Journal of
Applied Crystallography
ISSN 0021-8898
Editor: **Anke R. Pyzalla**

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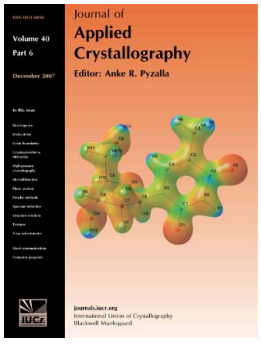
J. Appl. Cryst. (2009). **42**, 279–283



Journal of
Applied Crystallography
ISSN 0021-8898

Received 4 December 2008
Accepted 28 January 2009

Agarose gel facilitates enzyme crystal soaking with a ligand analog

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Orthorhombic crystals of the enzyme aspartyl-tRNA synthetase (AspRS) were prepared in agarose gel, a chemical alternative to microgravity or nano-volume drops. Besides providing a convection-free medium, the network of the polysaccharide improved the stability of the crystalline lattice during soaking with L-aspartol adenylate, a synthetic and non-hydrolysable analog of the catalytic intermediate aspartyl adenylate. When crystals were embedded in the polysaccharide matrix the ligand reached their surfaces more uniformly. Gel-grown crystals exhibited well defined reflections even at high resolution and low mosaicity values, despite their fairly high solvent content and the relatively harsh flash cooling procedure. By contrast, soaked AspRS crystals prepared in solution broke apart within 10–30 s after the ligand was introduced into the mother liquor, and subsequently these fragments became an amorphous precipitate. A general objection to the use of gels in protein crystallization is that chemical interactions may occur between the polysaccharide matrix and proteins or ligands. The example of AspRS shows that this is not a major concern. This method may be generally applicable to crystal soaking with other small molecules or heavy atoms.

1. Introduction

Convection in solution can be avoided by placing liquid samples in an unusual environment, such as weightlessness or a strong magnetic field (see *e.g.* Meyer *et al.*, 2008; Poodt *et al.*, 2006, respectively). Another way is to reduce at least one dimension of the container to less than 100 µm (see *e.g.* Tabeling, 2005). The latter situation exists inside very thin capillary tubes, nano-volume drops or microfluidic channels (see *e.g.* Sauter *et al.*, 2007). An alternative to these physical approaches consists in adding to the solution a chemical or biochemical compound that forms a gel (Gonzalez-Ramirez *et al.*, 2008).

Protein crystallization inside the hydrogel network formed by the algal polysaccharide agarose has been shown to have many advantages over that in pure solution. For instance, the loose carbohydrate mesh existing at low agarose gel concentration favors the growth of well ordered crystals that produce X-ray diffraction patterns with sharper and more intense reflections (*e.g.* Robert & Lefaucheux, 1988; Lorber, Sauter, Ng *et al.*, 1999; Moreno *et al.*, 2005).

Here we report on a novel application of an agarose gel property that was used in the course of the structural study of the catalytic site of the bacterial-type aspartyl-tRNA synthetase (AspRS-1) from *Thermus thermophilus*. AspRS-1 coexists in the same bacterium with an archaeal-type enzyme, AspRS-2 (Charron *et al.*, 2003). Whereas the former specifically charges aspartic acid onto the 3′ end of $tRNA^{Asp}$, AspRS-2 recognizes also $tRNA^{Asn}$ as a substrate. In both cases, tRNA aspartylation is a two-step reaction that involves the formation of the unstable intermediate aspartyl adenylate (Fig. 1).

The aim of this study was to prepare an isomorphous derivative by soaking crystals with L-aspartol adenylate, a synthetic and non-hydrolyzable analog of aspartyl adenylate. In the absence of the gel, the rapid entry of the small ligand into the crystalline lattice disrupted the latter and led to complete fracture. Conversely, crystals prepared in 0.2%(*m*/*v*) agarose gel could be soaked without damage and were suitable for structure determination at atomic resolution. The advantages of crystallizing enzymes in gels are discussed.

