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Adenovirus proteinase: crystallization and preliminary X-ray diffraction studies to atomic resolution

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Adenovirus proteinase (AVP) is required for the synthesis of infectious virus and is a target for antiviral therapy. The enzyme requires two viral cofactors for activation: pVIc, an 11-amino acid peptide, and the viral DNA. The structure of the enzyme in the absence of cofactors has not been observed. Single crystals of AVP were obtained *via* microseeding using the hanging-drop vapour-diffusion method with sodium acetate and sodium citrate as precipitants. At the National Synchrotron Light Source at Brookhaven National Laboratory, the native crystal diffracted to a resolution of 0.98 Å and an isomorphous heavy-atom derivative diffracted to 1.9 Å. Comparison of the structure of AVP with that of the AVP–pVIc complex should reveal the structural basis of activation of the enzyme by pVIc.

1. Introduction

Many medically important viruses contain a gene for a virus-coded proteinase whose activity is essential for the synthesis of infectious virus. Among these is human adenovirus (Weber & Tihanyi, 1994). Late in adenovirus infection, the adenovirus proteinase (AVP) becomes activated inside young virions and cleaves multiple copies of six virion precursor proteins. L3 23k is the gene for AVP (Yeh-Kai *et al.*, 1983) and it has been cloned, expressed in *Escherichia coli* (Anderson, 1993; Mangel *et al.*, 1993; Tihanyi *et al.*, 1993) or baculovirus-infected insect cells (Webster *et al.*, 1993) and the resultant 204 amino-acid protein purified.

Recombinant AVP alone is relatively inactive. Two viral cofactors were discovered that stimulate proteinase activity. One cofactor is pVIc, the 11-amino-acid peptide from the C-terminus of adenovirus precursor protein pVI (Baniecki *et al.*, 2001; Mangel *et al.*, 1993; Webster *et al.*, 1993). Its sequence is GVQSLKRRRCF. Cys104 of AVP can form a disulfide bond with Cys10′ of pVIc *in vitro* (Ding *et al.*, 1996; McGrath, Baniecki, Peters *et al.*, 2001) and does so *in vivo* in the virus particle (McGrath, Aherne *et al.*, 2002; McGrath, Ding *et al.*, 2002). A second cofactor is the viral DNA (Mangel *et al.*, 1993; McGrath, Baniecki, Li *et al.*, 2001). The cofactors increase the specificity constant (k_{cat}/K_m) for substrate hydrolysis (McGrath, Baniecki, Li *et al.*, 2001). In the presence of pVIc, k_{cat}/K_m for AVP increases 1130-fold; in the presence of Ad2 DNA, k_{cat}/K_m for AVP increases 110-fold. With all three components together, AVP, pVIc and Ad2 DNA, k_{cat}/K_m increases 34 100-fold compared with that of AVP alone.

The disulfide-linked complex AVP–pVIc has been crystallized (Keefe *et al.*, 1995; McGrath *et al.*, 1996) and its structure determined at 2.6 Å resolution (Ding *et al.*, 1996). More recently, a 1.6 Å resolution structure of AVP–pVIc has been determined (McGrath, Aherne *et al.*, 2002; McGrath, Ding *et al.*, 2002). AVP–pVIc is a cysteine proteinase. The order of the amino acids involved in catalysis along the polypeptide chain is unique, indicating AVP–pVIc to be the first member of a new class of cysteine proteinases. However, the amino acids involved in catalysis by AVP can be superimposed with similar residues involved in catalysis by papain. Upon superimposing AVP and papain, Cys122 in AVP is in an identical position to the nucleophilic Cys25 of papain. Furthermore, two other residues of AVP, His54 and Glu71, are in identical positions to those of His159 and Asn175, the two other residues of papain that constitute the charge-relay system (Blow *et al.*, 1969). Gln19 of papain (Drenth *et al.*, 1976), presumed to participate in formation of an oxyanion hole (Robertus *et al.*, 1972), and Gln115 of AVP also overlap. This remarkable juxtaposition of catalytic elements strongly suggests that AVP employs the same catalytic mechanism as papain (Polgar, 1974). Because the fold of AVP is different from the fold of papain, yet the positions of the residues involved in catalysis are the same, AVP is an example of convergent evolution. More recently, other proteinases have been added to the adenovirus proteinase family: Ulp1, a proteinase involved in sumoylation (Li &

Hochstrasser, 1999), the African swine fever virus proteinase (Andres *et al.*, 2001), a proteinase involved in processing virion precursor proteins, and YopJ, a proteinase involved in the inhibition of mitogen-activated protein kinase and nuclear factor κB signalling in animal cells and in the induction of localized cell death in plants (Orth *et al.*, 2000).

