Crystallization of *Escherichia coli* Aspartyl-tRNA synthetase in its free state and in a complex with yeast tRNA^{Asp}

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Abstract

Overexpressed dimeric *E. coli* aspartyl-tRNA synthetase (AspRS) has been crystallized in its free state and complexed with yeast tRNA^{Asp}. Triclinic crystals of the enzyme alone ($a = 104.4$, $b = 107.4$, $c = 135.0$ Å, $\alpha = 102.9$, $\beta = 101.0$, $\gamma = 106.3°$), have been grown using ammonium sulfate as the precipitant and monoclinic crystals ($a = 127.1$, $b = 163.6$, $c = 140.1$ Å, $\beta = 111.7°$), space group C2, have been grown using polyethylene glycol 6000. They diffract to 2.8 and 3.0 Å, respectively. Crystals of the heterologous complex between *E. coli* AspRS and yeast tRNA have been obtained using ammonium sulfate as the precipitant and 2-propanol as the nucleation agent. They belong to the monoclinic space group $P2_1$ ($a = 76.2$, $b = 227.3$, $c = 82.3$ Å, $\beta = 111.7°$) and diffract to 2.7 Å.

1. Introduction

Aminoacyl-tRNA synthetases (aaRS's) catalyse the aminoacylation of their cognate tRNA molecules in a highly specific two-step reaction. These enzymes can be partitioned into two classes of ten members, each class being characterized by a different mode of ATP recognition associated with active-site domains of distinct topology (for review see Carter, 1993; Moras, 1992).

Aspartyl-tRNA synthetases (AspRS's) are class II enzymes and aminoacylate their cognate tRNA's on the 3' hydroxyl group of the terminal ribose (Eriani, Delarue, Poch, Gangloff & Moras, 1990). The crystal structures of yeast AspRS complexed with yeast tRNA^{Asp} (Cavarelli, Rees, Ruff, Thierry & Moras, 1993; Ruff *et al.*, 1991) and of *Thermus thermophilus* AspRS alone (Delarue *et al.*, 1994) confirm the modular organization of these enzymes already shown by sequence analyses and three-dimensional structures of other aaRS's.

In addition to the active-site domain, common to all class II aaRS's, two domains can be identified. An N-terminal domain, which is common to both eukaryotic and prokaryotic AspRS's, is involved in the specific recognition of the anticodon loop of tRNA^{Asp}. An additional domain of variable size, whose function is only partially understood, is inserted between two conserved motifs of the catalytic module.

Recently, major functional and structural differences have been observed between eukaryotic and prokaryotic AspRS's (Cavarelli *et al.*, 1994; Poterszman *et al.*, 1993). Yeast AspRS will efficiently aspartylate *E. coli* tRNA^{Asp}, but *E. coli* AspRS is almost completely inactive in the presence of yeast tRNA^{Asp} (Martin *et al.*, 1993). All prokaryotic AspRS's have been shown to possess a large additional domain absent in eukaryotic enzymes (Delarue *et al.*, 1994).

Structural data derived from the crystals presented here will complement our present knowledge on AspRS's since high-resolution diffracting crystals of *E. coli* AspRS complexed with *E. coli* tRNA^{Asp} and yeast AspRS complexed with yeast tRNA^{Asp} have already been obtained and their structures solved (Eiler *et al.*, 1992; Ruff *et al.*, 1991). Structures of the native enzyme alone, of the homologous complexes and of the poorly efficient heterologous complex with yeast tRNA^{Asp} are essential to elucidate why eukaryotic and prokaryotic aspartylation systems behave differently and to design molecules specifically recognizing prokaryotic AspRS's.

2. Experimental

2.1. Materials

For the production of *E. coli* AspRS, the *E. coli* strain JM83 was transformed with the plasmid pBluescript-*aspS* containing the *E. coli aspS* gene and its flanking regions (Eriani, Dirheimer & Gangloff, 1990). DEAE Sephacel was from Pharmacia-LKB and TSK HW65(S) from Merck. Yeast tRNA^{Asp} was purified from bulk tRNA isolated from the brewer's yeast *Saccharomyces cerevisiae* (Boehringer Mannheim) using counter-current separation technique and two chromatographic steps (Dock *et al.*, 1984): a DEAE column followed by an inverse ammonium sulfate gradient using Phenomenex Porex 5C$_4$ preparative scale high-pressure liquid-chromatography column.

