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Effects of macromolecular impurities and of crystallization method on the quality of eubacterial aspartyl-tRNA synthetase crystals

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Although macromolecular purity is thought to be essential for the growth of flawless protein crystals, only a few studies have investigated how contaminants alter the crystallization process and crystal quality. Likewise, the outcome of a crystallization process may vary with the crystallization method. Here, it is reported how these two variables affect the crystallogenesis of aspartyl-tRNA synthetase from the eubacterium *Thermus thermophilus*. This homodimeric enzyme (M_r = 130 000) possesses a multi-domain architecture and crystallizes either in a monoclinic or an orthorhombic habit. Minute amounts of protein impurities alter to a different extent the growth of each crystal form. The best synthetase crystals are only obtained when the crystallizing solution is either enclosed in capillaries or immobilized in agarose gel. In these two environments convection is reduced with regard to that existing in an unconstrained solution.

1. Introduction

Frequently single protein crystals of adequate size and of optimal quality for X-ray analysis are difficult to grow. Despite novel methods such as the automated search for crystallization conditions in nano-droplets, crystal quality must generally be optimized before a detailed crystallographic analysis can be undertaken. To reach this goal, growth parameters must be varied to modulate the quality of the crystal habit. Apart from the cases of a few small proteins (see articles published in the proceedings of previous ICCBMs), the individual contributions of most physical and chemical variables are not well understood because of a lack of experimental data (Ducruix & Giegé, 1999; McPherson, 1999).

Purity is the first variable that is essential to obtain good crystals (*e.g.* Giegé *et al.*, 1986; Lorber *et al.*, 1987; Skouri *et al.*, 1995; Rosenberger *et al.*, 1996). Macromolecular contaminants and micro-heterogeneities that are present within a protein batch poison the faces of growing crystals (*e.g.* Anderson *et al.*, 1988; van der Laan *et al.*, 1989; Vekilov & Rosenberger, 1996) and alter the crystal packing (Sauter *et al.*, 2001). Reduced convection is another variable that is thought to produce crystals of superior quality. For instance, beneficial effects have been attributed to agarose and silica gels (Robert & Lefaucheux, 1988; Lorber *et al.*, 1999; Biertümpfel *et al.*, 2002; Sauter *et al.*, 2002; Robert *et al.*, 2003) and to capillary forces (García-Ruiz, 2003; Ng *et al.*, 2003).

Here we report relevant observations made with aspartyl-tRNA synthetase (DRS-1) from the eubacterium *Thermus thermophilus*. The architecture of this homodimeric enzyme (M_r = 130 000) encompasses several domains that are indispensable for the catalysis of $tRNA^{Asp}$ esterification by aspartic acid during protein biosynthesis (Giegé & Rees, 2005). Two crystal forms of the free enzyme are known that are distinguished by their diffraction properties. An orthorhombic form grows in an ammonium sulfate solution (Ng *et al.*, 1996) and a monoclinic one in a polyethylene glycol (PEG) solution that contains agarose gel (Zhu *et al.*, 2001). These crystals have led to structure models at 2.0 Å (Ng *et al.*, 2002) and 2.65 Å resolution (Charron *et al.*, 2001), respectively. Afterwards, the contacts in both packings were compared (Charron *et al.*, 2001) and mutants engineered (Charron *et al.*, 2002). In the present study, comparative experiments on two batches of DRS-1 give an insight into how protein purity influences crystallization and crystal quality. The

analysis of crystals prepared either in solutions or in gels equilibrated by vapor diffusion and in solutions contained in capillary tubes equilibrated by gel acupuncture indicates that best crystallographic quality is reached when convection is low.