There is extensive contact between AVP and its cofactor pVIc: 26 hydrogen bonds, four ion pairs and a disulfide bond between Cys104 of AVP and Cys10′ of pVIc (McGrath, Aherne *et al.*, 2002; McGrath, Ding *et al.*, 2002). Surprisingly, pVIc, which exerts powerful control on the rate of catalysis by AVP, binds quite far from the active-site residues involved in catalysis; Cys104 of AVP, which forms the disulfide bond with Cys10′ of pVIc, is 32 Å from Cys122. The AVP–pVIc complex consists of two domains. Cys122 resides in one domain, while His54 and Glu71 reside in the other. pVIc appears to form a 'strap' that may help position the two domains in a configuration for optimal catalysis.

Comparison of the crystal structure of AVP with the crystal structure of AVP–pVIc (Ding *et al.*, 1996; McGrath, Aherne *et al.*, 2002; McGrath, Ding *et al.*, 2002) should reveal why the enzyme is relatively inactive in the absence of cofactors and how the binding of pVIc activates AVP. Comparison of the structures should also provide insights towards answering the following questions. What is the structure of the pVIc-binding site in the crystal structure of AVP? Are the two domains of AVP separated compared with their relative positions in AVP-pVIc? Does binding of pVIc bring the two domains together? Some of the amino-acid residues in the active site may not be in optimal alignment for efficient catalysis, because AVP is relatively inactive. What structural signals are transduced towards the active site upon the binding of pVIc?

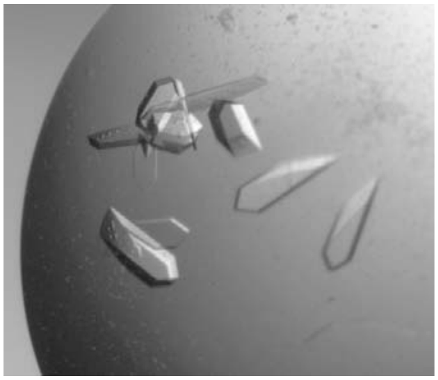

Figure 1
Photomicrograph of crystals of AVP. Prism-shaped crystals of AVP obtained by microseeding were used for X-ray diffraction. The typical crystal dimensions were 0.05 × 0.15 × 0.50 mm.

2. Materials, methods, results and future refinement

2.1. Cloning and purification

The L3 23k gene was cloned and expressed in *E. coli* strain BL21 (DE3pLysS) and the resultant AVP purified as previously described (Mangel *et al.*, 1996). The final step in the purification procedure involved dialysis against nitrogen-saturated 10 m*M* HEPES pH 8.0, 5 m*M* NaCl and 0.1 m*M* EDTA. AVP was stored at a concentration of 300 µ*M* at 193 K.

2.2. Crystallization

The hanging-drop vapor-diffusion technique (McPherson, 1990) was used to crystallize AVP in 24-well VDX plates (Hampton Research). Crystals grew after 2–3 months at 298 K in drops that contained 6.5 µl of enzyme and 3.5 µl of reservoir solution (1.0 *M* sodium citrate pH 5.0 and 1.8 *M* sodium acetate). These crystals were small and spherulitic in appearance and were not suitable for X-ray analysis. Crystal quality could not be improved by varying the precipitant, protein concentration, pH, buffer, additives or drop volume. However, crystal quality improved dramatically on using a microseeding procedure (Ducruix & Giegé, 1992) in which small crystals were crushed and inoculated using a cat whisker into drops consisting of 6.5 µl of enzyme and 3.5 µl reservoir solution. Crystals suitable for X-ray analysis grew within 3–5 d. For AVP, their dimensions were 0.05 × 0.15 × 0.50 mm and their appearance was prismatic (Fig. 1).

Table 1
Data-collection statistics for crystals of AVP and its platinyl derivative.

Values in parentheses are for the highest resolution shell.

	AVP	AVP + K_2PtCl_4
Space group	$P2_1$	$P2_1$
Unique reflections	92247 (6483)	12808 (1269)
Resolution (Å)	30.0–0.98 (1.0–0.98)	30–1.9 (1.97–1.90)
Completeness (%)	99.1 (97.1)	99.7 (99.8)
Average $I/\sigma(I)$	25.4 (1.6)	31.4 (20.6)
$R_{\rm merge}$† (%)	5.7 (59.4)	3.8 (5.7)
$R_{\rm iso}$‡ (%)		13.1

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where $\langle I\rangle$ is the average intensity. ‡ $R_{\rm iso} = \sum\big||F_{PH}| - |F_P|\big|/\sum|F_P|$, where F_P is the native protein structure amplitude and $|F_{PH}|$ is the platinum-derivative structure amplitude.

