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Crystallization and preliminary X-ray crystallographic analysis of adenosine 5′-monophosphate deaminase (AMPD) from *Arabidopsis thaliana* in complex with coformycin 5′-phosphate

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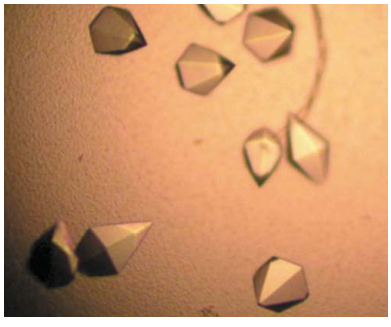

Adenosine 5′-monophosphate deaminase (AMPD) is a eukaryotic enzyme that converts adenosine 5′-monophosphate (AMP) to inosine 5′-monophosphate (IMP) and ammonia. AMPD from *Arabidopsis thaliana* (AtAMPD) was cloned into the baculoviral transfer vector p2Bac and co-transfected along with a modified baculoviral genome into *Spodoptera frugiperda* (Sf9) cells. The resulting recombinant baculovirus were plaque-purified, amplified and used to overexpress recombinant AtAMPD. Crystals of purified AtAMPD have been obtained to which coformycin 5′-phosphate, a transition-state inhibitor, is bound. Crystals belong to space group $P6_222$, with unit-cell parameters $a = b = 131.325$, $c = 208.254$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Diffraction data were collected to 3.34 Å resolution from a crystal in complex with coformycin 5′-phosphate and to 4.05 Å resolution from a crystal of a mercury derivative.

1. Introduction

Adenosine 5′-monophosphate (AMP) deaminase (AMPD; adenylate deaminase; AMP aminohydrolase; EC 3.5.4.6) catalyses the hydrolytic deamination of AMP to inosine 5′-monophosphate (IMP) and retains the purine-ring structure at the nucleotide level (Merkler *et al.*, 1993). The relative concentrations of adenine nucleotides (AMP, ADP and ATP) are greatly affected by this enzyme, so AMPD plays a major role in regulating the quantitative ‘energy charge’ in the organism.

AMPD is a ubiquitous enzyme in eukaryotic cells. AtAMPD is moderate in size, comprising 839 amino acids and including a putative transmembrane helix near the N-terminus. While higher eukaryotes of the animal kingdom contain three AMPD genes, plants and lower eukaryotes of the animal kingdom contain only one AMPD gene. In higher eukaryotes, tissue-specific AMPD isoforms are produced by differential expression of the three genes that encode this enzyme activity as well as by alternative splicing of each primary transcript (reviewed in Mahnke-Zizelman & Sabina, 2000). Adenosine deaminase (ADA) can also deaminate the amino group at the 6-position of the adenine-ring structure in all species of the animal kingdom, but this enzyme activity is undetectable in plants (Le Floc’h *et al.*, 1982; Butters *et al.*, 1985; Yabuki & Ashihara, 1991; Dancer *et al.*, 1997). Therefore, AMPD may play a more important role in plant adenine-nucleotide metabolism.

Coformycin 5′-phosphate is an analogue of inosine and has many of the structural features of a transition-state analogue of AMP deamination (Lienhard, 1973); it showed higher inhibitory activity against mammalian AMPD from skeletal muscle (Bzowska & Shugar, 1989) and heart (Thakkar *et al.*, 1993) than coformycin itself. We have crystallized AtAMPD in complex with coformycin 5′-phosphate.

2. Experimental methods

2.1. Construction of recombinant baculoviral transfer vector and expression of AtAMPD

In order to produce soluble enzyme, an N-truncated AtAMPD expression plasmid was engineered by oligonucleotide site-directed

mutagenesis from the wild-type cDNA, which was cloned into the SmaI(5′)–SacII(3′) restriction endonuclease sites immediately upstream of the P10 promoter in the baculoviral transfer vector p2Bac (Stratagene). Briefly, a BsiWI restriction-endonuclease site was added to the PCR product immediately upstream of a single base change in codon 139 [from leucine (at**a**) to methionine (at**g**)]. The resulting approximately 2.1 kbp *Bsi*W1–*Sac*II fragment was then subcloned back into the appropriate sites of the baculoviral transfer vector. The resulting ΔI139M recombinant plasmid was co-transfected into *Spodoptera frugiperda* (Sf9) cells together with a modified baculoviral genome (BaculoGold; Pharmingen). Viral plaques were purified, amplified and used to infect Sf9 cells.