2. Materials and methods

2.1. Protein preparation and characterization

T. thermophilus DRS-1 was overproduced in *Escherichia coli* and purified according the original protocol of Poterszman *et al.* (1993) with slight modifications. The partially purified extract from 40 g of cells was first fractionated on an anion-exchange column (TSK-gel DEAE-5PW, Tosohaas). Fractions containing DRS-1 activity were pooled and applied onto a hydroxyapatite column (CHT Ceramic hydroxyapatite, Biorad). The synthetase elutes as a single activity peak and is pure according to standard criteria. In the following, the pooled proteins from this activity peak will be termed 'batch P' (P for pure). Additional chromatographies on an ion-exchange column (UnoQ, Biorad) and on a size-exclusion column (Bio-Prep SE 100/17, Biorad) remove less than 1% of the protein material as seen after polyacrylamide gel electrophoresis (PAGE) performed under denaturing conditions in the presence of sodium dodecyl sulfate. This batch will be termed 'batch HP' (HP for highly pure). Protein concentration was determined from UV absorbance assuming the extinction coefficient $E_{280} = 1.0\ \mathrm{l\ g^{-1}\ cm^{-1}}$ when $A_{280}/A_{260} > 1.5$.

2.2. Biochemical characterization of protein impurities

In order to analyze the protein content of DRS-1 crystals by PAGE, the latter were withdrawn from mother liquor, washed with protein-free precipitant solution, dissolved in a small volume of 100 m*M* Tris–HCl pH 7.2 (orthorhombic crystals) or pH 7.8 (monoclinic crystals) and filtered on membranes with a porosity of 0.22 µm (UltrafreeMC, catalog No. UFC 30GV00, Millipore). The polypeptides revealed after staining with Coomassie Blue were transferred onto a polyvinylidene fluoride membrane prior to N-terminal sequencing by the Edman degradation method in an automated sequencer (model 492 ProciseCLC, Perkin–Elmer Applied Biosystems). The amino-acid sequences were compared to those in the SwissProt database (http://www.expasy.org) using the *BLAST* algorithm (Altschul *et al.*, 1997) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence alignments were performed with *LALIGN* (http://www.ch.embnet.org).

2.3. Crystallization of DRS-1

All solutions were made with ultrapure water and filtered on 0.22 µm porosity Millex filters (Millipore). A 2%(*w*/*v*) solution of low-gelling point agarose (T_{gel} = 301 K, So.Bi.Gel, Hendaye, France) was prepared and filtered to remove dust and insoluble particles. Protein samples were filtered on UltrafreeMC membranes. Crystallization was conducted at 293 K either by vapor diffusion inside Linbro plates or in capillaries using the set-up of the gel-acupuncture method (García-Ruiz & Moreno, 1994).

In hanging drops, DRS-1 was at 20 mg $\mathrm{ml^{-1}}$ in 0.1 *M* Tris–HCl, 10 m*M* $MgCl_2$ and 0.5 m*M* dithioerythritol at pH 7.2 (Ng *et al.*, 1996). Drops were made by mixing 2–6 ml DRS-1 solution with the same volume of precipitant solution. The appropriate volume of agarose stock solution was added to reach a final concentration of 0.2%(*w*/*v*). In controls, agarose was replaced by water. Drops were equilibrated over 750 µl reservoirs containing either 4 *M* sodium formate or 10%(*v*/*v*) PEG 8000 to grow orthorhombic and monoclinic crystals, respectively. The respective crystals were obtained after 14 and 7 d in solution and 20–30 d in gel.

In the gel-acupuncture method (GAME), protein solutions were filled in 50 mm long glass capillary tubes of <0.5 mm inner diameter cut from X-ray diffraction capillaries (Glas, Berlin, Germany). One end of the tube was closed with Plasticine and the open end was stuck vertically (at a depth of 5 or 10 mm) into a layer of silica gel that was contained in the bottom of a Granada Crystallization Box (García-Ruiz *et al.*, 2002). The silica gel was prepared by adjusting a sodium silicate solution having a density of 1.1 g $\mathrm{cm^{-3}}$ to pH 6.5 with acetic acid. The precipitant solution (that was twice as concentrated as in the reservoirs of the vapor diffusion plates) was then poured over the surface of the gel. Monoclinic and orthorhombic crystals were obtained after 60 and 45 d, respectively.