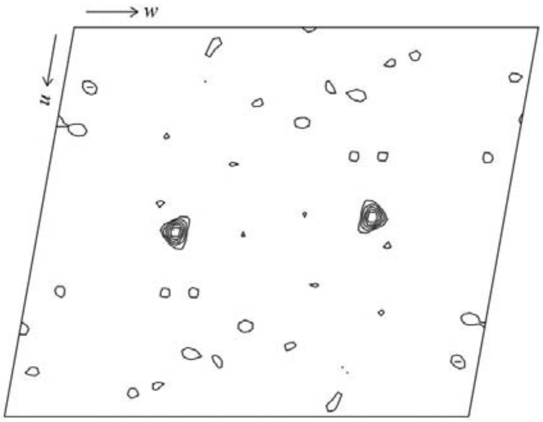

Figure 2
Harker section ($v = \frac{1}{2}$) of the isomorphous difference Patterson for the Pt derivative of AVP contoured at increments of 1σ from 2σ.

2.3. X-ray diffraction data collection and analysis

Crystals of AVP were prepared for freezing in cryobuffer by transfer into a drop containing reservoir solution (1.0 *M* sodium citrate pH 5.0 and 1.8 *M* sodium acetate). Then, five 1 µl aliquots of synthetic mother liquor (1.0 *M* sodium citrate pH 5.0, 1.8 *M* sodium acetate and 30% glycerol) were added stepwise; the time interval between each addition was 4 min. The crystals were flash-frozen in the liquid-nitrogen stream of an Oxford Cryostream Cooler. X-ray intensities were recorded on a Brandeis 4k CCD Detector (Strauss *et al.*, 1990; Phillips *et al.*, 2000) at beamline X25 at the National Synchrotron Light Source, Brookhaven National Laboratory. Several passes, using a different exposure time, distance and beam attenuation for each pass, were undertaken and the data merged together, resulting in a very complete data set. The X-ray wavelength was 0.986 Å (Table 1).

2.4. Heavy-atom derivatization

A crystal of AVP was soaked for 24 h at room temperature in mother liquor containing 6 m*M* K_2PtCl_4. It was then put into cryobuffer, flash-frozen and a data set collected. The distance between the crystal and detector was 99.5 mm and the X-ray wavelength was 1.064 Å. A data set was obtained to 99.7% completeness to 1.9 Å. The crystal was isomorphous to the native crystal and $R_{\rm iso}$ = 13.1% (Table 1). A single prominent Pt site could be identified from

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the isomorphous difference Patterson map (Fig. 2).

2.5. Results

The X-ray diffraction data were processed using *DENZO* and *SCALEPACK* from the *HKL* software suite (Otwinowski & Minor, 1997). Analysis of the X-ray diffraction data indicated that the AVP crystal belonged to space group $P2_1$, with unit-cell parameters $a = 36.2$, $b = 54.1$, $c = 42.1$ Å, $\beta = 100.5^\circ$. The unit-cell parameters and mass of the molecular species in the crystal were consistent with there being one 23 087 Da monomer of AVP per asymmetric unit, which gives a Matthews coefficient (Matthews, 1968) of 1.75 $\text{Å}^3\,\text{Da}^{-1}$, corresponding to a solvent content of 29.2%. The low solvent content may be the reason why this crystal diffracted to such high resolution (Dauter *et al.*, 1997; Teeter *et al.*, 1993). A data set was collected to 0.98 Å resolution from a single AVP crystal; the statistics are listed in Table 1.

2.6. Approach to solving the structure

Initially, molecular replacement will be attempted using as a search model the 1.6 Å structure of AVP–pVIc or a portion of it (McGrath, Aherne *et al.*, 2002; McGrath, Ding *et al.*, 2002). A successful rotation and translation search would mean that only rigid-body refinement should be necessary for an adequate starting model. However, if molecular replacement fails, the data set with the Pt derivative of AVP, collected with the objective of obtaining heavy-atom phasing, will be used as previously (Ding *et al.*, 1996). Since the resolution of the data extended beyond 1.0 Å, the structure will eventually be refined anisotropically with the program *SHELXL* (Sheldrick & Schneider, 1997).

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