaminase (E.C. 3.5.4.1) catalyzes the deamination of cytosine to uracil and ammonia. Cells expressing cytosine deaminase are sensitive to the nucleoside analog 5-fluorocytosine (5FC) owing to their ability to convert that substrate analogue to the toxic metabolite 5-fluorouracil (5FU). 5FU and its deoxyribonucleoside (fluorodeoxyuridine, FUdR) are potent inhibitors of DNA synthesis in their triphosphorylated forms and are widely used in cancer treatment (DiPiro *et al.*, 1997). Therefore, cytosine deaminase is being investigated for gene-therapy applications owing to the absence of this gene in humans (Austin & Huber, 1992; Hirschowitz *et al.*, 1995; Huber *et al.*, 1993; Kievit *et al.*, 1999; Mullen *et al.*, 1992). In addition, since most fungi express cytosine deaminase, 5-FC is commonly used as an antifungal drug (DiPiro *et al.*, 1997).

Cytosine deaminase is a distinct enzyme from cytidine deaminase, as that enzyme is completely dissimilar in sequence, size, substrate preferences and kinetic properties (Erbs *et al.*, 1997; Ipata & Cercignani, 1978; Kurtz *et al.*, 1999). A comparision of *E. coli* and *Saccharomyces cerevisiae* cytosine deaminases also reveals significant differences between these enzymes at the primary amino-acid sequence, monomer molecular mass, quaternary structure and substrate-affinity level, indicating that they are distinct enzymes (Erbs *et al.*, 1997; Kievit *et al.*, 1999). The bacterial enzyme (bCD) from *E. coli* is a hexamer of approximately 426 amino acids per subunit (see §2 below), giving a mass of approximately 300 kDa for the active enzyme complex. The yeast enzyme (yCD) is a homodimer consisting of 17 kDa per subunit. Several studies have indicated that the yeast enzyme is more efficient at catalyzing the conversion of 5FC to 5FU, in part because of its lower K_M for the 5FC substrate (0.8 m*M* for yeast, 17.9 m*M* for *E. coli*; Hayden *et al.*, 1998; Kievit *et al.*, 1999). These same studies have also indicated that the bacterial enzyme may be more thermostable than its yeast counterpart, but precise comparisons have not been reported and recent studies have not supported this observation (Peter Senter, private communication).

bCD is a metalloenzyme that has been reported to be dependent on Fe^{2+} for maximum activity (Porter & Austin, 1993). Removal of the ferrous ion by treatment with *o*-phenanthrolein results in inactivation of the enzyme; activity is fully restored by addition of free Fe^{2+}. Substitution with zinc for the active-site iron also restores activity, but only to approximately 10% of its maximal velocity and k_{cat}/K_M values (Porter, 2000). The enzyme is capable of deaminating a wide range of cytosine derivatives with varying efficiency, including cytosine, 2-thiocytosine, 6-azacytosine, 4-azacytosine and 5-fluorocytosine (Porter, 2000).

2. Methods and results

2.1. Subcloning, expression and purification

The *E. coli* cytosine deaminase gene was amplified by PCR from pCD2, a plasmid kindly provided by R. Michael Blaese. The primers for PCR were 5′-GTTATTCGC-CATGGCTAGCCT-3′ (forward with an *Nco*I site at the start codon) and 5′-GTCGTTCAAGCTTTGTAATCG-3′ (reverse with *Hin*dIII site just 3′ to the stop codon). The uncut PCR product was initially cloned into pCR2.1-TOPO (Invitrogen) and the cytosine deaminase gene was then subcloned as an 1.3 kbp *Nco*I/*Hin*dIII fragment into pET-HT (Brady *et al.*, 1996). Genes cloned in frame into the *Nco*I site of pET-HT are fused with sequences to provide a histidine tag at the N-terminus of expressed proteins. The resulting plasmid was sequenced and designated pETHT:bCD and contains an alteration in the bCD start codons from Val-Ser to Met-Ala. For protein expression, pETHT:bCD was transformed into the *E. coli* strain BL21(DE3).