2.2. Purification of aspartyl-tRNA synthetase

An overnight culture of 4 l of LB medium containing 200 µg ml^{-1} ampicilin yielded 20 g of cells (wet weight) which were suspended in 100 ml of a buffer solution containing 100 mM Tris–HCl pH 8, 10 mM MgCl$_2$ and 1 mM EDTA and then disrupted by sonication. After centrifugation (105 000g for 120 min), *E. coli* AspRS represented about 30% of the total amount of protein. The extract was loaded on a DEAE Sephacel column ($\varphi = 55$ mm, $h = 125$ mm), previously equilibrated with 20 mM potassium phosphate pH 7.5 at a flow rate of 1 ml min^{-1} and washed with one column volume of the equilibration buffer. The proteins were then eluted by a linear salt gradient of 1600 ml (20 to 250 mM potassium phosphate pH 7.5) at a flow rate of 2 ml min^{-1}. The fractions from the DEAE column showing AspRS activity (230 mg of total protein) were pooled and concentrated to 12.5 ml in a 50 mM Tris–HCl pH 7.5 buffer containing 1.6 M ammonium sulfate using an Amicon concentrator with a YM30 membrane. The concentrated protein solution was then loaded at a flow rate of 1 ml min^{-1} onto a Fractogel TSK HW 65(S) column ($\varphi = 26$ mm, $h = 320$ mm) equilibrated with a

buffer solution containing 50 mM Tris–HCl pH 7.5 and 2.4 M ammonium sulfate. The column was subsequently eluted at a flow rate of 2 ml min^{-1} with a linear reverse ammonium sulfate gradient from 2.4 to 0 M over 2400 ml. The AspRS fraction, eluted at 1.2 M ammonium sulfate, contained 120 mg of pure protein as judged from a highly loaded sodium dodecyl sulfate–polyacrylamide electrophoresis gel (SDS–PAGE). The enzyme is stored at 277 K after precipitation with 3 volumes of 3.6 M ammonium sulfate buffered at pH 7.5 with 200 mM Tris–H$_2$SO$_4$.

(a)

(b)

(c)

(d)

(e)

(f)

Fig. 1. Influence of NaCl concentration on the crystallization of *E. coli* AspRS using PEG 6000 as the precipitant. Results obtained from 240 assays by screening [AspRS], [PEG], [NaCl] and pH showed that [NaCl] was a sensitive parameter. The pictures show typical results obtained at pH 7 with fixed [AspRS] (50 mg ml^{-1}), [PEG] (16%) and various [NaCl]: (a) [NaCl] = 100–400 mM, (b) [NaCl] = 200–500 mM, (c) [NaCl] = 400–800 mM, (d) [NaCl] = 600–1200 mM, (e) [NaCl] = 1000–1600 mM, (f) [NaCl] = 1400–2000 mM.

2.3. *Crystallization and crystal analysis*

Crystallization experiments were performed using the hanging-drop vapour-diffusion technique, at 290 K in Linbro plates. Preliminary X-ray data were collected at 273 K either on a Siemens area detector using a Rigaku rotating anode ($\lambda = 1.54$ Å) or on an MAR Research imaging plate using the W32 synchrotron beamline at LURE ($\lambda = 0.90$ Å). For X-ray studies performed at 113 K, crystals were mounted on a free-standing film of reservoir solution containing 20% glycerol (Teng, 1990). Data were collected on an MAR Research imaging plate using the X11 synchrotron beamline at DESY ($\lambda = 0.93$ Å). Cell parameters were determined and refined using the *MARXDS* program system (Kabsch, 1988).

3. Results and discussion

3.1. *Crystallization and preliminary crystallographic analysis of free E. coli AspRS*

Conventional precipitants alone, such as MPD, PEG or ammonium sulfate, at different concentrations under a broad range of pH, temperature and protein concentrations did not yield any crystals. Various additives were then tried in combination with the previously tested precipitants, two of which promoted nucleation: 2-propanol added to ammonium sulphate and NaCl added to PEG 6000. Varying the concentrations of both the precipitant and the additive led to two different sets of conditions yielding crystals suitable for X-ray investigation.

