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Crystallization of PNMT, the adrenaline-synthesizing enzyme, is critically dependent on a high protein concentration

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Phenylethanolamine *N*-methyltransferase, PNMT, utilizes the methylating cofactor *S*-adenosyl-L-methionine to catalyse the synthesis of adrenaline. Human PNMT has been crystallized in complex with an inhibitor and the cofactor product *S*-adenosyl-L-homocysteine using the hanging-drop technique with PEG 6000 and lithium chloride as precipitant. A critical requirement for crystallization was a high enzyme concentration (>90 mg ml^{-1}) and cryocrystallography was used for high-quality data measurement. Diffraction data measured from a cryocooled crystal extend to a resolution of 2.3 Å. Cryocooled crystals belong to space group $P4_32_12$ and have unit-cell parameters $a = b = 94.3$, $c = 187.7$ Å.

1. Introduction

Adrenaline, also known as epinephrine, is a catecholamine produced in the adrenal glands and in the CNS to induce specific biologic effects. In the periphery, the effects of adrenaline are well known. Its release from the adrenal medulla is triggered by excitement or physical stress and induces the 'flight–fight' response, so called because it prepares the body for immediate and energetic activity (Bowman & Rand, 1980). The response results in an increase in heart rate, blood pressure, blood sugar, metabolic rate and bronchodilation. In the CNS, the effects of adrenaline are not so well understood, though there is strong evidence that it is involved in the central control of blood pressure (Ruggiero & Reis, 1987; Liang *et al.*, 1984).

Adrenaline is synthesized *in vivo* by phenylethanolamine *N*-methyltransferase (PNMT; EC 2.1.1.28). PNMT is a 282-residue (30.7 kDa) protein that binds *S*-adenosyl-L-methionine (AdoMet) and the substrate noradrenaline. In the reaction catalysed by PNMT, AdoMet donates a methyl group to the amine of noradrenaline and thereby produces adrenaline. Here, we describe the crystallization of human PNMT in complex with a PNMT inhibitor and the cofactor product *S*-adenosyl homocysteine (AdoHcy). Crystals grown in this way were used for determining the three-dimensional structure of PNMT by X-ray diffraction (Martin *et al.*, 2001).

2. Crystallization

Recombinant human PNMT was prepared and purified as described previously (Caine *et al.*, 1996). Homogeneity and protein concentration were assessed by SDS–PAGE analysis and protein concentration was quantitated using an empirical formula based on absorption measurements at 260 and 280 nm (Kalb & Bernlohr, 1977). Absorption of samples was measured on a Shimadzu UV-1201 spectrophotometer.

Crystallization trials were performed by the hanging-drop vapour-diffusion method and were attempted with PNMT alone, with PNMT in the presence of an inhibitor, 7-sulfonamide tetrahydroisoquinoline (SK&F 29661), and with PNMT in the presence of both SK&F 29661 and the cofactor product, AdoHcy. PNMT samples were prepared in 20 m*M* Tris buffer pH 7.2 with 1 m*M* ethylenediamine tetraacetic acid (EDTA) and 0.5 m*M* dithiothreitol (DTT). For crystallization, additives (additional DTT to a final concentration of 10 m*M*, SK&F 29661, AdoHcy) were pre-incubated with the enzyme for 30 min prior to setting up trials. A PNMT concentration of 16 mg ml^{-1} and a drop size of 2 µl of protein and 2 µl of reservoir were used in the initial sparse-matrix screening conditions (Jancarik & Kim, 1991), which were set up using commercially available kits from Hampton Research. Several polyethylene glycol (PEG) conditions gave granular precipitate, but no crystals or crystalline precipitate were produced from the sparse-matrix screens. Dozens of subsequent trials were then set up to investigate the effect of varying the PEG molecular weight and concentration, varying the temperature (277 and 293 K), the pH (5–7) and the protein concentration (16–130 mg ml^{-1}).

A critical variable for crystal growth was found to be protein concentration (Fig. 1). At concentrations of 16 mg ml^{-1}, PEG conditions generally gave a fine granular precipitate.