Cells constitutively expressing bCD were grown in LB media at 310 K until an OD_{600} greater than 1.5 was achieved. The cells were harvested by centrifugation at 2000*g* for 10 min at 277 K and the cell pellets were resuspended in 50 ml lysis buffer (300 m*M* NaCl, 20 m*M* Tris–HCl pH 7.0, 5 m*M* imidazole, 0.2% Triton X-100, 0.5 m*M* PMSF). Lysozyme was added to 0.5 mg ml^{-1}, followed by 30 min incubation on ice. Cells were sonicated using a Branson sonifier 250 equipped with a macrotip set to pulse for 5–8 min at 277 K with the duty cycle set to 40%. The cell lysates were clarified by centrifugation at 20 000*g* for 30 min at 277 K and the clarified supernatant was passed through a 0.45 µm syringe filter. The filtered supernatant was added to 5 ml packed bed volume of TALON metal affinity resin (Clontech) equilibrated with ten bed volumes lysis buffer and incubated at 295 K for 30 min with rocking at 10 rev min^{-1}. The affinity resin was washed three times with ten bed volumes of wash buffer (300 m*M* NaCl, 20 m*M* Tris–HCl pH 7.0, 10 m*M* imidazole, 0.5 m*M* PMSF) and the bCD protein was eluted from the resin with 3 × 15 ml elution buffer (300 m*M* NaCl, 150 m*M* imidazole, 20 m*M* Tris–HCl pH 7.0, 0.5 m*M* PMSF). The eluted fractions were concentrated and dialyzed into storage buffer (100 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0, 1 m*M* DTT, 1 m*M* EDTA) prior to loading onto a Superdex 200 HiLoad16/60 sizing column (Pharmacia) equilibrated with storage buffer. Peak bCD fractions were analyzed by SDS–PAGE, pooled and concentrated to 30 mg ml^{-1}, with typical yields of 60–70 mg of highly pure His-tagged bCD (∼99%) obtained per litre of bacterial culture. Thrombin cleavage of the N-terminal histidine tag was conducted for 3 h at 310 K using 0.5 U biotinylated thrombin (Novagen) per milligram of bCD protein in thrombin cleavage buffer (150 m*M* NaCl, Tris–HCl pH 8.4, 2.5 m*M* $CaCl_2$). Biotinylated thrombin was removed by 30 min incubation at 296 K with 10 µl streptavidin-conjugated agarose beads per unit of thrombin followed by gravity flow-through column purification. The column flowthrough was dialysed into storage buffer and the protein concentration estimated by Bradford assay. Analysis of the protein molecular weight by size-exclusion chromatography against a variety of protein standards indicate that the protein is a functional hexamer (Fig. 1), rather than a tetramer or pentamer as previously hypothesized (Katsuragi *et al.*, 1986; Kievit *et al.*, 1999). This result is consistent with the space group and asymmetric unit of the crystallized protein (see below) and has been confirmed as part of the crystallographic structure determination (data not shown).

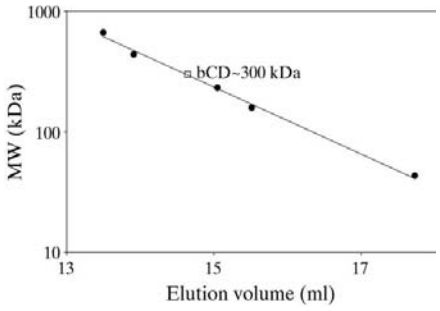

Figure 1
Size-exclusion chromatographic analysis of *E. coli* cytosine deaminase. Size standards from Pharmacia: thyroglobulin (669 kDa), apoferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), ovalbumin (43 kDa).

For inducible expression of the selenomethionine-derivatized protein, pETHT:bCD was digested with *Xba*I/*Xho*I and the fragment containing the fusion protein was subcloned into pET15b (Novagen). Semethionine-containing bCD (SeMet-bCD) was expressed in minimal media from *E. coli* strain BL21(DE3) adapted for growth with methionine-pathway inhibition (Doublie, 1997). Cells were grown in minimal media at 310 K to an OD_{600} of 0.6 and the following amino acids were added to inhibit methionine biosynthesis: 100 mg l^{-1} lysine, 100 mg l^{-1} threonine, 100 mg l^{-1} phenylalanine, 50 mg l^{-1} leucine, 50 mg l^{-1} isoleucine, 50 mg l^{-1} valine and 75 mg l^{-1} selenomethionine. Following 15 min incubation at 310 K, isopropyl-thio-β-D-galactosidase (IPTG) was added to a final concentration of 0.5 m*M* and the cultures were grown at 310 K for 12 h post-induction. SeMet-bCD was purified analogously to native bCD. Incorporation of selenium was verified by mass spectrometry.

Table 1
Native data statistics.

Values in parentheses refer to the 1.56–1.50 Å resolution bin.

