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Cloning, preparation and preliminary crystallographic studies of penicillin V acylase autoproteolytic processing mutants

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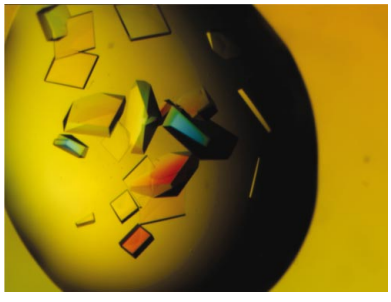


The crystallization of three catalytically inactive mutants of penicillin V acylase (PVA) from *Bacillus sphaericus* in precursor and processed forms is reported. The mutant proteins crystallize in different primitive monoclinic space groups that are distinct from the crystal forms for the native enzyme. Directed mutants and clone constructs were designed to study the post-translational autoproteolytic processing of PVA. The catalytically inactive mutants will provide three-dimensional structures of precursor PVA forms, plus open a route to the study of enzyme–substrate complexes for this industrially important enzyme.

1. Introduction

Penicillin V acylase (PVA) from *Bacillus sphaericus* (Pundle & SivaRaman, 1997) is a homotetrameric protein of 37.5 kDa subunits. It is industrially used in the hydrolysis of penicillin V to produce 6-aminopenicillanic acid (6-APA), which is the precursor molecule for semi-synthetic β-lactam antibiotics (Shewale & Sudhakaran, 1997). The crystal structure of PVA (Suresh *et al.*, 1999) placed this protein in the N-terminal nucleophile (Ntn) hydrolase superfamily (Brannigan *et al.*, 1995). The structures of this family share a characteristic $\alpha\beta\beta\alpha$ fold, with the catalytic centre being the side chain of an amino-terminal residue (Cys, Ser or Thr) incorporated in the central β-sheet as the nucleophile for catalytic attack at the carbonyl C atom of the substrate. Currently known Ntn hydrolases (Pei & Grishin, 2003) include proteins that are active on a range of substrates and that display diverse quaternary organizations. However, all share the common feature that the active-site nucleophile must be unmasked by a post-translational processing event. In the crystal structure of PVA, which is a single chain, the active-site cysteine was found to be the N-terminal residue (Suresh *et al.*, 1999), whereas the gene encoding PVA has an extra tripeptide (Met-Leu-Gly) as part of the reading frame preceding cysteine (Olsson & Uhlen, 1986). The presence of Cys at the N-terminus is explained by assuming that PVA has undergone post-translational modification to remove the pro-sequence, similar to that observed in other mechanistically related enzymes such as penicillin G acylase (PGA) from *Escherichia coli* (Duggleby *et al.*, 1995) and cephalosporin acylase (CPA; Kim *et al.*, 2000). However, this processing event is simpler in PVA compared with that in PGA and CPA, as in both of the latter systems a spacer peptide is removed from the middle of the peptide chain (Hewitt *et al.*, 2000; Kim *et al.*, 2002, 2003) whereby the polypeptide chain of the active enzyme splits into two.

We have designed clone constructs and site-directed mutants that were predicted to lead to PVA-processing defects by substitution of the active-site Cys in the presence of the tripeptide pro-sequence. Superposition of the PVA and PGA structures suggest a conserved topology for the 'oxyanion-hole' residue that balances the negative charge on the tetrahedral reaction intermediate. Substitution of this residue in PVA (Asn175) with alanine was performed to mimic the study on PGA B-chain residue Asn241, which allowed processing to occur but yielded a catalytically inactive protein (McVey *et al.*, 2001). Mutants were also prepared that lacked the PVA pro-sequence. It was presumed that the initiator formylmethionine residue could be removed by a methionine aminopeptidase, thus unmasking the nucleophile in a manner similar to glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase from *B. subtilis* (Smith *et al.*,

Table 1
Crystallization conditions for mutant PVA proteins.

The well-solution components and the cryoprotectant used are tabulated for each mutant. PB, 200 m*M* sodium phosphate buffer pH 6.4; AS, saturated ammonium sulfate; S, 10%(*w*/*v*) sucrose; M, 2 *M* maltose; DMSO, dimethyl sulfoxide; NC, 2 *M* nickel chloride in PB; HXT, 1,2,6-hexanetriol.

