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Crystallization of a complex of *Caenorhabditis elegans* diadenosine tetraphosphate hydrolase and a non-hydrolysable substrate analogue, AppCH$_2$ppA

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The molecule diadenosine tetraphosphate (Ap$_4$A) has been suggested to be a component of the cellular response to metabolic stress and/or, *via* the intracellular Ap$_3$A/Ap$_4$A ratio, to be involved in differentiation and apoptosis. Thus, the enzyme Ap$_4$A hydrolase has a key metabolic role through regulation of the intracellular Ap$_4$A levels. Crystals of this enzyme from the nematode *Caenorhabditis elegans* have been obtained in the presence of a non-hydrolysable substrate analogue, AppCH$_2$ppA. The crystals belong to space group $P2_1$, unit-cell parameters $a = 57.6$, $b = 36.8$, $c = 68.9$ Å, $\beta = 114.2°$, and diffract to approximately 2.0 Å. Determination of the structure of this complex will provide insights into the substrate specificity and catalytic activity of this class of enzymes.

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1. Introduction

The Ap$_4$A hydrolase from the nematode *C. elegans* is a member of the Nudix hydrolase enzyme family and hydrolyses a diphosphate (pyrophosphate) linkage in diadenosine 5′-5‴-P^1,P^n-polyphosphates with four or more phosphate groups, always producing ATP as one of the products (McLennan, 1999; Guranowski, 2000). It is generally most active with Ap$_4$A as substrate, but can also hydrolyse other dinucleoside polyphosphates such as Gp$_4$G (Warner & Finamore, 1965; Prescott *et al.*, 1989). Asymmetrically cleaving Ap$_4$A hydrolases have been isolated from a variety of sources and have been classified on the basis of sequence into two distinct groups: the 'animal-type' Ap$_4$A hydrolases,which contain the animal and archaeal sequences, and the 'plant-type' Ap$_4$A hydrolases, which contain the plant and proteobacterial sequences (Cartwright *et al.*, 1999; Abdelghany *et al.*, 2001).

Ap$_4$A is synthesized predominantly by aminoacyl-tRNA synthetases by the addition of the AMP moiety from an aminoacyl-AMP to ATP (Brevet *et al.*, 1989). The precise function of Ap$_4$A is still unclear. On the one hand, it may be an unavoidable byproduct of protein synthesis that has to be cleared from the cell before it attains a potentially toxic concentration, particularly under conditions of metabolic stress (McLennan, 2000). In this case, Ap$_4$A hydrolase would perform the housecleaning role proposed to be one function of members of the Nudix hydrolase family (Bessman *et al.*, 1996). On the other hand, there is mounting evidence that the intracellular ratio of Ap$_4$A to the related compound Ap$_3$A may influence several cellular functions (McLennan, 2000; Kisselev *et al.*, 1998). For example, differentiation and apoptosis of cultured cells have been reported to have significant and opposite correlations with this ratio, differentiation being associated with a decrease in the ratio and apoptosis with an increase (Vartanian *et al.*, 1997). Additionally, Ap$_4$A may also be an important ligand of the Fhit tumour suppressor protein (Murphy *et al.*, 2000). The Fhit protein is an Ap$_3$A hydrolase and a member of the histidine triad (HIT) protein family. It has been suggested that the Fhit–Ap$_3$A complex is a component of an antiproliferative signalling pathway (Pace *et al.*, 1998). As Ap$_4$A is known to be associated with cellular proliferation (Rapaport & Zamecnik, 1976) and since Fhit can also bind Ap$_4$A, Ap$_4$A may serve as an antagonistic proliferative signal in this proposed pathway. As Ap$_4$A hydrolase is believed to regulate the levels of Ap$_4$A and hence the Ap$_3$A:Ap$_4$A ratio, this enzyme would play an important role in all the above schemes.

The purification and crystallization of the free enzyme form of *C. elegans* Ap$_4$A hydrolase has been reported elsewhere (Abdelghany *et al.*, 2001), but we present here a different purification protocol and an alternative crystal form of the enzyme grown in the presence of a non-hydrolysable substrate analogue, 5′-5‴-(P^2,P^3-methylene)-P^1,P^4-tetraphosphate diadenosine (AppCH$_2$ppA; Blackburn *et al.*, 1992). The structure of the complex will be essential in determining the substrate specificity and catalytic activity of this group of Nudix hydrolases.