2. Materials and methods

2.1. Protein and chemicals

AspRS-1 (a heterodimer with subunit Mr 66030, Swissprot sequence code number Q5SKD2) was overproduced in

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Escherichia coli and purified as reported by Ng *et al.* (2002). L-Aspartol adenylate was synthesized by condensation of 2′,3,-isopropylideneadenosine with the L-aspartol derivative *t*-Bu-O_2C-CH_2-CH(NHBoc)CH_2OH and purified as reported by Bernier *et al.* (2005). Agarose with a gelling temperature of 301 K was obtained from SO.BI.GEL (Hendaye, France). A 6 *M* sodium formate stock solution was prepared by adjusting the pH of a formic acid solution with sodium hydroxide. All solutions that were in contact with the protein were prepared with ultrapure and sterile water (Cooper, France) and filtered over Millex or Ultra-Free membranes with a porosity of 0.22 µm (Millipore).

2.2. AspRS crystallization and crystal soaking with inhibitor

Orthorhombic AspRS-1 crystals were grown at 293 K in a range of conditions. Hanging drops were prepared in Linbro plates by mixing 2–6 µl of a protein solution at 8–20 mg ml^{-1} in 10 m*M* magnesium chloride, 0.5 m*M* dithiothreitol and 100 m*M* Tris–HCl at pH 7.2 with the same volume of reservoir solution. They were equilibrated over 750 µl reservoirs containing 4 *M* sodium formate at 293 K. Agarose powder was mixed with water and heated to 363 K to prepare a 2%(*m*/*v*) solution. This solution was then cooled to 308 K before it was added to the drops at a final concentration of 0.2%(*m*/*v*). The gel was absent in controls. For comparative analyses crystals prepared at the same protein and precipitant concentrations and with a length of about 400 µm were treated in parallel. They were soaked by adding to gelified drops 1 µl aliquots of mother liquor containing 5 m*M* L-aspartol adenylate until a concentration of 2 m*M* was reached in the mother liquor after 14 (1) d.

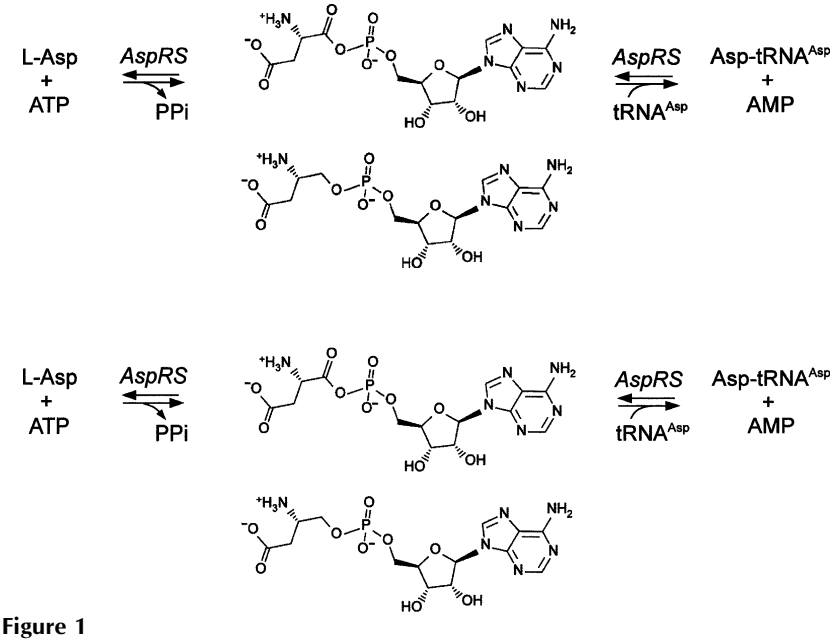

Figure 1
Schematic view of the tRNA aspartylation. (Top) Two-step reaction occurring in AspRS active site with the catalytic intermediate aspartyl adenylate shown in the center. This mechanism is common to all aminoacyl-tRNA synthetases. (Bottom) Chemical formula of L-aspartol adenylate, the non-hydrolysable analog of the natural intermediate used in these investigations.

