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Crystallization and preliminary crystallographic studies of five crystal forms of *Escherichia coli* L-asparaginase II (Asp90Glu mutant)

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L-Asparaginase II from *Escherichia coli* with an Asp90Glu mutation in the active site has been crystallized in five polymorphic forms. Crystals of all polymorphs suitable for X-ray diffraction experiments were obtained by the vapour-diffusion method. Crystals of form I belong to the monoclinic system (space group $C2$), have unit-cell parameters $a = 73.1$, $b = 133.1$, $c = 62.6$ Å, $\beta = 108.8^\circ$ and diffract to 2.27 Å resolution. Three of the crystal forms are orthorhombic, with unit-cell parameters $a = 225.4$, $b = 128.0$, $c = 62.6$ Å (form II, $P2_12_12$), $a = 59.9$, $b = 71.2$, $c = 130.6$ Å (form III, primitive cell) and $a = 73.8$, $b = 122.1$, $c = 124.2$ Å (form IV, $P2_12_12_1$ or $P2_12_12$); the crystals diffract to 2.33, 3.5 and 1.7 Å, respectively. Polymorph V is trigonal, space group $P3_121$, with unit-cell paramters $a = 123.1$, $c = 83.8$ Å; the crystals diffract to 2.65 Å resolution.

1. Introduction

L-Asparaginases (E.C. 3.5.1.1) are enzymes that catalyze the hydrolysis of L-asparagine to L-aspartate with the release of ammonia. They were first discovered in guinea pig blood by Clementi (1922). In *E. coli*, two forms of this enzyme, termed type I (cytosolic, with low substrate affinity) and type II (periplasmic, with high affinity towards the substrate), have been found. Type II enzymes, with high substrate affinity ($K_M \simeq 10^{-5}$ M), isolated from *Erwinia chrysanthemi* (ErA) and *E. coli* (EcAII) have been employed for many years as effective drugs in the treatment of acute lymphoblastic leukaemia, leukaemic lymphosarcoma and lymphosarcoma (Hill *et al.*, 1967; Chakrabarti, 1997).

Several crystal structures of type II bacterial asparaginases are known. Among them is the enzyme from *E. coli* with bound aspartate (Swain *et al.*, 1993), its active-site Thr89Val mutant with covalently bound product (Palm *et al.*, 1996) and the Tyr25Phe mutant (Jaskólski *et al.*, 2001). The enzymes from *Erwinia chrysanthemi* (ErA; Miller *et al.*, 1993; Aghaiypour *et al.*, 2001), *Wolinella succinogenes* (WsA; Lubkowski *et al.*, 1996), *Acinetobacter glutaminasificans* (AGA; Lubkowski, Wlodawer, Ammon *et al.*, 1994) and *Pseudomonas* 7A (PGA; Lubkowski, Wlodawer, Housset *et al.*, 1994; Jakob *et al.*, 1997) have also been characterized crystallographically. The asparaginase molecule is a homotetramer with nearly ideal 222 symmetry composed of four identical subunits (326 amino-acid residues each in EcAII) denoted *A*, *B*, *C* and *D*. The asparaginase tetramer can be treated as a dimer of dimers because the active site (Thr12, Tyr25, Ser58, Thr89, Asp90, Lys162, Asn248 and Glu283 in the EcAII sequence) is created by subunits *A* and *C* or subunits *B* and *D*. The role of the catalytic triad of serine proteases (Ser-His-Asp) can be played in L-asparaginases by a similar triad, Thr89-Lys162-Asp90 (in the EcAII sequence), which is conserved in the sequences and three-dimensional structures of all bacterial asparaginases (Bonthron & Jaskólski, 1997; Dodson & Wlodawer, 1998). Access to the active-site cavity is controlled by a flexible loop between residues 10 and 40. In uncomplexed crystals this loop is typically not visible in electron density, leading to the assumption that it is disordered in an open conformation. The flexible loop has been observed in the 'closed' conformation when the active site is occupied by the reaction product or its analogue. The situation in the active site and at the flexible loop is influenced by the pH of crystallization. Binding of the reaction product (L-aspartate) requires acidic pH. When the pH is in the basic range the product is expelled from the active site, leading to unlocking of the flexible loop. To date, the native and mutated EcAII protein has been reported in as many as 12 crystal forms. Among them is an intriguing case of a hexagonal space group that forms an enantiomorphic pair with one of the crystal forms of the closely related ErA protein (Jaskólski *et al.*, 2001).