2. Materials and methods

2.1. Expression and purification

The cloning and expression of *C. elegans* Ap$_4$A hydrolase is described elsewhere

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.06–2.01 Å).

Resolution (Å)	20–2.01
No. of observations	36397
Unique reflections	16982
Data completeness (%)	86.3 (94.8)
$I/\sigma(I) > 3$ (%)	74.2 (47.0)
Multiplicity	2.4 (2.2)
$R_{\rm merge}$ (%)†	7.4 (27.3)

† $R_{\rm merge} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$, where I is the intensity of a reflection and $\langle I \rangle$ is the average intensity for multiple observations of its symmetry-related reflections.

(Abdelghany *et al.*, 2001). For purification, the cells were suspended in buffer *A* (50 m*M* Tris–HCl pH 8.0), lysed by sonication and the supernatant clarified by centrifugation at 20 000 rev $\rm min^{-1}$. The resulting lysate was then applied to a 40 ml Fast Flow DEAE-Sepharose Column and the protein eluted with a 0–0.5 *M* NaCl gradient developed over 150 ml. Fractions containing the hydrolase were pooled before addition of 4 *M* ammonium sulfate to a final concentration of 1.7 *M*. This solution was loaded onto a Butyl-Toyopearl column equilibrated with buffer *A* plus 1.7 *M* ammonium sulfate and the protein eluted with a 1.7–0.6 *M* ammonium sulfate gradient developed over 150 ml. The peak fractions were pooled and concentrated by ultrafiltration to 1 ml (Vivaspin 20) and then applied to a Superdex 200 Hi-load gel-filtration column equilibrated with phosphate-buffered saline. The final protein was over 99% pure as judged by SDS–PAGE Coomassie Brilliant Blue staining and electrospray mass spectrometry. Thus, the purification protocol reported here differs markedly from that reported by Abdelghany *et al.* (2001). The earlier protocol proceeds *via* two steps, an Ultrogel Aca4 column followed by step elution from a Mono Q column, whereas the protocol here consists of the three steps described above.

2.2. Crystallization

For crystallization, the protein was dialysed into 10 m*M* Tris–HCl pH 8.0 and concentrated to 10 mg $\rm ml^{-1}$ by ultrafiltration (Vivaspin 20) before addition of 10 m*M* magnesium chloride and 10 m*M* $\rm AppCH_2ppA$. Initial crystallization conditions were sought by the sitting-drop method of vapour diffusion using sparse-matrix kits from Hampton Research (Jancarik & Kim, 1991; Hampton Research, USA). After one week, one condition from Hampton Crystal Screen 1 [30%(*w*/*v*) PEG 4000, 0.2 *M* magnesium chloride and 0.1 *M* Tris–HCl pH 8.5] produced several needle-shaped crystals with maximum dimensions 0.1 × 0.05 × 0.6 mm. Attempts to optimize these initial crystallization conditions failed to produce any crystals of greater diffraction quality. No crystals were obtained under these conditions in the absence of $\rm AppCH_2ppA$.

2.3. X-ray analysis

For diffraction experiments, the $\rm AppCH_2ppA$ cocrystals were taken directly from the drop and cooled in a stream of nitrogen gas at 100 K (Oxford Cryosystems Cryostream). A preliminary data set was collected to a resolution of 2.0 Å (Fig. 1) from a single crystal on a MAR 345 image-plate scanner mounted on a Rigaku RU-200 X-ray generator with Yale focusing mirror optics. Processing and scaling of the data using the *HKL* suite of programs (Otwinowski & Minor, 1997) showed the crystals have a primitive monoclinic lattice, with unit-cell parameters a = 57.6, b = 36.8, c = 68.9 Å, β = 114.2° and an overall $R_{\rm merge}$ of 7.4%. Complete data-collection statistics are shown in Table 1. Examination of the diffraction pattern using the program *HKLVIEW* (Collaborative Computational Project, Number 4, 1994) on the $0kl$ zone revealed a pattern of systematic absences along the $0k0$ axis consistent with the condition $k = 2n$, indicating the space group probably to be $P2_1$.