2.3. Crystallographic analyses

Prior to flash freezing in liquid ethane, crystals were transferred in cryo-protectant solution composed of 4 *M* sodium formate, 100 m*M* Tris–HCl pH 7.2 and 30%(*v*/*v*) glycerol. X-ray diffraction analyses were performed on beamline ID14-1 at the ESRF (Grenoble, France). Sets of 260 and 270 images were collected from two single crystals (labeled C1 and C2 in Table 1) on an ADSC Q4 detector (wavelength 0.934 Å, oscillation angle 0.5°, 10 s exposure, detector distance 235 and 210 mm, respectively). 50 supplementary images were collected on the second crystal with an oscillation angle of 3°, an exposure of 10 s, and a detector distance of 386 mm. Data were processed with *XDS* and *XSCALE* (Kabsch, 2001), and indexed intensities were converted to structure factors using *TRUNCATE* from the *CCP4* package (Collaborative Computational Project, Number 4, 1994). The structure of AspRS-1 at a resolution of 2.0 Å (Protein Data Bank code 1l0w; Ng *et al.*, 2002) served as a model for molecular replacement using *PHENIX* (Adams *et al.*, 2002).

3. Results and discussion

3.1. Crystal derivatization with small substrates

In the study of catalytic pathways, small molecules that are substrates are frequently added in co-crystallization assays or soaked into pre-existing enzyme crystals (Hassell *et al.*, 2007). Aminoacyl-tRNA synthetases activate amino acids using adenosine triphosphate (ATP) before transferring the amino acid unit onto the 3′ end of tRNAs. ATP and amino acid are common additives used in crystallization assays [see the review by Giegé *et al.* (2008)]. The reaction intermediate adenylate generated *in situ* can be visualized inside the catalytic cleft (*e.g.* Schmitt *et al.*, 1998; Retailleau *et al.*, 2001). Since this chemical entity is highly reactive and unstable, non-hydrolyzable analogs have been synthesized to facilitate the derivatization of crystals of seryl-tRNA synthetase (Belrhali *et al.*, 1994). Nowadays such compounds replace the natural substrates.

When the crystallization medium or the mother liquor in which pure enzyme crystals have grown is supplemented with small substrates for co-crystallization or soaking experiments, these compounds are not always found in the electron density or occupy only part of the sites. In the worst cases, as was observed for example with aminoacyl-tRNA synthetases, soaking with ligands leads to crystal dissolution or to stress and strain

Table 1
X-ray analysis of AspRS-1 crystals soaked with L-aspartol adenylate.

The labels C1 and C2 are explained in §2.3. Values in parentheses are for the highest-resolution shell. For comparison, the cell parameters of native AspRS-1 crystals analyzed at 100 K are a = 60.2, b = 154.5 and c = 173.7 Å.

Crystal	C1	C2
Beamline	ID14-1/ESRF	
Temperature (K)	100	
Wavelength (Å)	0.934	
Space group	$P2_12_12_1$	
Unit-cell parameters (Å)	a = 60.1, b = 154.6, c = 173.8	a = 60.1, b = 154.7, c = 173.6
Crystal mosaicity (°)	0.19	0.15
Resolution range (Å)	20–2.16 (2.21–2.16)	30–1.85 (1.9–1.85)
No. of observations	389 692	640 906
No. of unique reflections	83 783	122 782
Completeness (%)	94.8 (72.4)	93.2 (61.8)
Multiplicity	4.65 (2.6)	5.21 (2.0)
$R_{\rm merge}$† (%)	3.3 (15.5)	5.3 (41.3)
$R_{\rm meas}$ (%)	3.7 (18.3)	5.8 (54.1)
$\langle I/\sigma(I)\rangle$	26.9 (6.5)	18.6 (3.0)
B factor from Wilson plot (Å^2)	37.8	32.5
Matthews coefficient (Å^3 Da^{-1})	3.1	
Solvent content (%)	60	
Asymmetric unit content	1 dimer + 2 analogs	

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$ and $R_{\rm meas}$, the redundancy-independent indicator, as defined by Diederichs & Karplus (1997).

