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Crystallization and preliminary crystallographic characterization of catechol-O-methyltransferase in complex with its cosubstrate and an inhibitor

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Catechol-O-methyltransferase (COMT) is involved in the metabolism of catecholamines, catechol steroids and xenobiotic catechols. A precise knowledge of the enzyme–inhibitor structural interactions could help in the design of better inhibitors. Soluble rat COMT was expressed in *Escherichia coli* and the recombinant protein was crystallized with a new tight-binding inhibitor, BIA 3-335 [1-(3,4-dihydroxy-5-nitrophenyl)-3-(*n*-3′-trifluoromethylphenyl)piperazine-1-propanone dihydrochloride]. The crystals were obtained by the sitting-drop vapour-diffusion method using PEG 6K as a precipitant. These crystals diffracted to better than 1.9 Å and belong to the trigonal space group $P3_221$. The unit-cell parameters for the crystal measured at room temperature were $a = b = 51.5$, $c = 168.3$ Å; each shrank by about 1 Å on freezing.

1. Introduction

Catechol-O-methyltransferase (COMT; E.C. 2.1.1.6) catalyses the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to a hydroxyl group on a catechol substrate in the presence of magnesium (Axelrod & Tomchick, 1958). COMT is widely distributed in mammalian tissues, with the highest activities being found in the liver, kidney and gut wall (Guldberg & Marsden, 1975; Männistö *et al.*, 1992). Two distinct forms of COMT have been characterized based on their subcellular location: a rough-endoplasmic reticulum membrane-bound form (MB-COMT) and a soluble form (S-COMT) present in the cytoplasm and possibly in the nucleus (Roth, 1992; Ulmanen *et al.*, 1997; Weisz *et al.*, 1998, 2000). The characterization of rat and human S-COMT cDNAs revealed that S-COMT has 221 amino acids in both species, with 81% sequence identity (Lundström *et al.*, 1991; Salminen *et al.*, 1990).

COMT plays an important role in the metabolism of catecholamines and catechol steroids and also in the detoxification of xenobiotic catechols (Guldberg & Marsden, 1975). One important COMT substrate is L-3,4-dihydroxyphenylalanine (L-DOPA), presently the most effective drug used in the therapy of Parkinson's disease (Männistö *et al.*, 1992), which makes COMT an additional therapeutic target for the treatment of this disease. COMT inhibitors such as tolcapone (Zürcher *et al.*, 1990) and entacapone (Männistö *et al.*, 1988) were shown to enhance the bioavailability of L-DOPA and to increase its half-life by 30–50% in human volunteers (Bonifati & Meco, 1999). Moreover, both inhibitors enhance and extend the therapeutic effect of L-DOPA in patients with advanced and fluctuating Parkinson's disease (Rajput *et al.*, 1997; Rinne *et al.*, 1998).

The structure of COMT as a complex with SAM, 3,5-dinitrocatechol and a magnesium ion has already been determined (Vidgren *et al.*, 1994). However, this failed to show the interaction of the carbonyl group present in compounds such as tolcapone. The three-dimensional structure determination of complexes of the enzyme with SAM and other inhibitors is expected to allow the identification and recognition of the active enzyme residues sensitive to these new inhibitors. This knowledge may improve the design of new COMT inhibitors and the development of innovative strategies for the treatment of disorders of the brain.

Here, we report the purification, crystallization and preliminary crystallographic analysis of recombinant rat COMT complexed with a new tight-binding inhibitor, BIA 3-335 [1-(3,4-dihydroxy-5-nitrophenyl)-3-(*n*-3′-trifluoromethylphenyl) piperazine-1-propanone dihydrochloride].