Space group	*R*32
Unit-cell parameters (Å)	$a = b = 109$, $c = 240$
Resolution (Å)	1.5
Total reflections	715529
Unique reflections	170364
Redundancy	4.2
Completeness	100.0 (100.0)
Average $I/\sigma(I)$	40.7 (12.0)
$R_{\rm merge}$	0.041 (0.114)

2.2. Crystallization and preliminary X-ray analysis.

Initial crystals of bCD were grown from Hampton sparse-matrix screens (Jancarik & Kim, 1991) by the method of vapour diffusion in hanging drops at room temperature. Crystals were optimized around the initial conditions using grids that varied protein and precipitant concentrations, pH and ionic strength. The optimized crystallization conditions produced large crystals from a protein solution at 10 mg ml^{-1} equilibrated against a reservoir solution consisting of 11–14% PEG 8000, 0.1 *M* HEPES pH 7.3–7.7 and 0.2 *M* $MgCl_2$. Crystals grew as large 0.4 × 0.4 × 0.5 mm rhombs in 7–10 d in approximately 2% of the drops. Crystals were flash-cooled for data collection after sequential transfers to a final artificial mother liquor containing 30% glycerol, 15% PEG 8000, 0.1 *M* HEPES pH 7.5, 0.2 *M* $MgCl_2$.

The crystals diffract to high resolution on beamline 5.0.2 at the Advanced Light Source Synchrotron facility, with spots visible to beyond 1.5 Å resolution. A native data set was collected at that beamline using a 2 × 2 CCD area detector (ADSC). Data were processed and scaled using the *DENZO*/*SCALEPACK* program packages (Otwinowski & Minor, 1997). The crystals belong to space group *R*32, with unit-cell parameters $a = b = 109.1$, $c = 240$ Å (hexagonal convention). Statistics for the native data set are shown in Table 1. Assuming that the asymmetric unit corresponds to a single protein subunit (and that the protein

hexamer is produced by crystallographic threefold and dyad symmetry axes), the Matthews coefficient V_M (Matthews, 1968) is approximately 2.8 Å^3 Da^{-1}, corresponding to a solvent content of 56%. This assignment has been confirmed by determination of the number of methionine residues in the asymmetric unit during the initial stages of MAD data collection and phase calculations, with nine selenomethionine peaks observed per asymmetric unit, in agreement with the protein subunit sequence (unpublished data). The presence of a single protein subunit in the asymmetric unit of space group *R*32 appears to preclude a pentameric protein assembly.

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References

Austin, E. A. & Huber, B. E. (1992). *Mol. Pharmacol.* **43**, 380–387.
Brady, W. A., Kokoris, M. S., Fitzgibbon, M. & Black, M. E. (1996). *J. Biol. Chem.* **271**, 16734–16740.
DiPiro, J. T., Talbert, R. L., Yee, G. C., Matzke, G. R., Wells, B. G. & Posey, L. M. (1997). *Pharmacotherapy: A Pathophysiologic Approach.* Stamford, CT: Appleton & Lange.
Doublie, S. (1997). *Methods Enzymol.* **276**, 523–530.
Erbs, P., Exinger, F. & Jund, R. (1997). *Curr. Genet.* **31**, 1–6.
Hayden, M. S., Linsley, P. S., Wallace, A. R., Marquardt, H. & Kerr, D. E. (1998). *Protein Expr. Purif.* **12**, 173–184.
Hirschowitz, E. A., Ohwada, A., Pascal, W. R., Russi, T. J. & Crystal, R. G. (1995). *Hum. Gene Ther.* **6**, 1055–1063.
Huber, B. E., Austin, E. A., Good, S. S., Knick, V. C., Tibbels, S. & Richards, C. A. (1993). *Cancer Res.* **53**, 4619–4626.
Ipata, P. L. & Cercignani, G. (1978). *Methods Enzymol.* **51**, 394–400.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Katsuragi, T., Sakai, T., Matsumoto, K., Tonomura, K. (1986). *Agric. Biol. Chem.* **50**, 1721–1730.
Kievit, E., Bershad, E., Ng, E., Sethna, P., Dev, I., Lawrence, T. S. & Rehemtulla, A. (1999). *Cancer Res.* **59**, 1417–1421.
Kurtz, J. E., Exinger, F., Erbs, P. & Jund, R. (1999). *Curr. Genet.* **36**, 130–136.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mullen, C. A., Kolstrup, M. & Blaese, R. M. (1992). *Proc. Natl Acad Sci. USA*, **89**, 33–37.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Porter, D. J. (2000). *Biochim. Biophys. Acta*, **1476**, 239–252.
Porter, D. J. & Austin, E. A. (1993). *J. Biol. Chem.* **268**, 24005–24011.