Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of a new L-aminopeptidase-D-amidase/D-esterase activated by a Gly–Ser peptide bond hydrolysis

Coralie Bompard-Gilles,[a] Vincent Villeret,[a] Laurence Fanuel,[b] Bernard Joris,[b] Jean-Marie Frère[b] and Jozef Van Beeumen[a]*

[a]Laboratorium voor Eiwitbiochemie en Eiwitengineering, Universiteit Gent, K. L. Ledeganckstraat, 35, B-9000 Gent, Belgium, and [b]Laboratoire d'Enzymologie et Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000 Sart-Tilman, Belgium

Correspondence e-mail: jozef.vanbeeumen@rug.ac.be

Received 22 July 1998
Accepted 24 November 1998

Ochrobactrum anthropi possesses an L-aminopeptidase (DmpA) also able to act as a D-amidase/D-esterase. DmpA (40 kDa) is activated by auto-catalyzed protein splicing liberating an α-amino group presumably used as a general base in the catalytic mechanism. Two crystal forms were obtained at 294 K in 13–16% PEG 2000 monomethylether at pH 9.0, adding either 0.2 *M* magnesium chloride or 1 *M* lithium chloride. Crystals of the first form belong to the space group $C222_1$ and diffract to 3.0 Å resolution, whereas crystals of the second form belong to the space group $P2_12_12$ and diffract to 2.3 Å resolution. Initial screening for heavy-atom derivatives on form II crystals, has led to a well substituted Hg derivative.

1. Introduction

Aminopeptidases (alpha aminoacyl-peptide hydrolase) are exopeptidases that catalyze the hydrolysis of the amino-terminal residue from polypeptide substrates. Most of them recognize L-amino acids and are of critical biological and medical importance because of their key role in protein modification and degradation. They are mostly divalent cation-dependent or thiol enzymes (Taylor, 1993). Only three aminopeptidases active on peptides containing N-terminal D-residues have been isolated so far, all of them from the bacterium *Ochrobactrum anthropi*. The first two peptidases, DAP and DmpB, are two homologous strict D-stereospecific aminopeptidases, isolated, respectively, from strains SCRC C1-38 and LMG7991 (Asano *et al.*, 1989; Fanuel, 1997). Although of yet unknown biological role, these enzymes show about 25% sequence identity with *Streptomyces* R61 DD-carboxypeptidase. The third one, DmpA, is an L-aminopeptidase showing the unique feature to also hydrolyze D-amides and D-esters (also isolated from strain LMG7991; Fanuel, 1997).

The DmpA gene has been cloned, overexpressed in *Escherichia coli* and purified as previously described (Fanuel, 1997). It has been completely sequenced on both strands. The deduced amino-acid sequence does not exhibit significant isology with known aminopeptidases but shows 28% identity with the deduced sequence of ORF B1549_C2_208 from *Mycobacterium leprae* B1549 cosmid, whose translation product has not yet been characterized. As mentioned, DmpA is an L-aminopeptidase but it also shows D-amidasic and D-esterasic activities on peptides containing an N-terminal D-alanine residue (Fanuel, 1997). This enzyme is one of the rare aminopeptidases that are not metalloenzymes, and the only one known to be specific for both D- and L-aminoacids. DmpA is synthesized as a single polypeptide precursor. The active form consists of two different peptides resulting from the unique cleavage of the Gly249–Ser250 peptide bond of the precursor polypeptide. Site-directed mutagenesis studies revealed that both residues are essential for protein maturation and catalysis (Fanuel, 1997). The cleavage site is recognized in both *O. anthropi* and *E. coli* and is similar to that found in enzymes of the N-terminal nucleophile (Ntn) hydrolase family which includes penicillin acylase (Duggleby *et al.*, 1995), the proteasome β-subunits (Löwe *et al.*, 1995), the glycosyl asparaginase (Guan *et al.*, 1996) and the glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase (Smith *et al.*, 1994). Their functions, modes of activation and fold have been described previously (Artymuiuk, 1995; Brannigan *et al.*, 1995; Shao & Kent, 1997). Ntn hydrolase enzymes are amidohydrolases characterized by their unusual use of an N-terminal nucleophile (threonine, serine or cysteine) that appears to utilize its own α-amino group as a general base in its catalytic mechanism. This catalytic N-terminal residue is produced by a self catalyzed protein splicing (Shao & Kent, 1997) and is situated at the extremity of a β-strand. They share a common fold consisting in a core of two stacked antiparallel β-sheets flanked on both sides by helices which provide both the capacity for nucleophilic attack and the possibility of autocatalytic processing (Brannigan *et al.*, 1995). Fanuel (1997) suggested that DmpA

may belong to this family, a proposal that should ultimately be confirmed by the three-dimensional determination of this protein.