2. Experimental

2.1. Protein expression and purification

Rat S-COMT was expressed as a fusion protein in *E. coli* BL21 (DE3) with a calmodulin-binding peptide (CBP) tag at the N-terminus. Details of this procedure will be published elsewhere (Bonifácio *et al.*, 2001). In brief, cells were grown at 310 K in LB medium containing 50 mg l^{-1} carbanecillin and protein expression was induced by 0.5 m*M* isopropyl-

β-D-thiogalactopyranoside for 2 h at 310 K. Cells were harvested by centrifugation and lysed in calcium-binding buffer (300 m*M* NaCl, 10 m*M* DTT, 1 m*M* $MgCl_2$, 1 m*M* imidazole, 2 m*M* $CaCl_2$, 50 m*M* Tris pH 8.0) with protease inhibitors (5 µg ml^{-1} leupeptin and 0.7 µg ml^{-1} pepstatin) by freeze–thaw cycles and homogenization. The bacterial soluble extract was isolated by centrifugation and loaded onto a calmodulin-affinity resin column equilibrated in calcium-binding buffer with 10% glycerol. Recombinant S-COMT was eluted with 300 m*M* NaCl, 2 m*M* EGTA in 50 m*M* Tris buffer pH 8.0 containing 10 m*M* DTT and 10% glycerol. After concentration it was subjected to gel filtration on a Superdex-75 column equilibrated with 20 m*M* Tris buffer pH 8.5 containing 150 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* DTT and 10% glycerol. To remove the CBP tag, the recombinant S-COMT was digested with enterokinase in the gel-filtration buffer with 2 m*M* $CaCl_2$ and 0.1% Tween 20. The digested preparation was further subjected to ion-exchange chromatography on a Resource Q column (Amersham Pharmacia Biotech) equilibrated in 20 m*M* bis-tris propane pH 7.1, 10 m*M* DTT, 1 m*M* $MgCl_2$, 10% glycerol. S-COMT was eluted with a linear gradient of NaCl (0–0.25 *M*).

2.2. Crystallization

The purified recombinant COMT solution was concentrated using an Amicon concentrator with a YM-10 membrane to 5 mg ml^{-1} in 10 m*M* MES buffer pH 6.5 containing 2.5 m*M* $MgCl_2$ and 1 m*M* DTT. Prior to the crystallization assays, the cosubstrate SAM and the inhibitor BIA 3-335 were added at 3 and 1.5 times the molar amount of the enzyme, respectively. A concentrated solution (~20 m*M*) of the inhibitor was freshly prepared using ethanol and DMSO (1:1) as solvents, which showed a yellow colour.

Crystallization trials were set up at room temperature (~293 K) using the sitting-drop vapour-diffusion method. An initial screening of crystallization conditions was performed using PEG 6K as a precipitant. Droplets containing protein and precipitant solutions in equal amounts (2 µl) were equilibrated against 0.7 ml of reservoir solution. In some experiments, the reservoir solution contained 0.5 *M* NaCl.

Crystals suitable for diffraction measurements were grown from 8–10% PEG 6K, 0.1 *M* MES at pH 5.5–6.5. Yellow crystals appeared after 3 d and reached maximum dimensions of around 0.3 × 0.2 × 0.15 mm (Fig. 1).

2.3. X-ray data collection and processing

Two diffraction data sets were collected in-house with a 300 mm MAR Research image-plate detector mounted on an Enraf–Nonius rotating-anode generator with Cu $K\alpha$ radiation.

A crystal grown from 10% PEG 6K, 0.1 *M* MES pH 5.5 was mounted on a glass capillary tube and diffraction data were recorded to 2.0 Å resolution (crystal *A* in Table 1). Since this crystal suffered some radiation damage, we decided to measure data from a frozen crystal. Therefore, a crystal obtained using 8% PEG 6K, 0.1 *M* MES pH 6.5 (with a reservoir solution of 0.5 *M* NaCl) was transferred into a cryoprotectant solution consisting of 20% PEG 6K, 0.1 *M* MES pH 6.5 and 15% glycerol. The crystal was then immediately flash-frozen in a cold nitrogen stream (Oxford Cryosystems) and X-ray diffraction data were measured at a temperature of 115 K (crystal *B* in Table 1) to 1.86 Å. Data were integrated and scaled using *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997). Relevant data-collection and processing statistics are presented in Table 1.