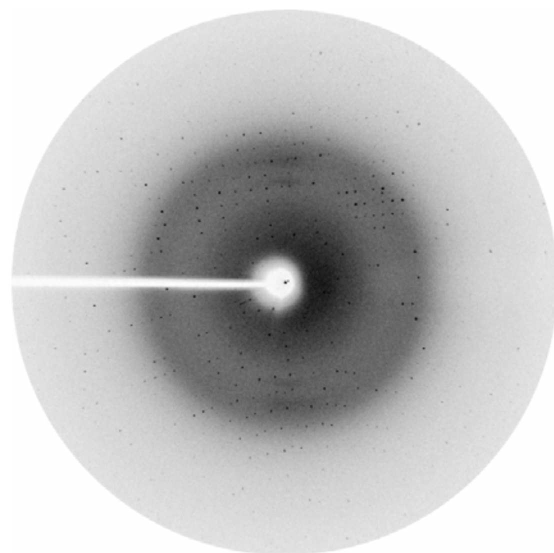

Figure 1
A 1° rotation diffraction pattern from a cocrystal of $\rm Ap_4A$ hydrolase. The resolution is 1.9 Å at the edge of the plate.

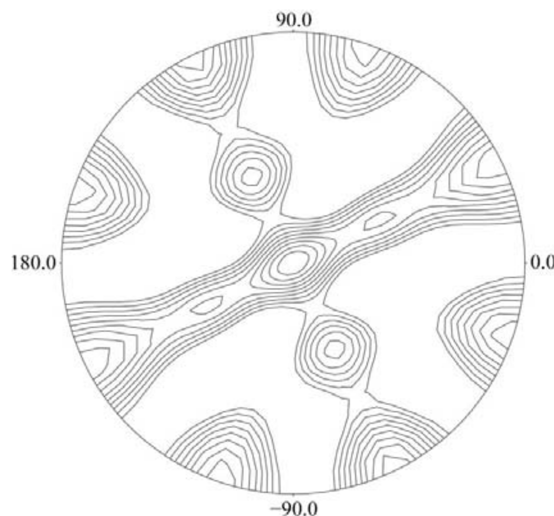

Figure 2
A stereographic projection of the κ = 180° section of the self-rotation function of $\rm Ap_4A$ hydrolase cocrystals calculated with the orthogonalization c, $b^* \times c$, b^*. The peak corresponding to the crystallographic twofold axis can be seen at the centre of the plot (ω = 0°). Further sets of peaks occur at ω values of 45 and 90° for a φ value of 25° and every 90° thereafter, as well as at an ω value of 90° for a φ value of 70° and every 90° thereafter. These additional peaks can be accounted for by the combination of a crystallographic and a non-crystallographic twofold axis.

3. Results

Given a monomer molecular weight of 16 088 Da, packing-density calculations for the $\rm AppCH_2ppA$ cocrystals suggest that a monomer in the asymmetric unit gives a $V_{\rm M}$ of 4.14 $\rm Å^3\,Da^{-1}$ and a corresponding 70% solvent content. As these values are fairly high (Matthews, 1968), it is possible that two molecules of the hydrolase constitute the asymmetric unit ($V_{\rm M}$ = 2.07 $\rm Å^3\,Da^{-1}$ and solvent content = 41%). Calculation of a self-rotation function using the program *POLARRFN* (Kabsch, unpublished work; Collaborative Computational Project, Number 4, 1994) reveals the presence of additional non-crystallographic twofold symmetry (Fig. 2). Therefore, it seems likely that the asymmetric unit contains two molecules of $\rm Ap_4A$ hydrolase related by a non-crystallographic twofold axis. Gel-filtration studies of *C. elegans* $\rm Ap_4A$ hydrolase have suggested that the protein is monomeric (Abdelghany *et al.*, 2001).

Protein phase information is currently being sought by using the recently released coordinates of the NMR structure of the *L. angustifolius* Ap_4A hydrolase (Swarbrick *et al.*, 2000) for molecular replacement and by heavy-atom derivatization as part of a multiple isomorphous replacement approach.

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