Here, we report the crystallographic characterization of an active-site mutant of EcAII

with Asp90Glu substitution. The protein has been crystallized in five polymorphic modifications, three of which have not been observed previously.

2. Crystallization

A sample of the EcAII Asp90Glu mutant was a generous gift from Professor Klaus Röhm, Philipps Universität, Marburg, Germany. The protein suspension was desalted and then concentrated using Centricon-10 concentrators; the final concentration was determined by UV absorption at 280 nm. Crystals in five forms were obtained using new crystallization conditions as well as condition reported earlier for other EcAII mutants. All crystallization experiments were conducted at room temperature using the vapour-diffusion method and the hanging-drop technique (McPherson, 1982).

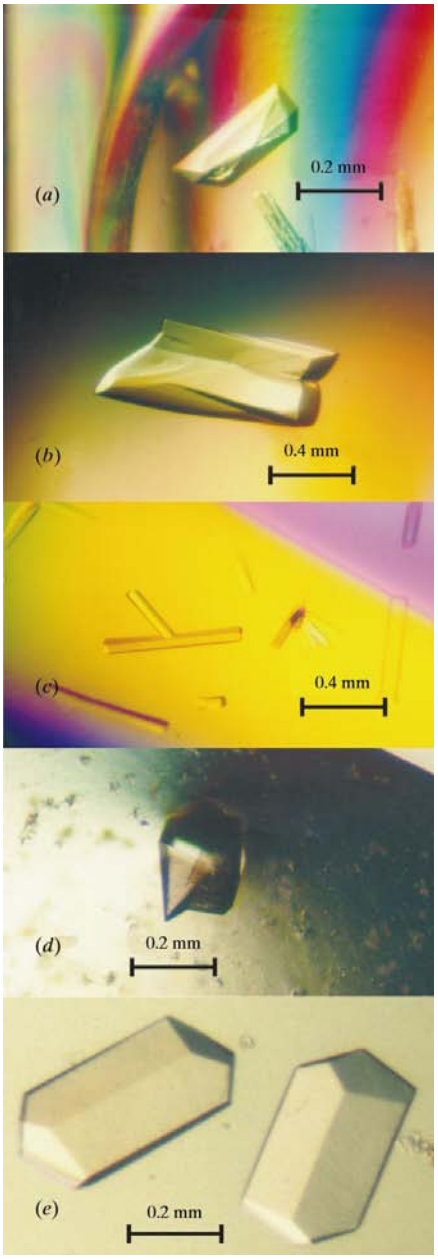

Figure 1
Single crystals of the Asp90Glu mutant of *E. coli* L-asparaginase II: (*a*) form I, (*b*) form II, (*c*) form III, (*d*) form IV and (*e*) form V.

Table 1
Summary of crystal data and data-collection parameters.

Values in parentheses are for the last resolution shell.

	Form I	Form II	Form III	Form IV	Form V
Space group	*C*2	$P2_12_12$	Orthorhombic *P* lattice	$P2_12_12_1$ or $P2_12_12$	$P3_121$
Unit-cell parameters					
a (Å)	73.1	225.4	59.9	73.8	123.1
b (Å)	133.1	128.0	71.2	122.1	
c (Å)	62.6	62.6	130.6	124.2	83.8
β (°)	108.8				
Temperature (K)	100	293	293	293	293
Wavelength (Å)	1.104	1.54178	1.54178	1.54178	1.54178
Oscillation range (°)	1.0	0.5	1.0	0.4, 0.6, 1.2	1.2
Resolution (Å)	25.0–2.27	25–2.5	20.0–3.5	20–2.35	20–2.65
Total No. of reflections	129052	156965	8767	47606	63384
No. of unique reflections	26057	60001	4462	27104	21080
Completeness (%)	98.6 (87.3)	94.1 (92.8)	59.3 (62.4)	57.2 (57.5)	97.3 (96.4)
$R_{\rm merge}$	0.098 (0.143)	0.094 (0.585)	0.149 (0.422)	0.110 (0.406)	0.109 (0.542)
$I/\sigma(I)$	28.9 (6.2)	14.2 (2.3)	5.5 (2.1)	17.0 (1.9)	11.2 (2.2)