Table 1
X-ray data-collection and processing statistics.

Values in parentheses refer to the outer resolution shell: 2.09–2.02 Å for crystal *A* and 1.93–1.86 Å for crystal *B*.

	Crystal *A*	Crystal *B*
X-ray source	Enraf–Nonius, 4.5 kV	Enraf–Nonius, 4.2 kV
Wavelength (Å)	1.5418	1.5418
Detector	MAR Research IP (30 cm)	MAR Research IP (30 cm)
Space group	$P3_221$	$P3_221$
Unit-cell parameters (Å,°)	a = 51.49, b = 51.49, c = 168.29, α = 90, β = 90, γ = 120	a = 50.55, b = 50.55, c = 167.13, α = 90, β = 90, γ = 120
Mosaicity (°)	0.25–0.40	0.35
Total No. of reflections	131542	222059
No. of unique reflections	17663	21316
Redundancy	7.4	10.4
Resolution range (Å)	25.75–2.02	21.62–1.86
Completeness (%)	98.9 (95.3)	98.0 (91.8)
$I/\sigma(I)$	9.2 (2.2)	25.7 (3.1)
R_{merge} (%)	11.3 (48.3)	4.4 (29.7)

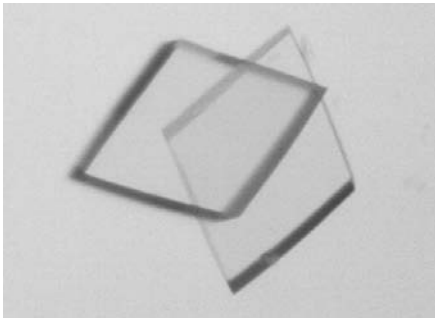

Figure 1
Crystals of recombinant rat soluble catechol-O-methyltransferase complexed with *S*-adenosyl-L-methionine (SAM) and inhibitor BIA 3-335. The largest dimension is 0.3 mm.

3. Results and discussion

Rat S-COMT was expressed in *E. coli* as a fusion protein with a CBP tag at its amino terminus. The recombinant protein, which constituted 30–40% of the total cellular protein, was purified by affinity chromatography followed by gel filtration. The CBP tag was cut by enzymatic digestion with enterokinase and S-COMT was recovered from the digested preparation by ion-exchange chromatography. Up to 7 mg of pure S-COMT was obtained per litre of culture.

The initial crystallization approach of using PEG as precipitant at around pH 6.5 was based on previously reported results for the complex of COMT with 3,5-dinitrocatechol (Vidgren *et al.*, 1999). Attempts to crystallize the apo-protein (without cosubstrate and inhibitor) only yielded very thin needles. Good-quality crystals of COMT complexed with SAM and BIA 3-335 were easily obtained. In contrast, a similar screening was pursued for many other inhibitors, which produced only needle-like and plate-like crystals unsuitable for diffraction studies. Therefore, the nature of the inhibitor compound greatly influences the quality of the crystals.

The crystal mounted in a capillary tube (crystal *A* in Table 1) belongs to space group $P3_221$, with unit-cell parameters $a = b = 51.5$, c = 168.3 Å. The frozen crystal (crystal *B*) showed a contraction upon freezing of ~1 Å in each unit-cell dimension. The diffraction data set collected from crystal *B* has better statistics than crystal *A* and the maximum resolution is marginally improved from 2.02 to 1.86 Å. The calculated V_M for the crystals is 2.6 Å^3 Da^{-1} (Matthews, 1968), which corresponds to a solvent content of approximately 53% and one monomer per asymmetric unit.

The crystallographic refinement using the model of the complexed COMT (PDB code 1vid) is in progress.

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