		Crystallization conditions (µl component in well solution)						
PVA mutant	Protein conc. (mg ml^{-1}) in H_2O	PB	AS	S	M	DMSO	NC	Cryoprotectant [30%(*v*/*v*)]
Pre-Cys1Ala	25	700	300	100	50	—	—	HXT
Cys1Ala	20	700	300	100	—	—	—	Glycerol
Pre-Cys1Ser	20	700	300	100	—	50	—	HXT
Cys1Ser	20	700	300	100	—	—	—	HXT
Pre-Asn175Ala	25	650	350	100	—	—	—	HXT
Asn175Ala	25	750	250	100	—	—	25	HXT

Table 2
Protein-sequence analysis of PVA mutant enzymes.

'Pre-' indicates the presence of precursor tripeptide. Pre-Asn175Ala showed ~50% processing activity based on the products detected by N-terminal sequencing. The proteins were pre-derivatized with acrylamide to allow detection of a stable Cys-adduct. The site of mutation at the N-terminus is highlighted in bold. $_f$Met indicates that processing had occurred, probably by simple removal of the initiator methionine residue.

	N-terminal sequence		Activity	
PVA/mutant	Cloned gene	Mature peptide	Processed	Catalytic
Native	MLG**C**SS...	**C**SS...	Yes	Yes
Pre-Cys1Ala	MLG**A**SS...	MLG**A**SS...	No	No
Pre-Cys1Ser	MLG**S**SS...	MLG**S**SS...	No	No
Pre-Asn175Ala	MLG**C**SS...	MLG**C**SS... + **C**SS...	50%	No
Cys1Ser	M**S**SS...	**S**SS...	$_f$Met	No
Cys1Ala	M**A**SS...	**A**SS...	$_f$Met	No
Asn175Ala	M**C**SS...	**C**SS...	$_f$Met	No

1994) and so bypass normal PVA processing. These designed mutants were overexpressed in *E. coli*, purified, crystallized and characterized using X-ray crystallographic techniques to probe the mechanism of autoproteolytic post-translational processing of PVA.

2. Materials and methods

2.1. Cloning

The PVA gene and flanking DNA sequence from *B. sphaericus* NCIMB 9370 was amplified from chromosomal DNA by PCR using oligonucleotide primers that incorporated the restriction-endonuclease sites *Bam*HI and *Eco*RI at the termini of the 1164 bp PCR fragment. Digested PCR product was cloned into the phagemid pBluescript SK such that the gene encoding PVA was placed downstream of the *lac* promoter. The DNA sequence was consistent with that reported (Olsson & Uhlen, 1986) except for a C to G transversion that alters the coding sequence at position 98 of the mature protein and leads to an amino-acid substitution (ACA Thr to AGA Arg). This construct was used as a template to prepare the precursor mutants Pre-Asn175Ala (Pre-N175A), Pre-Cys1Ser (Pre-C1S) and Pre-Cys1Ala (Pre-C1A) using the QuikChange site-directed mutagenesis kit (Stratagene). Mutants lacking the three-amino-acid presequence were cloned into pET vectors and expressed in *E. coli* BL21(DE3) cells. The mutants N175A and C1S were produced with a C-terminal histidine tag to aid purification.

2.2. Expression and purification

Protein expression was performed by growing the transformed *E. coli* cells at a temperature of 310 K in Luria–Bertani medium containing kanamycin (30 µg ml^{-1}) for pET-based plasmids and ampicillin (100 µg ml^{-1}) for the pBS phagemids. When the OD_{660} of the culture reached about 0.6, IPTG was added to a final concentration of 1 m*M*. The cells were harvested 4 h post-induction and disrupted using sonication. After centrifugation, the supernatant was mixed with streptomycin sulfate to remove nucleic acids and 56%(*w*/*v*) ammonium sulfate (AS) was added. Precipitated protein was dissolved in a minimum volume of buffer (0.05 *M* sodium phosphate pH 6.5, 10 m*M* EDTA) and dialyzed overnight. AS was added to the dialyzed protein to a final concentration of 24%(*w*/*v*) before loading onto an Octyl-Sepharose column (Pharmacia) pre-equilibrated with 24%(*w*/*v*) AS. This column was used for the purification of C1A, Pre-N175A, Pre-C1S and Pre-C1A mutants. Since both N175A and C1S mutants have a His tag at their C-termini, purification was carried out using Ni^{2+}-bound chelating resin (Pharmacia). The purity of the final protein preparations was confirmed using SDS–PAGE, in which each preparation showed a single band. The yield of the mutant proteins was 20 mg per litre of culture. Penicillin V acylase activity was measured by reacting the 6-amino group of the product 6-APA with *p*-dimethylaminobenzaldehyde to yield a chromogenic Schiff base (Shewale *et al.*, 1987).