within the crystal lattice. The latter may either lead to a space-group transition (Berthet-Colominas *et al.*, 1998) or be deleterious to diffraction (Sherlin & Perona, 2003). Minor conformational changes (associated with ligand binding and amplified by the periodicity along the three axes of the crystal) may affect the packing and result in visible alterations like cracks propagating as the ligand enters the lattice (Geremia *et al.*, 2006). Co-crystallization is an alternative in which the enzyme–substrate complex is formed before crystal growth, but this approach may produce only native crystals or not be applicable at all (see *e.g.* Sauter, 1999; Sherlin & Perona, 2003).

In the peculiar case of AspRS-1, co-crystallization with L-aspartol adenylate failed to produce crystals and soaking damaged them. To overcome these difficulties, we have implemented a novel strategy in which the preformed enzyme crystal lattice is immobilized in a gel matrix before the ligand diffuses through it. This 'smooth soaking technique' (meaning without sudden, violent movements) relies on the fact that the macromolecular crystal is entirely embedded in the gel and that gel fibers are trapped inside its channels during the growth process (Gavira & García-Ruiz, 2002).

3.2. More stable protein crystal grown in agarose gel

AspRS-1 was first crystallized in an orthorhombic system which led to a native structure determination and to that of an adenylate-bound enzyme after soaking with aspartic acid and ATP (Delarue *et al.*, 1994; Poterszman *et al.*, 1994). Afterwards, a monoclinic crystal form was found in a different growth condition (Zhu *et al.*, 2001), but diffraction data could only be obtained under cryogenic conditions with crystals grown in the presence of a low concentration of agarose gel. This showed that the latter stabilized the crystal lattice and preserved its near-to-original properties upon freezing (Charron *et al.*, 2001).

In the present work, we wished to solve the structure of orthorhombic crystals of AspRS-1 having an analog of the catalytic intermediate in the active site. Since co-crystallization did not produce crystals and native crystals prepared in solution did not withstand the soaking step, native crystals were grown in the presence of 0.2%(*m*/*v*) agarose. Gel-grown crystals had exactly the same prismatic habit and exhibited the same birefringence in polarized light as crystals prepared in solution. The major difference was that crystals prepared in solution broke apart within 10–30 s after the ligand was introduced into the mother liquor (Fig. 2*a*). Furthermore, the crystal fragments were not stable; in a few minutes they were replaced by an amorphous precipitate. The crystalline state could not be recovered. This is in contrast to the situation reported in the case of lysozyme crystals subjected to a strong hypertonic shock (López-Jaramillo *et al.*, 2002). AspRS-1 crystal deterioration was independent of crystal size but