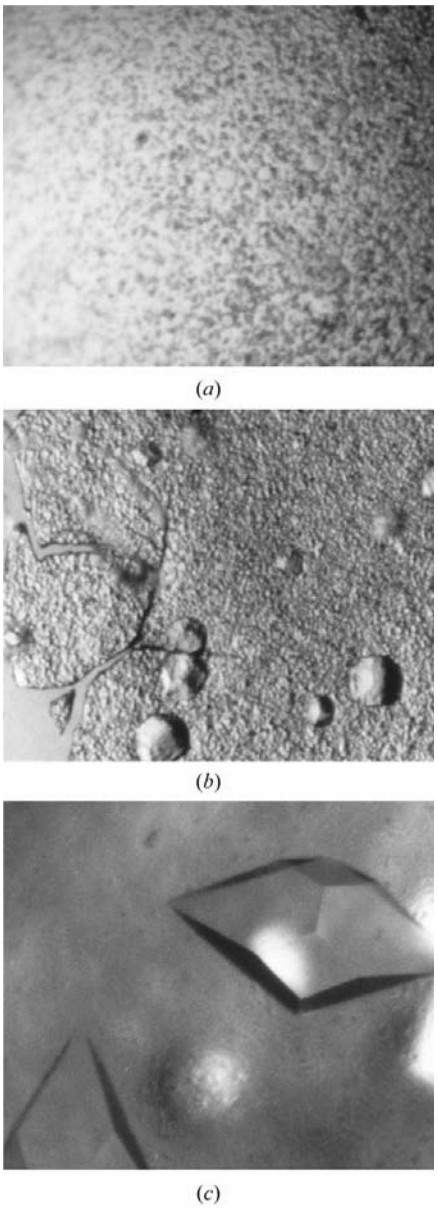

Figure 1
Effect of protein concentration on PNMT crystallization at room temperature using as precipitant 7–10% PEG 6K, 0.25 *M* LiCl, 0.1 *M* cacodylate buffer pH 5.85 or 6.0. (*a*) 16 mg ml^{-1} PNMT produces granular precipitate. (*b*) 60 mg ml^{-1} PNMT produces tiny irregular crystals and a glass-like granular precipitate. (*c*) 130 mg ml^{-1} PNMT produces large well formed single crystals that diffract to 2.3 Å.

Increasing the protein concentration to 60 mg ml^{-1} yielded a glass-like granular precipitate and tiny irregular crystals. At 90–130 mg ml^{-1} large single crystals were obtained. These grew at 293 K from a protein mixture consisting of 90–130 mg ml^{-1} PNMT, 15 m*M* SK&F 29661, 2 m*M* AdoHcy, 10 m*M* DTT in 20 m*M* Tris pH 7.2 and 1 m*M* EDTA. The precipitant contained 5–10% polyethylene glycol 6000 (PEG 6K), 0.1 *M* cacodylate buffer pH 5.65–6.25 and 0.25 *M* lithium chloride. The crystallization drop was prepared by combining the protein mixture and the reservoir precipitant in a 1:1 ratio and using drop volumes of 4–10 µl. Crystals of PNMT appeared from very thick precipitate after two weeks and continued to grow over four weeks to an average size of 0.4 × 0.3 × 0.3 mm.

3. Diffraction data measurement

PNMT crystals grow from a thick and very sticky precipitate. A needle was used to remove the precipitate from the crystals before cryocooling or mounting in quartz capillary tubes. PNMT crystals are radiation sensitive: the diffraction pattern deteriorates rapidly in the X-ray beam at room temperature. The best data measured from PNMT crystals at room temperature extended to a maximal resolution of 3 Å. However, using cryocrystallography the PNMT crystals are protected from radiation damage and the measured data were of much better quality and extended to higher resolution (2.3 Å). Crystallographic statistics for room temperature and cryocrystallographic data are summarized in Table 1. The cryoprotectant solution used was similar to the precipitant solution and incorporated 20–25% glycerol. The crystal was soaked in the cryoprotectant solution for 30–60 s prior to flash-freezing in a gaseous nitrogen stream at 100 K (Oxford Cryosystems Cryostream). All X-ray diffraction experiments were carried out using a Rigaku RU-200 Cu $K\alpha$ rotating-anode X-ray generator operating at 46 kV and 60 mA and equipped with Yale focusing mirror optics. X-ray diffraction data were recorded on an R-AXIS IIC imaging-plate area detector and were integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

Analysis of systematic absences in the crystallographic data indicate that the crystals belong to one of the enantiomorphic space groups $P4_12_12$ or $P4_32_12$. Our recent structure determination of this crystal form by multiple isomorphous replacement (Martin *et al.*, 2001) resolved the ambiguity, indicating a space group of $P4_32_12$. The unit-cell parameters are $a = b = 94.3$, $c = 187.7$ Å.

Table 1
Data-collection statistics for PNMT crystals.

Values in parentheses represent the value for the outer shell of data. Completeness indicates the number of unique reflections divided by the theoretical number of reflections.

Temperature (K)	290	100
Unit-cell parameters		
a (Å)	95.2	94.3
b (Å)	95.2	94.3
c (Å)	188.4	187.7
α (°)	90	90
β (°)	90	90
γ (°)	90	90
Resolution (Å)	3.0	2.3
Outer shell (Å)	3.0–3.5	2.3–2.38
Completeness (%)	67 (60)	90 (54)
No. observations [$I > 0\sigma(I)$]	28476	164774
No. unique [$I > 0\sigma(I)$]	12100	34845
$\langle I\rangle/\langle\sigma(I)\rangle$	6.7 (3.1)	18.9 (2.1)
$R_{\rm merge}$†	0.086 (0.216)	0.051 (0.274)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$.

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