(i) Crystals with dimensions $200 \times 400 \times 1200$ µm were first obtained at 277 K by equilibrating 20 µl drops containing 5 mg ml^{-1} protein in 50 mM Bis Tris propane pH 7 and 1.2 M ammonium sulfate against 400 µl reservoirs containing 1.8 M ammonium sulfate, 75 mM Bis Tris propane pH 7 and 1% isopropanol. Crystals grew over a period of 3 to 5 months.

(ii) Crystals have also been obtained in PEG 6000 at 288 K. As illustrated in Fig. 1, NaCl is crucial for the crystallization of *E. coli* AspRS: in combination with PEG 6000 its concentration influences both the nature and size of the objects obtained in the drops. Single crystals suitable for X-ray diffraction studies were obtained by equilibrating 10 µl drops containing 20 mg ml^{-1} protein 5.0% PEG 6000, 530 mM NaCl and 33 mM Bis Tris pH 7 buffer against 400 ml of a solution containing 100 mM Bis Tris pH 7, 16% PEG 6000 and 1.6 M NaCl. Under such conditions, 1 to 5 crystals with maximum dimensions $400 \times 800 \times 1600$ µm were observed within 10 to 20 d.

AspRS from dissolved crystals obtained under both conditions exhibits the same catalytic efficiency and mobility on SDS–PAGE as the protein that has not been crystallized. Although diffraction patterns from crystals obtained in either ammonium sulfate or PEG 6000 are visually indistinguishable, they belong to different space groups.

Crystals grown in ammonium sulfate are triclinic with cell parameters $a = 104.4$, $b = 107.4$, $c = 135.0$ Å, $\alpha = 102.9$, $\beta = 101.0$, $\gamma = 106.3°$ and they diffract to 2.8 Å resolution (Fig. 2).

Crystals grown in PEG 6000 belong to the monoclinic space group $C2$, with cell parameters $a = 127.1$, $b = 163.6$, $c = 140.1$ Å, $\beta = 111.7$ and diffract to 3.0 Å. This unit cell can be transformed into a triclinic primitive cell with parameters $a = 103.7$, $b = 103.9$, $c = 140.0$ Å, $\alpha = 103.1$, $\beta = 103.1$ and $\gamma = 104.5°$ which are close to those observed for the $P1$

crystal form. This observation suggests that the crystal packing is similar in the two crystal forms with three monomers (3×66 kDa) in the asymmetric unit for crystals belonging to the monoclinic form (a dimeric molecule in the general position and one on the twofold axis) and three dimers (3×66 kDa) in the triclinic cell. The V_m values are 3.42 and 3.44 Å^{3}Da^{-1} respectively and are close to the V_m value obtained in the case of the crystals of AspRS from *T. thermophilus* (Poterszman *et al.*, 1993). The number of monomers in the asymmetric units of both crystal forms has recently been confirmed by a molecular-replacement search using *T. thermophilus* AspRS as the model structure (results not shown).

3.2. *Crystallization and preliminary crystallographic analysis of the eterologous complex E. coli AspRS–yeast tRNA^{Asp}*

Microcrystals of the heterologous complex between *E. coli* AspRS and yeast tRNA^{Asp} were obtained at 293 K with 2.5 molecules of tRNA per molecule of AspRS dimer using ammonium sulfate as the precipitant and 2-propanol to trigger nucleation.

Single crystals suitable for X-ray analysis were grown by macroseeding using the microcrystals. 10 µl hanging drops containing 2 mg ml^{-1} AspRS, 1 mg ml^{-1} tRNA, 1 mM MgCl$_2$, 1.5 M ammonium sulfate, 75 mM Bis Tris propane pH 6.7, 0.5 mM AMP–PCP and 1 mM L-aspartic acid were equilibrated against reservoirs containing 1.9 M ammonium sulfate, 100 mM Bis Tris propane pH 6.7 and 1 to 3%(v/v) 2-propanol. After one week, reservoir solutions are replaced with identical solutions without 2-propanol and the drops are seeded with 10 to 30 µm single crystals washed in 2.4 M ammonium sulfate reservoir solution in order to
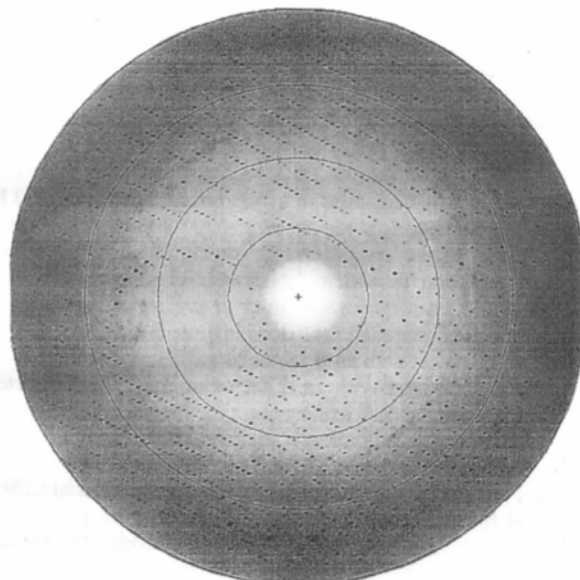