2.2. Purification and coformycin 5′-phosphate complex formation

AtAMPD recombinant enzyme was purified by phosphocellulose chromatography using a previously described protocol that includes sequential potassium chloride and potassium phosphate gradient elution and a final ammonium sulfate precipitation step (Mahnke-Zizelman & Sabina, 2001). Leupeptin was included in all extraction and storage buffers to minimize N-terminal proteolysis, as previously described (Haas & Sabina, 2003).

Purified AtAMPD protein was resuspended in 20 m*M* Tris–HCl pH 8.0, 90 m*M* KCl, 100 m*M* ammonium sulfate, mixed with 2 m*M* coformycin 5′-phosphate and incubated on ice for 1 h. The coformycin 5′-phosphate stock solution was prepared at 19.23 m*M* in H_2O. The complex solution was centrifuged at 16 100*g* for 10 min prior to the crystallization setup.

2.3. Crystallization

Initial screening for crystallization was performed on AtAMPD in complex with coformycin 5′-phosphate by the sitting-drop vapour-diffusion method using 384-well crystallization plates (Corning) at 295 K. The protein concentration was 8.0 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 90 m*M* KCl and 100 m*M* ammonium sulfate. A sitting drop was prepared by mixing 1.5 µl each of the protein solution and the reservoir solution and was equilibrated against 50 µl reservoir solution. The initial search for crystallization conditions was performed using Crystal Screen crystallization solutions (Hampton Research) and UW-192 screening solutions. Out of the 240 conditions screened, several crystals were obtained after 3–5 d: single crystals from Crystal Screen No. 11 (1.0 *M* monoammonium dihydrogen phosphate, 0.1 *M* trisodium citrate dihydrate pH 5.6), UW-192 No. 50 [17.6%(*w*/*v*) polyethylene glycol 4000, 0.1 *M* MES pH 6.0, 0.16 *M* sodium chloride], UW-192 No. 55 [10.4%(*w*/*v*) polyethylene glycol 4000, 0.1 *M* HEPES pH 7.5, 0.4 *M* sodium chloride), UW-192 No. 58 [20.0%(*w*/*v*) polyethylene glycol 4000, 0.1 *M* HEPPS pH 8.5, 0.08 *M* calcium chloride], UW-192 No. 74 [18.4%(*w*/*v*) polyethylene glycol 8000, 0.1 *M* MES pH 6.0, 0.12 *M* potassium/sodium phosphate] and UW-192 No. 82 [17.6%(*w*/*v*) polyethylene glycol 8000, 0.1 *M* TE pH 8.0, 0.42 *M* tetramethyl ammonium chloride].

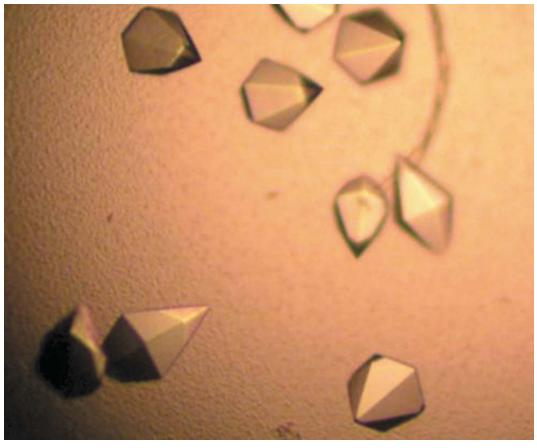

Figure 1
Crystals of *A. thaliana* AMP deaminase grown in the presence of coformycin 5′-phosphate, with approximate dimensions of 0.2 × 0.2 × 0.3 mm.

These conditions were optimized using hanging-drop vapour-diffusion experiments. Each hanging drop was prepared by mixing 2 µl each of the protein solution and the reservoir solution and was equilibrated over 1 ml reservoir solution. Diffraction-quality AtAMPD crystals in complex with coformycin 5′-phosphate were obtained with a reservoir solution consisting of 0.4 *M* monoammonium dihydrogen phosphate, 0.1 *M* trisodium citrate pH 5.6 and 10%(*v*/*v*) ethanol.