2. Materials and methods

The DmpA gene was cloned, expressed in *E. coli* DH5a and purified as described in Fanuel (1997).

2.1. Crystallization experiments

Crystallizations were initiated at a protein concentration of about 20 mg ml^{-1}. Prior to the experiments, the protein was briefly centrifuged to remove insoluble material. Preliminary crystallization trials were conducted with 4 µl hanging drops, at 294 K following the vapor-diffusion sparse-matrix sampling method (Jancarik & Kim, 1991) as implemented in Hampton Research Screens. This method allowed us to identify two sets of initial crystallization conditions: PEG 2000 mono-methyl-ether (MME)/magnesium chloride and PEG 2000 MME/lithium chloride. They resulted in two crystal forms. Crystals of form I were grown at 294 K with a reservoir solution containing 15% (*w*/*v*) PEG 2000 MME, 0.2 *M* magnesium chloride and 100 m*M* Bicine buffer, pH 9. Crystals of form II were obtained at 294 K in 13–16% (*w*/*v*) PEG 2000 MME in 100 m*M* Bicine buffer, pH 9, but using 1 *M* lithium chloride instead of magnesium chloride. A ratio of 2:1 for protein to reservoir solution was found to produce the largest crystals. For both crystal forms, a crystalline precipitate appears 1 d after setting up the crystallization assays. A few large crystals of the second form appear and reach their maximum size (0.9 × 0.6 × 0.5 mm) within 2 d, partially dissolving the precipitate (Fig. 1). On the other hand numerous small crystals of form I were obtained after 3 d in the precipitate (approximate size of 0.3 × 0.1 × 0.1 mm). All attempts to increase the size of these crystals or to decrease the nucleation in crystallization drops did not lead to any improvements.

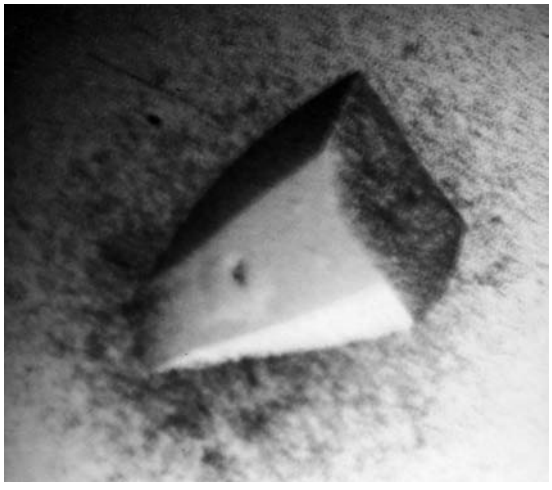

Figure 1
Micrograph of a DmpA crystal of form II. These crystals typically grew to a size of 0.9 × 0.6 × 0.5 mm.

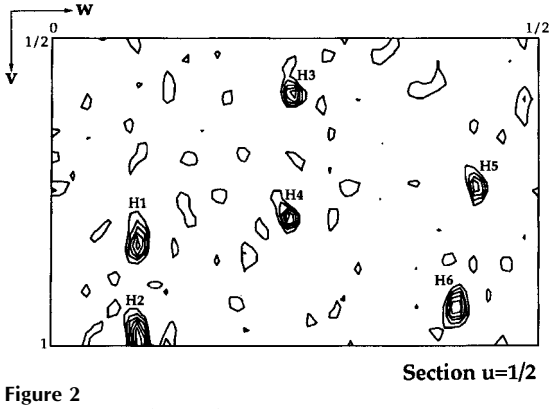

Figure 2
Harker section ($u = 1/2$) from the difference Patterson map contoured at 1σ intervals starting at 1σ above the mean density. The map has been calculated with all data from the form II crystals, lying between 15 and 3.5 Å resolution. Mercury difference Harker vectors from the six atom, initially interpreted from this Harker plane and the other Harker planes for this space group ($v = 1/2$ and $w = 0$), are labelled H1 to H6.