2.3. Crystallization and data collection

Crystals used in data collection were grown using the hanging-drop vapour-diffusion method, mixing an equal amount (1 µl) of protein at a concentration of 20–25 mg ml^{-1} with the well solutions. Crystallization studies were performed with a number of commercial screens, including Crystal Screens (Hampton) and Clear Strategy Screens (Molecular Dynamics Ltd). Beautiful crystals were obtained in CSS-I condition 8 [0.2 *M* lithium sulfate and 15%(*w*/*v*) PEG 4K], but had poor diffraction properties. The crystals of mutant PVA proteins were successfully grown from conditions based on those used for native PVA. The well solution contained ~700 µl 0.2 *M* sodium phosphate buffer pH 6.4 with ~300 µl saturated AS and 100 µl 10%(*w*/*v*) sucrose solution. In some cases, the use of additional additives gave rise to improved quality crystals (Table 1). The crystallization temperature was 292 K. Diffraction data were collected using synchrotron radiation at the European Synchrotron Radiation Source (ESRF, Grenoble, France) or Synchrotron Radiation Source (SRS, Daresbury, Warrington, UK) using CCD detectors. All data were collected at 100 K under liquid nitrogen from crystals flash-cooled in the presence of 30%(*v*/*v*) glycerol or 1,2,6-hexanetriol (Table 1). The diffraction data were processed and scaled using the *DENZO* and *SCALEPACK* modules of the *HKL* package (Otwinowski & Minor, 1997).

3. Results and discussion

The two residues targeted for mutation were Cys1 and Asn175. Information based on studies of other Ntn hydrolases allowed us to identify cysteine as acting as the nucleophile in PVA and thus it was assumed that its mutation would affect the catalytic properties of the enzyme. Structural comparison with other members of the family helped to identify Asn175 in PVA as the oxyanion-hole residue stabilizing the transition-state complex during catalysis. Mutating these two functional residues to alanine resulted in loss of activity towards penicillin V. Inclusion of the N-terminal tripeptide of the precursor in each mutant was intended to assess the individual roles of the mutated amino acids in the post-translational processing of PVA. As stated in §1, some Ntn hydrolases are functional with a Ser or Thr in the place of Cys as their catalytic centre. To test whether a serine residue can functionally replace the cysteine in PVA, we prepared a third pair of mutants by mutating Cys1 to Ser. Surpris-

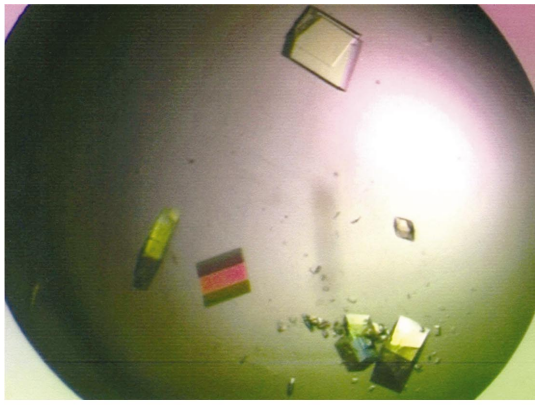
Table 3
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