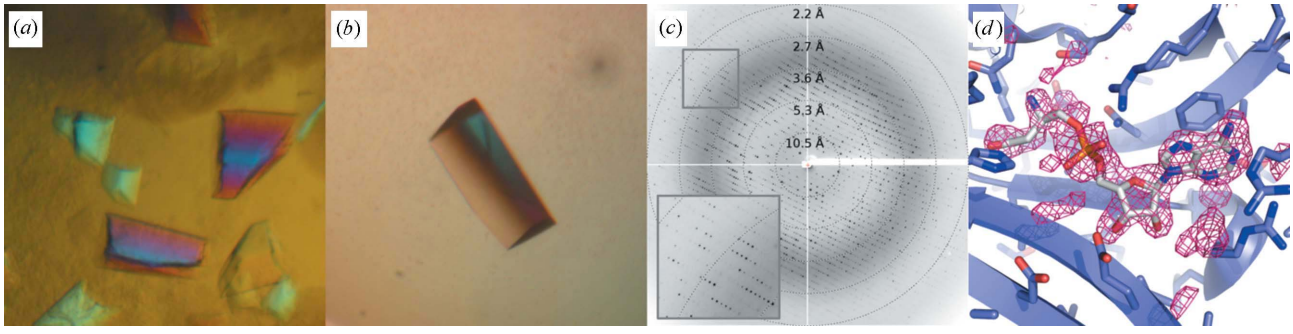

Figure 2
Soaking and X-ray analysis of AspRS-1 crystals grown in agarose gel. (*a*) Crystals prepared in solution broke into pieces shortly after the start of the soaking with L-aspartol adenylate. A few minutes later the fragments were replaced by a precipitate. (*b*) Intact crystals prepared in 0.2%(*m*/*v*) agarose after soaking. (*c*) Diffraction pattern of a crystal grown in gel and soaked with the substrate analog. The inset displays a close-up view of the diffraction pattern of crystal C2 in Table 1, with reflections extending beyond 2 Å resolution. (*d*) $F_{\rm obs} - F_{\rm calc}$ difference electron density map in the catalytic site of both monomers of AspRS-1 (contour level 3σ), computed before including the substrate in the model, confirming the presence of the analog. Additional peripheral density clearly indicates that several amino acid side chains undergo conformational changes upon substrate binding. The images in panels (*a*) and (*b*) are displayed on the same scale, and the crystal in panel (*b*) is about 400 µm long.

appeared to be controlled by transport phenomena. On the other hand, crystals prepared in agarose were stable during the entire soaking process, which took at least two weeks (Fig. 2*b*). It was noticed that the result was independent of the rate at which the concentration of analog was increased in the mother liquor.

Visual inspection under a binocular microscope revealed that the soaking step occurred differently when the drop containing the crystals was a solution or a hydrogel. In the first case, the addition of the ligand was accompanied by turbulences and convectional flow in solution, visible as variations of refractive index and minor liquid movements. One can anticipate that, in such a situation, the local ligand concentration at the surface of the crystal facets fluctuated strongly. In the presence of the gel, these events were not observed to occur, or at least they were much weaker. The ligand diffused gently and homogeneously through the agarose matrix and thus reached the crystal surfaces more uniformly. Agarose at a concentration as low as 0.2%(*m*/*v*) is sufficient to suppress density differences in solution and hamper crystal sedimentation (Garcia-Ruiz *et al.*, 2001).

Furthermore, the gel-grown AspRS-1 crystals diffract X-rays at high resolution like native crystals (Fig. 2*c*) and are isomorphous. In spite of their fairly high solvent content and the harsh flash cooling procedure to which they have been subjected, these crystals exhibit low mosaicity values (< 0.2°) and well defined reflections even at high resolution (Fig. 2*c*, inset). The beam smearing values on beamline ID14 (horizontal and vertical beam divergences of 10.7 and 3.2 µrad or 2.2 and 0.66 arc seconds, respectively, and spectral spread $\Delta\lambda/\lambda \simeq 3.\ 10^{-4}$) are much smaller than 0.2° or 720 arc seconds. When deconvoluted out these would not significantly alter the mosaicity estimate since a very tightly collimated X-ray undulator and pure spectral spread has been used (see Colapietro *et al.*, 1992). Thus, the viscoelastic hydrogel contributes to increase the mechanical resistance of the enzyme lattice and maintains its cohesion during soaking and cryo-cooling.

The presence of the adenylate analog is confirmed by the difference electron density map (Fig. 2*d*). Conformational changes affecting one loop and a few side chains in the neighborhood of the catalytic cleft are also observed. A detailed structural analysis (to be published elsewhere) shows that the amino acid and the ATP bind to the catalytic pocket of AspRS *via* a lock-and-key mechanism. Furthermore, two loops in the periphery participate in the selection of the substrates, including the tRNA (Cavarelli *et al.*, 1994; Poterszman *et al.*, 1994; Schmitt *et al.*, 1998). Many enzymes of the aminoacyl-tRNA synthetase family undergo such subtle conformational changes when they bind their natural ligands (Ibba *et al.*, 2005). The structural flexibility of these loops upon ligand binding likely explains the sensitivity of AspRS-1 crystals to soaking in the absence of a protective gel matrix. Furthermore, the diffraction limit of gel-grown and derivatized AspRS-1 crystals is comparable to that of native crystals grown in a convection-free solution under microgravity (Ng *et al.*, 2002). An additional advantage of the gel is that it makes unnecessary the crosslinking with glutaraldehyde (Hassell *et al.*, 2007).