2.4. X-ray diffraction analysis

For the purpose of this comparative study, a standard data-collection procedure was adopted. Prior to data collection under cryogenic conditions, all crystals of similar dimensions were soaked for 45–60 s in precipitant solution containing 30%(*v*/*v*) glycerol, mounted in nylon loops (Hampton Research) and flash-frozen in liquid ethane. Preliminary crystal characterization was performed in-house on a Nonius diffractometer equipped with a rotating copper anode generator (operated at 45 kV, 90 mA) and a DIP 200 MacScience image plate. Sets of 72 frames (λ = 1.534 Å, 1° oscillation, crystal-to-detector distance 150 mm) were recorded. Further X-ray analysis was carried out on beamline BM30 (ESRF, Grenoble, France) equipped with a MAR CCD detector (MAR Research, Hamburg, Germany). For each crystal grown by a different method two data sets of 240 frames (0.5° oscillation, λ = 1.005 Å, crystal-to-detector distance of 190 mm) were collected at 2.5 Å resolution. The data were indexed and reduced using the *HKL* Package (Otwinowski & Minor, 1997). Further data manipulation [structure-factor calculations, $I/\sigma(I)$ and Wilson plot analysis] was performed using *TRUNCATE* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

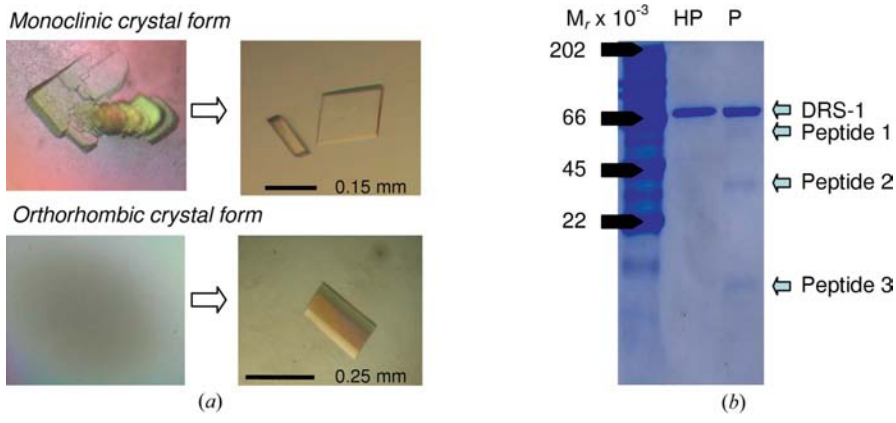

Figure 1
Characterization of protein impurities and their effects on DRS-1 crystallization. (*a*) Effect of purification on the growth of the monoclinic and orthorhombic crystal forms of DRS-1. (*b*) Electrophoretic analysis of the two DRS-1 batches (H and HP). The first lane shows the M_r markers, the second lane the purest DRS batch (HP) and the third one the less pure batch containing three minor polypeptides (P).

3. Results and discussion

3.1. How protein impurities alter the crystallizability of DRS-1

Pure DRS-1 crystallizes either in an orthorhombic or a monoclinic crystal form according to solvent composition (Zhu *et al.*, 2001; Charron *et al.*, 2002). The same crystals can be prepared in the presence of a low concentration of agarose gel (Fig. 1*a*). When crystallization assays were prepared with batch P of DRS-1 (that was purified according to the simplified protocol), an amorphous precipitate was obtained instead of orthorhombic crystals and monoclinic crystals had the shape of dendrites (Fig. 1*a*). Electrophoresis under denaturing conditions revealed that this batch actually contains next to the synthetase subunit (M_r = 65000), three minor polypeptides with relatives masses of ~55000, 30000, and 15000 that represent altogether <1% of the total protein material (Fig. 1*b*).

Once these contaminants were removed by two additional chromatographic steps, DRS-1 batch HP crystallized as reported originally. Further, when droplets were set up with batch HP contaminated on purpose with batch P in a 1:1 ratio, only dendrites and ill-shaped polymorphs could be obtained under the conditions where the most pure DRS (batch HP) yielded monoclinic crystals. On the other hand, no crystals were obtained in contaminated droplets under the conditions producing the orthorhombic form and under conditions mixing batch HP with batch P in a ratio 0.5:1, while controls prepared with purest protein gave well shaped single crystals.