Fig. 2. A screenless rotation image from a crystal of *E. coli* AspRS grown in ammonium sulfate, recorded on an MAR Research imaging plate using the W32 beamline of LURE. The oscillation range was 1.0° and the exposure time 180 s. The crystal-to-film distance was 270 mm, giving a resolution limit of 2.8 Å at the edge of the photograph. Circles corresponding to a resolution of 11.2, 5.6 and 3.7 Å are drawn.

Fig. 3. A screenless rotation photograph from a crystal of the heterologous complex of *E. coli* AspRS and yeast tRNA^{Asp} obtained in ammonium sulfate, recorded at 113 K on an MAR Research imaging plate using the X11 beamline of DESY. The oscillation range was 0.5° and the exposure time 360 s. The crystal-to-film distance was 400 mm, giving a resolution limit of 2.6 Å at the edge of the photograph.

at 113 K. At this temperature it was possible to collect data on the same crystal to a resolution better than 2.7 Å for more than 36 h without significant decay (see Fig. 3). Crystals belong to the monoclinic space group $P2_1$ with cell parameters $a = 76.2$, $b = 227.3$, $c = 82.3$ Å and $\beta = 111.7°$. Assuming that each asymmetric unit contains one AspRS dimer (2×66 kDa) and two tRNA molecules (2×25 kDa), the specific volume (V_m) is 3.66 Å^{3}Da^{-1}, a value well in the range of those already observed for AspRS–tRNA^{Asp} complexes already crystallized (Eiler *et al.*, 1992; Ruff *et al.*, 1988). The width of the rocking curve remains within the usually observed limits. It changes from 0.20° for crystals at room temperature to 0.45° after flash freezing, so the increase in mosaic spread is not a problem for data collection. The rapid disruption of the crystal in presence of 20% glycerol at 273 K suggests that the improved diffraction is correlated with the lower temperature and not with the solvent modification introduced prior to freezing.

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eliminate microseeds. Growth of the crystals is controlled by increasing the ammonium sulfate concentration in the reservoirs to 2.2 *M* by 50 m*M* steps over two weeks; the crystals thus attained a size of about $250 \times 350 \times 700$ μm. The presence of both AspRS and tRNA was checked by SDS and 8 *M* urea/polyacrylamide gel electrophoresis as well as aminoacylation tests. These tests were performed both on crystals dissolved in 20 m*M* Tris–HCl pH 7.5 after several washes in a 2.4 *M* ammonium sulfate reservoir solution and on the last washing solution as control.

The crystals diffract to 3.0 Å resolution and decay after a few minutes when exposed to synchrotron radiation at 273 K. Flash-freezing and low-temperature data collection increased the lifetime of the crystals in the synchrotron beam as well as the resolution to which data could be collected. As will be published elsewhere (Moulinier *et al.*, in preparation), at 273 K it was possible, all reflexions included, to collect a 3.2 Å resolution data set with an *R* factor of 8.0% using five crystals. At 110 K, a data set with an *R* factor of 3.2% was collected to a resolution of 2.7 Å using only one crystal. Though rather exceptional, such an increase in diffraction limit has been observed in other systems (Rodgers, 1994). The crystals were quickly transferred to a reservoir solution containing 20% glycerol for 30 s, mounted in loop made from very fine fiber of polyamide and then flash-frozen in a nitrogen gas stream