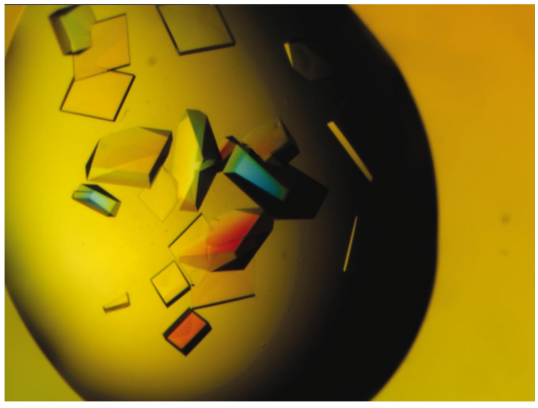
Mutant	Asn175Ala	Cys1Ala	Cys1Ser	Pre-N175A	Pre-C1S	Pre-C1A
X-ray source	ESRF ID14.3	ESRF ID14.4	ESRF ID14.3	Daresbury 9.6	Daresbury 14.2	ESRF ID14.1
Crystal size (mm)	0.3 × 0.2 × 0.1	0.3 × 0.2 × 0.1	0.1 × 0.1 × 0.1	0.3 × 0.3 × 0.1	0.3 × 0.3 × 0.1	0.3 × 0.3 × 0.1
Space group	$P2_1$ (form I)	$P2_1$ (form I)	$P2_12_12_1$	$P2_1$ (form II)	$P2_1$ (form II)	$P2_1$ (form II)
Unit-cell parameters						
a (Å)	47.28	47.86	90.93	103.66	102.64	103.30
b (Å)	379.38	381.89	129.42	92.52	90.09	89.88
c (Å)	102.01	102.89	158.78	103.84	102.30	103.60
β (°)	93.5	94.1	—	101.8	102.1	100.6
Max. resolution (Å)	1.7	2.1	1.95	1.9	2.5	2.5
Total No. reflections	1293348	206635	470208	1130879	236110	150271
Unique reflections	370615	196267	132141	129730	62997	72123
Completeness	94.6 (64.7)	97.1 (86.4)	96.7 (97.9)	99.0 (94.7)	99.9 (99.4)	93.3 (79.8)
$I/\sigma(I)$	19.6 (2.2)	13.1 (6.3)	10.3 (1.4)	11.0 (1.8)	16.5 (4.4)	10.4 (1.7)
$R_{\rm merge}$† (%)	6.9 (48.6)	9.0 (16.0)	11.8 (72.8)	9.2 (46.4)	8.6 (29.2)	7.8 (48)
Unit-cell volume (Å^3)	1825765	1873652	1867113	969527	931873	945673
Matthews coefficient (Å^3 Da^{-1})	3.0	3.1	3.1	3.2	3.2	3.2
Solvent content	59.6	60.6	60.5	61.6	61.7	61.0
No. tetramers per asymmetric unit	2	2	1	1	1	1

† $R_{\rm merge} = 100 \times \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

ingly, this mutant also showed no activity towards penicillin V. Table 2 is a summary of N-terminal protein-sequence analysis of the purified mutant proteins. Recombinant PVA is processed normally when expressed in *E. coli*, yielding a preparation whose specific activity (30 U mg^{-1}) resembled that of purified enzyme from native sources (Pundle & SivaRaman, 1997). All mutant proteins with the pro-sequence retain the three amino acids Met-Leu-Gly and all mutant constructs lacking the pro-sequence appear to have their initiation formylmethionyl ($_{\rm f}$Met) residue removed. None of the mutant proteins yielded active PVA and the N175A mutant led to both processing and activity defects.



(*a*)



(*b*)

Figure 1
(*a*) Monoclinic (form II) crystals of Pre-N175A precursor and (*b*) (form I) crystals of N175A mutant proteins of *B. sphaericus* PVA.

Initially, several different precipitants and buffers were used in crystallization attempts. Often, the crystals were unstable or their diffraction quality was poor. The only condition found that worked well for all the mutants was 30% saturated AS as the precipitant in sodium phosphate buffer pH 6.4 supplemented with sucrose (Table 1). In the case of all three pairs of mutants, the crystals (Fig. 1) usually appeared within a few days. The details of data collection and crystal parameters for the mutants are summarized in Table 3. Curiously, all the mutants crystallized in space groups that were different from that of the native enzyme, although the crystallization conditions of the wild-type enzyme and the mutants were almost identical. The crystals of PVA mutant proteins (Fig. 1) are monoclinic, whereas native PVA crystallized in space groups $P6_5$ and $P1$ (Suresh *et al.*, 1999). They diffracted to various resolutions (Table 3). Molecular-replacement calculations using the PVA structure (PDB code 2pva) as a search model showed that the asymmetric unit was constituted of a tetramer. The crystals with a large unit cell (monoclinic form I, Table 3) contained two tetramers in the asymmetric unit. Based on the above information, the calculation of the Matthews coefficient (Matthews, 1968) gave values almost equal in magnitude for all crystals and the solvent content worked out as around 60% in all forms (Table 3). The refinement of the structures of all the mutants and investigations towards understanding the mechanism of autoproteolytic activation of PVA enzyme through structural analysis are in progress.

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