A biochemical analysis of the dendrite-like crystals indicated that they contain the three contaminants that are present in batch P. N-terminal sequencing of the contaminants yielded the following amino-acid sequences: RGNYDANIV for the peptide with $M_r \simeq$ 55 000, MNEQLNQQNN for the one with $M_r \simeq$ 30 000, and ENINYFG-SLRI for that with $M_r \simeq$ 15 000. Sequence comparison suggested that the latter peptide might be a fragment of DNA translocase ftsK from *E. coli* (SwissProt code Q8X5H9), a protein encompassing 1342 residues accounting for an M_r of 148 098. This sequence varies slightly from one bacterial strain to another (codes Q8FJC7 and P46889).

Altogether, these experiments show that protein impurities alter in a different way the nucleation and/or the growth of the well facetted orthorhombic and monoclinic DRS-1 crystals. In the first case, presumably nucleation and growth were both inhibited since no crystals could form in a range of impurity concentrations. In the second one, the impurities were incorporated in the same proportion as in the initial DRS sample.

These results share similarities with observations reported for other proteins. Traces of protein impurities have been shown to cause heterogeneous and secondary nucleation, lattice defects, including vacancies, strain, stress in selected growth sectors (Caylor *et al.*, 1999; Kurihara *et al.*, 1999; Yau *et al.*, 2001), breakage into mosaic blocks (Yau *et al.*, 2001), alteration of facet morphology (Vekilov *et al.*, 1995) and of unit-cell parameters (Hirschler & Fontecilla-Camps, 1996; Bhamidi *et al.*, 1999), modification of crystal habit (Chernov *et al.*, 2004), or termination of growth (Plomp *et al.*, 2003). Depending on the degree of relationship and on the affinity, adsorption, attachment and subsequent incorporation of impurities can be detectable either at a macroscopic or at a microscopic scale. While morphological changes are usually visible with the help of a low magnification microscope, lattice defects require a detailed analysis by more sophisticated methods such as atomic force microscopy (*e.g.* McPherson *et al.*, 1996), interferometry (*e.g.* Vekilov & Rosenberger, 1996) or X-ray topography (*e.g.* Robert *et al.*, 2001). Structural biologists should be aware that well shaped single crystals yielding a poor diffraction pattern (containing deformed or split diffraction spots or characterized by a high mosaicity or a low diffraction limit) may be a sign for a presence of impurities. One remedy for such a situation is to reconsider the purification protocol. A good example was the crystallization of tryptophanyl-tRNA synthetase from *Bacillus stearothermophilus* over expressed in *E. coli*. To obtain crystals of this protein, it was necessary to delete the homologous *trp* gene from the *E. coli* host cells so that to prevent contamination of the *B. stearothermophilus* synthetase by the closely related *E. coli* enzyme (Carter, 1988).

Table 1
Data-collection statistics for orthorhombic DRS-1 crystals grown by three different methods.

The crystallization methods were (i) classic vapor diffusion (Solution), (ii) gel acupuncture method with the protein solution enclosed in capillary tubes (GAME), and (iii) vapor diffusion in droplets to which agarose gel was added (Gel). For each method, two crystals have been analyzed in a strictly comparative manner.

	Solution		GAME		Gel	
Crystals	*a*	*b*	*c*	*d*	*e*	*f*
Crystallization temperature (K)	293					
Data-collection temperature (K)	100					
Space group	$P2_12_12_1$					
Unit-cell parameters (Å)						
a	60.3	60.2	60.3	60.2	60.2	60.2
b	154.9	154.7	154.9	154.9	154.8	154.8
c	174.1	174.5	174.2	173.8	173.7	173.7
Solvent content			45%			
Apparent mosaicity† (°)	0.34	0.91	0.38	0.66	0.29	0.38
Resolution range (Å)	30–2.5					
No. of observations	262449	178235	259881	195278	266122	259312
No. of reflections	55520	53830	50476	47007	55906	53079
Completeness (%)	96.8	93.8	88.2	82.2	97.8	92.8
R_{merge} (%)	5.3	4.5	4.0	3.7	3.6	6.3
$I/\sigma(I)$	18.2	19.2	26.0	23.4	27.2	16.5
High-resolution shell (Å)	2.56–2.5					
Completeness (%)	98.6	82.3	76.7	60.4	98.8	92.2
R_{merge} (%)	22.2	15.6	17.0	12.9	14.0	25.5
$I/\sigma(I)$	3.2	3.9	5.7	4.8	5.5	2.8
Average *B* factor‡ (Å^2)	43	45	47	48	48	48

† Estimated from *HKL* package. ‡ From Wilson plot.