Form I was crystallized using the sparse-matrix method (Jancarik & Kim, 1991) and Crystal Screen II (Hampton Research). Crystallization drops (6–10 µl) were prepared by mixing equal amounts of reservoir solution (25% PEG–MME 550, 100 m*M* MES pH 6.5, 10 m*M* $ZnSO_4$) and protein solution at 10–15 mg ml^{-1}. Crystals suitable for X-ray diffraction experiments (Fig. 1*a*) appeared after 2 d and reached maximum dimensions of about 0.3 × 0.2 × 0.15 mm after an additional 2 d.

Form II was obtained in a crystallization experiment based on the conditions reported by Kozak & Jaskólski (2000). 5 µl protein samples (10–15 mg ml^{-1}) were mixed on siliconized cover slips with equal amounts of reservoir solution containing 30–35% PEG–MME 550, 100 m*M* bicine pH 9.0 and 100 m*M* NaCl and were equilibrated against 1 ml reservoir solutions. Wedge-shaped crystals (Fig. 1*b*) appeared after 2–4 d and reached maximum dimensions of 0.6 × 0.4 × 0.2 mm within a week.

The crystallization conditions for form III were established using Crystal Screen from Hampton Research. The crystallization drop was prepared by mixing (1:1) 3 µl of protein solution (10 mg ml^{-1}) with reservoir solution (28% PEG 400, 100 m*M* sodium HEPES pH 7.5, 200 m*M* $CaCl_2$). Crystals (Fig. 1*c*) appeared after 24 h and reached maximum dimensions of 0.4 × 0.1 × 0.1 mm after an additional 2 d.

The crystallization conditions for form IV were also determined using the sparse-matrix method and Crystal Screen. Protein samples (2 µl) at a concentration of 15 mg ml^{-1} were mixed with equal amounts of reservoir solution (100 m*M* sodium HEPES pH 7.5, 1.4 *M* trisodium citrate). Bipyramidal crystals (Fig. 1*d*) grew in hanging drops at room temperature within 6 d to maximum dimensions of 0.3 × 0.2 × 0.2 mm.

Form V was grown using a modification of the crystallization conditions described for polymorph IV. The best crystals were obtained when the reservoir solution contained 1 *M* sodium HEPES pH 7.5 and a saturated solution of sodium citrate mixed in a 1:9 ratio. Prismatic crystals (Fig. 1*e*) appeared after 1–2 d and reached maximum dimensions of 0.5 × 0.2 × 0.2 mm after another 2 d.

Crystals for X-ray diffraction experiments at room temperature were mounted in thin-walled quartz capillaries with a small amount of mother liquor. Crystals for low-temperature data collection (form I) were mounted in a nylon fibre loop and flash-frozen in a nitrogen-gas stream (Teng, 1990).

3. Diffraction experiments

Diffraction data for crystals of forms II–V were collected at room temperature using a 300 mm image-plate scanner (MAR Research) and Cu $K\alpha$ radiation generated by an SRA2 rotating-anode generator (Siemens) operated at 45 kV and 112 mA. A summary of data-collection parameters is presented in Table 1. Crystals of form IV were very unstable in the X-ray beam at room temperature and consequently the diffraction data had to be measured using three specimens. The oscillation range was

increased from 0.4° for the first crystal to 1.2° for the last (Table 1). Fresh crystals diffracted to 1.7 Å but the resolution limit decreased rapidly with time. Low-temperature data were collected from two crystals of form I (cryoprotecting agent PEG–MME 550) at 100 K using synchrotron radiation (MAX Lab, Lund, Sweden, beamline 711, $\lambda = 1.104$ Å) and a 345 mm MAR Research image-plate scanner. The crystal-to-detector distances were 300 and 280 mm for the first and second crystal, respectively, with the same oscillation range of 1°.