3.3. Conclusion and perspectives for crystallogenesis

From a practical point of view, hydrogels are an advantageous, convenient and relatively inexpensive alternative to approaches like microgravity or nano-droplets. The loose network of a hydrogel eliminates convectional flow in solution, and this is favorable to the nucleation and growth of crystals with enhanced diffraction properties. Crystals grown in a gel such as agarose have fewer packing defects and a smaller mosaic spread than those prepared in solution because nucleation and growth proceed *via* a diffusive regime (see *e.g.* Lorber, Sauter, Ng *et al.*, 1999). A similar situation exists in solutions placed inside capillary tubes, such as for instance during counter-diffusion experiments (*e.g.* Otálora *et al.*, 1999; Boggon *et al.*, 2000). Crystallization conditions found in free solution can easily be transposed to the gel case. In practice, on the one hand agarose may be used in combination with various crystallization methods, like vapor diffusion, dialysis and counter-diffusion (Biertümpfel *et al.*, 2002). On the other hand, it is compatible with many crystallizing agents (Gonzalez-Ramirez *et al.*, 2008). A very low concentration may be sufficient to gel the crystallization medium and create a convection-free solution (Garcia-Ruiz *et al.*, 2001).

The results with aspartyl-tRNA synthetase demonstrate that crystals grown in gels are more resistant than crystals grown in solution to internal perturbations occurring during the soaking with a substrate analog. The implemented method may be applicable to other enzyme–ligand systems. Interestingly, it was shown with lysozyme crystals that the polysaccharide fibers of agarose are randomly distributed in the solvent channels (Gavira & García-Ruiz, 2002). On the one hand, this does not prevent crystals from diffracting X-rays at high resolution. On the other, and as demonstrated earlier and again here, it enhances the strength of the crystalline lattice.

Agarose gel was used to preserve the quality of thaumatin crystals grown under microgravity aboard the space shuttle since crystals produced in solution lost their optical and diffraction properties owing to mechanical shocks and possibly to temperature fluctuations during the re-entry and post-landing transportation across continents (Lorber, Sauter, Robert *et al.*, 1999). In this medium, crystals remained immobile at the place where they had nucleated, their nucleation was more synchronous than in solution, they did not sediment and they reached their optimal volume at a high growth rate (Lorber & Giegé, 2001). Since these crystals had contact neither with others nor with the container walls they did not suffer from abrasions or other damage prior to analysis (Lorber, Sauter, Robert *et al.*, 1999; Lorber & Giegé, 2001). This list of advantages is that usually attributed to microgravity [for a recent review see Snell & Helliwell (2005)], but it is also applicable to gels.

A possible objection to the use of gels in protein crystallization is that chemical interactions may occur between the polysaccharide matrix and proteins or ligands. The example of

AspRS shows that this is not a major concern. Furthermore, the method described above may be applicable to the soaking of crystals with other small molecules such as heavy atoms. Thus, there are numerous reasons to crystallize proteins in a gel like agarose.

We are indebted to D. Kern for the bacterial strain that overproduces AspRS-1 and to SO.BI.GEL for a generous gift of agarose. The authors are grateful to the ESRF for the beamtime allocated to this project and in particular to the team managing beamline ID14 for help with data collection. The authors also thank the referees for their constructive comments. This work was supported by a 'Projet International de Coopération Scientifique (PICS)' of the Centre National de la Recherche Scientifique (CNRS), by the French Ministry for Research (ACI 'BCMS' 04358), and by the grant 61.103 from 'Ministère des Relations Internationales du Québec' and 'Consulat Général de France' in the context of the program 'Coopération franco–québecoize de recherche scientifique et technologique'. AM thanks DGAPA-UNAM (Mexico) and CNRS for financial support during his sabbatical year in Strasbourg. CS was a recipient of a Marie Curie European Reintegration Grant (MERG-CT-2004-004898).

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