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

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

**Maciej Kozak**, b Dominika Borek, b Robert Janowski b and Mariusz Jaskólski b,c

Department of Macromolecular Physics, Faculty of Physics, A. Mickiewicz University, Poznań, Poland, bDepartment of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznań, Poland, and cCenter for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland

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1. Introduction

t-Asparaginases (E.C. 3.5.1.1) are enzymes that catalyze the hydrolysis of t-asparagine to t-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 \approx 10^{-3} 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 glutaminisicans* (AGA; Lubkowski, Wlodawer, Ammon et al., 1994) and *Pseudomonas* 7A (PGA; Lubkowski, Wlodawer, Houset 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 t-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 (t-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 enantiomorph 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 EcAlII 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 EcAlII mutants. All crystallization experiments were conducted at room temperature using the vapour-diffusion method and the hanging-drop technique (McPherson, 1982).

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 mM MES pH 6.5, 10 mM ZnSO4) and protein solution at 10–15 mg ml⁻¹. Crystals suitable for X-ray diffraction experiments (Fig. 1a) 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 & Jaskolski (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 mM bicarbonate pH 9.0 and 100 mM NaCl and were equilibrated against 1 ml reservoir solutions. Wedge-shaped crystals (Fig. 1b) 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 mM sodium HEPES pH 7.5, 200 mM CaCl2). Crystals (Fig. 1c) 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 mM sodium HEPES pH 7.5, 1.4 M trisodium citrate). Bipartite crystals (Fig. 1d) 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. 1e) 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 Kα 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...

Table 1

<table>
<thead>
<tr>
<th>Space group</th>
<th>Form I</th>
<th>Form II</th>
<th>Form III</th>
<th>Form IV</th>
<th>Form V</th>
</tr>
</thead>
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<tr>
<td>Unit-cell parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>73.1</td>
<td>225.4</td>
<td>59.9</td>
<td>73.9</td>
<td>123.1</td>
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<tr>
<td>b (Å)</td>
<td>133.1</td>
<td>128.0</td>
<td>71.2</td>
<td>122.1</td>
<td>83.8</td>
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<tr>
<td>c (Å)</td>
<td>62.6</td>
<td>62.6</td>
<td>130.6</td>
<td>124.2</td>
<td>123.1</td>
</tr>
<tr>
<td>β (°)</td>
<td>108.3</td>
<td>108.3</td>
<td>108.3</td>
<td>108.3</td>
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<td>Temperature (K)</td>
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<td>293</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>Oscillation range (°)</td>
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<td>1.5</td>
<td>0.4, 0.6, 1.2</td>
<td>1.2</td>
<td></td>
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<tr>
<td>Resolution (Å)</td>
<td>25.0–2.27</td>
<td>25–2.5</td>
<td>20.0–3.5</td>
<td>20–2.35</td>
<td>20–2.65</td>
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<tr>
<td>Total No. of reflections</td>
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<td>156.965</td>
<td>8767</td>
<td>47606</td>
<td>63384</td>
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<td>No. of unique reflections</td>
<td>26057</td>
<td>60001</td>
<td>4482</td>
<td>27104</td>
<td>22080</td>
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<tr>
<td>Completeness (%)</td>
<td>98.6 (87.3)</td>
<td>94.1 (92.8)</td>
<td>95.3 (92.4)</td>
<td>97.2 (97.7)</td>
<td>97.3 (96.4)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.098 (0.143)</td>
<td>0.094 (0.585)</td>
<td>0.149 (0.422)</td>
<td>0.110 (0.406)</td>
<td>0.109 (0.542)</td>
</tr>
<tr>
<td>R cryst</td>
<td>28.9 (6.2)</td>
<td>14.2 (2.3)</td>
<td>5.5 (2.1)</td>
<td>17.0 (1.9)</td>
<td>11.2 (2.2)</td>
</tr>
</tbody>
</table>
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, λ = 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 (Otwinski & Minor, 1997).

### Table 2

<table>
<thead>
<tr>
<th>Program</th>
<th>Search model</th>
<th>EPMR</th>
<th>AMoRe</th>
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<tr>
<td>Form I</td>
<td>Monomer A (PDB code 3eca)</td>
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<tr>
<td>Form V</td>
<td>Dimer AC (PDB code 3eca)</td>
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</tbody>
</table>

Space group C2  
P2₁2₁2₁  
P2₁2₁2₁  
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 Å³ Da⁻¹ and a solvent content of 38.4%. This precludes the possibility of the P2₁2₁2₁ 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 P2₁2₁2₁ or P2₁2₁2₂, 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 Å³ 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 P3₁2₁) 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 Å³ Da⁻¹ and a solvent content of 53.4% are obtained. The structure was solved by molecular replacement 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 P3₁2₁ 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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### References