2.2. Data collection

For both crystal forms, data were collected with a MacScience DIP2030 image-plate system, using the Cu $K\alpha$ radiation produced by a Nonius FR591 rotating-anode generator equipped with a double-mirror X-ray optical system and running at 100 mA, 45 kV. Rotating frames of 1° were collected with an exposure time of 15 min (form I crystals) or 10 min (form II) per frame. The diffraction data were auto-indexed, processed, scaled and merged using the programs *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997).

Form I crystals belong to the space group $C222_1$ with unit-cell parameters $a = 109.91$, $b = 147.47$ and $c = 91.69$ Å. Assuming a molecular mass of 40 302 Da, as determined by electrospray ionization mass spectrometry (Fanuel, 1997), and two molecules in the asymmetric unit, the V_m value is 2.3 Å^3 Da^{-1} corresponding to a solvent content of 46% (Matthews, 1968). A data set has been measured to 3 Å resolution. The data are 97.6% complete from 15.0 to 3 Å resolution (completeness on the last resolution shell, 3.11–3.00 Å: 98.5%) and the $R_{\rm merge}$ on intensities is 12.2% based on 73 305 measured observations. These data have been reduced to 14 796 unique reflections.

Crystals of the second form belong to the space group $P2_12_12$ and have unit-cell dimensions $a = 156.97$, $b = 96.22$ and $c = 154.41$ Å. Assuming six molecules in the asymmetric unit, the V_m value is 2.41 Å^3 Da^{-1} corresponding to a solvent content of 49%. These values are within the observed range for protein crystals (Matthews, 1968). A total of 473 750 diffraction intensities was collected to 2.3 Å; they were merged into 102 709 unique reflections. The data set is 98.8% complete (98.1% in the last shell), with an average value of $I/\sigma(I)$ above 16 and a merging R factor (on intensities) for symmetry-related reflections of 8.1%.

3. Results and discussion

The native Patterson function, calculated between 20 and 5 Å resolution on form II crystals, showed one significant peak (25% of the value of the origin peak) at fractional coordinates (0.5, 0.393, 0.0) indicating that some subunits are related by this translation vector. Using the program *AMoRe* (Navaza, 1994), a self-rotation function was calculated but no significant peak could be observed.

Heavy-atom screening was undertaken on form II crystals which are more reproducible, more easy to handle and do not deteriorate upon storage. Heavy-atom soaks were carried out at 294 K in reservoir solutions. One crystal was soaked in 10 m*M* parahydroxymercury sulfonic acid for 10 weeks. Difference Patterson synthesis, calculated using the *CCP*4 package (Collaborative Computational Project, Number 4, 1994), indicated six major Hg-binding sites in the asymmetric unit (Fig. 2). Six other sites could be subsequently identified using the difference Fourier technique. Initial refinement of coordinates and occupancies was performed with *MLPHARE* (Collaborative Computational Project, Number 4, 1994) leading to a Cullis R factor and a phasing power for centric reflections of 0.54 and 1.66, respectively. The six major binding sites have an occupancy between 2.2 and 1.54, while the six minor sites have an

occupancy between 0.52 and 0.25. Each low-occupancy binding site is close to one of the six major sites, suggesting that they are probably bound to the same subunit. Because of the presence of only one cysteine residue per molecule this result is consistent with the presence of six enzyme molecules in the asymmetric unit. Crystals of form II appear suitable for a three-dimensional structure determination by the multiple isomorphous replacement method and a search for other heavy-atom derivatives is well under way. This X-ray study will illustrate the structural motifs of a new member of the N-terminal nucleophile hydrolase family and will ultimately reveal molecular features allowing this L-aminopeptidase to also act as a D-amidase/D-esterase towards D-Ala substrates.

This work was supported by Grants of the Federal Programme IUAP/PAI IV3 and the Geconcerterde Onderzoeksactie 12050198 of the Flemish Government. CB is indebted to the FWO-Vlaanderen for a Visiting Postdoctoral Fellowship.

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