Indexing and integration of the images was performed in *DENZO* and scaling of the intensity data was performed in *SCALEPACK* from the *HKL* program package (Otwinowski & Minor, 1997).

4. Results

The crystals of forms I, II and V were stable in the X-ray beam, while the crystals of forms III and IV were unstable and prone to drying. A summary of crystallographic data for all crystal forms is presented in Table 1. Molecular-replacement calculations for forms I and V are summarized in Table 2.

The crystals of form I are monoclinic, space group $C2$, and have unit-cell parameters $a = 73.1$, $b = 133.1$, $c = 62.6$ Å, $\beta = 108.8°$. This crystal form is similar to the form obtained for native EcAII by Polikarpov *et al.* (1999). Assuming two subunits in the asymmetric unit, the Matthews volume (Matthews, 1968) is 2.1 Å^3 Da^{-1} and the solvent content is 40.5%. The structure was solved using molecular replacement and the *EPMR* program (Kissinger *et al.*, 1999). Monomer *A* of native EcAII (PDB code 3eca; Swain *et al.*, 1993) was used as a search model. The asymmetric unit contains the *AB* dimer. The active-site competent *AC* dimer and the complete tetramer are created by crystallographic twofold rotation. Refinement of this structure is in progress.

The crystals of form II are orthorhombic, space group $P2_12_12$, and have unit-cell parameters $a = 225.4$, $b = 128.0$, $c = 62.6$ Å. This form is almost identical to the crystal form reported for the Ser58Ala mutant of EcAII, with six protein molecules in the asymmetric unit arranged into one pseudo-222-symmetric homotetramer and an active-site competent dimer from which another homotetramer is generated by crystallographic symmetry (Kozak & Jaskólski, 2000).

Table 2
Summary of molecular-replacement calculations.

	Form I	Form V	
Program	*EPMR*	*AMoRe*	
Search model	Monomer *A* (PDB code 3eca)	Dimer *AC* (PDB code 3eca)	
Space group	$C2$	$P3_121$	$P3_221$
Correlation coefficient (%)	71.5	75.6	33.4
R factor	0.338	0.271	0.450

Form III is a new polymorphic modification of EcAII. The crystals belong to the orthorhombic system, with a primitive unit cell of dimensions $a = 59.9$, $b = 71.2$, $c = 130.6$ Å. Missing axial reflections prevent us from determining the space group unambiguously. Cell-contents analysis indicates two subunits in the asymmetric unit with a Matthews volume of 2.0 Å^3 Da^{-1} and a solvent content of 38.4%. This precludes the possibility of the $P2_12_12_1$ space group but leaves a choice of three other possibilities. The completeness of the data set (59%) is not sufficient for structure determination.

The crystals of form IV also represent a new polymorphic modification of the protein. They are orthorhombic, space group $P2_12_12_1$ or $P2_12_12$, and have unit-cell parameters $a = 73.8$, $b = 122.1$, $c = 124.2$ Å. Low completeness of the diffraction data and missing axial reflections do not allow an unambiguous identification of the space group. An analysis of the Matthews volume (Matthews, 1968) for this form indicates that the full tetramer could be accommodated in the asymmetric unit, corresponding to a Matthews coefficient of 2.0 Å^3 Da^{-1} and a solvent content of 38.7%. The completeness of the data set (57%) is not sufficient for structure determination.

The crystals of form V also represent a new crystal form of EcAII. They belong to the trigonal system (space group $P3_121$) and have unit-cell parameters $a = 123.1$, $c = 83.8$ Å. Assuming two molecules in the asymmetric unit, a Matthews coefficient of 2.7 Å^3 Da^{-1} and a solvent content of 53.4% are obtained. The structure was solved by molecular replecement using *AMoRe* (Navaza, 1994). The active *AC* dimer of the native asparaginase (PDB code 3eca) was used as a search model. The two possible enantiomorphic space groups were checked. The results clearly indicate that $P3_121$ is the correct space group (Table 2). The asymmetric unit contains the active-site dimer (*AC*) and the full tetramer is generated by the crystallographic twofold axis.

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