2.4. Heavy-atom derivatives and data collection

An AtAMPD crystal measuring 0.2 × 0.2 × 0.3 mm was grown from 0.4 *M* monoammonium dihydrogen phosphate, 0.1 *M* trisodium citrate pH 5.6 and 10%(*v*/*v*) ethanol in 7 d (Fig. 1) and was used for data collection. The crystal was transferred to the same solution with crystal-growth solution and the concentration of ethylene glycol was increased by adding continuously ethylene glycol solution at higher concentration. Finally, the crystal mounted in a cryoloop was transferred to cryoprotectant solution [0.4 *M* monoammonium dihydrogen phosphate, 0.1 *M* trisodium citrate pH 5.6 and 30%(*v*/*v*) ethylene glycerol] for 2 s, immediately dipped into liquid nitrogen and stored in liquid nitrogen until data collection.

To prepare the heavy-atom derivatives, a single crystal in complex with coformycin 5′-phosphate was transferred to the same solution as the crystal-growth solution and various heavy-atom solutions were added to the solution such as 5 m*M* thimerosal (Hg), 5 m*M* $KOsCl_6$, 5 m*M* K_2PtCl_4, 5 m*M* $K_2Pt(CN)_4$ and 5 m*M* $(CH_3)_3PbCl$. The crystals were soaked in the solution for times ranging between 2 h and overnight. The heavy-metal-soaked crystals were frozen in liquid nitrogen in the same way as the AtAMPD crystal in complex with coformycin 5′-phosphate.

X-ray diffraction data from an AtAMPD crystal in complex with coformycin 5′-phosphate were collected using a MAR CCD detector at 12.661 keV with a cryogenically cooled Si(220) monochromator at synchrotron beamline 22-ID at the Advanced Photon Source (APS) at Argonne National Laboratory (ANL) and X-ray diffraction data from the mercury derivative were collected using an APS-1 CCD detector at 12.344 keV with cryogenically cooled Si(111) monochromator optics at synchrotron beamline 19-ID at the APS at ANL. The crystal of AtAMPD in complex with coformycin-5′-phosphate was rotated through a total of 53° with 1.0° oscillation per frame and the crystal of the mercury derivative was rotated through a total of 150° with 0.5° oscillation per frame. The program *HKL*2000 (Otwinowski & Minor, 1997) was used for data processing and scaling.

3. Results and discussion

Deletion of the first 138 amino acids of *A. thaliana* AMPD followed by expression in insect cells produced soluble protein. Crystals of this truncation mutant in complex with coformycin 5′-phosphate were obtained within 7 d using a reservoir solution consisting of 0.4 *M* monoammonium dihydrogen phosphate, 0.1 *M* trisodium citrate pH 5.6 and 10%(*v*/*v*) ethanol.

Table 1
Data-collection statistics.

Values in parentheses are for the outermost resolution shell (3.42–3.34 Å for AtAMPD in complex with coformycin 5′-phosphate and 4.12–4.05 Å for the mercury derivative).

	AtAMPD in complex with coformycin 5′-phosphate	Mercury derivative (thimerosal)
X-ray wavelength (Å)	0.979 (22ID at APS)	1.0044 (19ID at APS)
Resolution range (Å)	50.0–3.34	50.0–4.05
Space group	$P6_222$	$P6_222$
Unit-cell parameters (Å, °)	$a = b = 131.325$, $c = 208.254$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 132.635$, $c = 207.623$, $\alpha = \beta = 90$, $\gamma = 120$
Total/unique reflections	98039/16001	158907/9357
Completeness (%)	99.5 (99.6)	99.9 (100)
Mean $I/\sigma(I)$ (%)	30.6 (4.1)	26.5 (10.3)
$R_{\rm merge}$† (%)	6.0 (48.9)	12.1 (35.9)

† $R_{\rm merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

The AtAMPD–coformycin 5′-phosphate crystal belongs to space group $P6_222$, with unit-cell parameters $a = b = 131.325$, $c = 208.254$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The data set from the complex is 99.5% complete to 3.34 Å resolution. The asymmetric unit contains one AtAMPD–coformycin 5′-phosphate molecule and the specific volume ($V_{\rm M}$) is 3.278 Å^3 Da^{-1}, with a solvent content of 62.5% (Matthews, 1968). Statistics of data collection are summarized in Table 1. Initial phases have been obtained and the solution of the structure is in progress.

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