3.2. How the crystallization method influences crystal quality

Crystals prepared with the purest batch of DRS-1 (batch HP) have served to investigate the effect triggered by the crystallization method on the diffraction properties of the crystals. For comparison, X-ray diffraction data sets have been collected on crystals of similar dimensions (length ~200 µm, thickness ~50 µm) that were grown by vapor diffusion either in free or gelled solutions as well as by GAME in solutions enclosed in capillary tubes. Standard procedures for the acquisition and the processing of these data have led to the statistics in Table 1. Differences in completeness are due to the non-optimal orientation of the crystals in the cryoloops and to the limited oscillation angle. The $I/\sigma(I)$ plots for the best crystal of each series are displayed in Fig. 2. Altogether, the results indicate that crystals grown in gel or in capillaries can be less mosaic and give a more intense diffraction than those prepared in solution. Moreover, the quality of the crystals that are prepared in the gel seems to be more reproducible than that of crystals prepared in solution within thin capillaries. The same trend was observed with the crystals that had been analyzed independently on an in-house X-ray generator (result not shown).

More than a decade ago, it was reported that horse serum albumin crystals of superior size and quality can be grown by limiting solutal convection either in a solution placed under microgravity or in an agarose gel under earth gravity (Miller *et al.*, 1992). Afterwards, other advantages of the gels have been recognized: crystals of small model

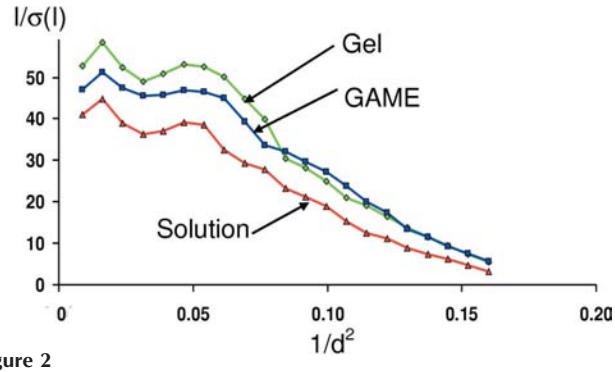

Figure 2
Comparison of $I/\sigma(I)$ plots of orthorhombic DRS-1 crystals prepared by three crystallization methods. All crystals were grown from the purest protein batch (HP) and had similar dimensions. For each method, only the data corresponding to the better of two crystals (of each series listed in Table 1) is represented. For experimental details, see §2.

proteins grown in agarose gel were always better than reference crystals grown in pure solution. The habits of the former can develop in three-dimensions and are well facetted because they are trapped in the gel network and cannot sediment. As a consequence, they have excellent optical properties and their lattices have fewer defects as indicated by their smaller mosaicity (Lorber *et al.*, 1999; Vidal *et al.*, 1999). In another instance growth in a gel suppressed twinning (Sica *et al.*, 1994). All these improvements are attributed to the facts that (i) mass transport is essentially controlled by diffusion within the gel and (ii) the protein-concentration gradient (or depletion zone) that is generated during the growth phase of each crystal is not disturbed by density-driven solutal flow or sedimentation. Despite the large molecular mass and the multi-domain architecture of DRS-1, the enhanced properties of its crystals confirm previous observations on small M_r proteins (mainly lysozyme) and demonstrate that the approach is of general interest.

3.3. Concluding remarks and perspectives

Experiments with DRS-1 from *T. thermophilus* illustrate that crystals of high quality that are useful for structural biology can only be achieved with highly pure protein. Furthermore, better crystals can be grown in a dilute agarose gel or in a capillary in which mass transport by convection is minimized as under microgravity (Thomas *et al.*, 2000). Many macromolecular crystals prepared in solution that are of insufficient quality for a crystallographic analysis could probably benefit from being prepared in gel or by the gel-acupuncture method.

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