Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray crystallographic analysis of yeast arginyl-tRNA synthetase–yeast $tRNA^{Arg}$ complexes

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Received 13 December 1999
Accepted 31 January 2000

Three different crystal forms of complexes between arginyl-tRNA synthetase from the yeast *Saccharomyces cerevisae* (yArgRS) and the yeast second major $tRNA^{Arg}$ ($tRNA^{Arg}_{ICG}$) isoacceptor have been crystallized by the hanging-drop vapour-diffusion method in the presence of ammonium sulfate. Crystal form II, which diffracts beyond 2.2 Å resolution at the European Synchrotron Radiation Facility ID14-4 beamline, belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 129.64, b = 107.47, c = 71.38 Å. This crystal form presents the highest resolution obtained for an active form of an aminoacyl-tRNA synthetase–tRNA complex. The estimated V_m of 2.6 $Å^3\ Da^{-1}$ indicates one molecule of complex in the asymmetric unit. The three crystal forms were solved by the molecular-replacement method using the coordinates of the free yArgRS.

1. Introduction

Aminoacyl-tRNA synthetases (aaRS) are a family of enzymes essential for gene expression. They allow the translation of series of mRNA codons into the corresponding polypeptide sequence by covalently linking the appropriate amino acid to the 3′-end of the correct tRNA. In general, each aaRS recognizes a unique amino acid and all isoacceptor tRNAs in a two-step catalytic reaction. The first step, which requires ATP and Mg^{2+} ions, leads to the formation of an enzyme-bound aminoacyladenylate and is followed by the transfer of the amino acid to the 3′-end of the tRNA to form an aminoacyl-tRNA. Extensive progress in understanding the structure–function relationship of this heterogenous family of proteins has been made during the last decade. Aminoacyl-tRNA synthetases now constitute the best textbook example of multidomain proteins including insertion and terminal functional modules appended to one of the two class-specific active-site domains (see Martinis *et al.*, 1999, for a recent review of the field). However, each new structure of aaRS, either in the free state or engaged in complexes with the other partners of the aminoacylation reaction, led to unexpected results which always refined our comprehension of the structure–function relationships and molecular-recognition principles. Moreover, complete sequencing of several archaeal genomes led to the discovery of novel pathways and enzymes for the synthesis of several aminoacyl-tRNAs. Phylogenetic analysis of the 20 aaRSs also revealed a complex evolutionary picture (Doolittle & Handy, 1998; Tumbula *et al.*, 1999; Wolf *et al.*, 1999). In this context, structures are essential in order to gain structural insights from sequence-block alignments and, therefore, to decipher the relationships between function, evolution and sequence.

The structures of nearly all class-II aaRSs and several class-I aaRSs have been determined by X-ray crystallography either in the free state or engaged in complexes with the small substrates of the aminoacylation reaction (ATP, amino acid, Mg^{2+} ions). However, for class-I aaRS, our present understanding of the second step of the aminoacylation reaction, which implies specific tRNA recognition, is essentially based on two crystal structures (Rould *et al.*, 1989; Silvian *et al.*, 1999). We present here the preliminary diffraction analysis of three complexes involving arginyl-tRNA synthetase from the yeast *S. cerevisae* (yArgRS) and its cognate isoacceptor $tRNA^{Arg}$. The crystal structure of yArgRS with L-arginine (L-Arg) bound to the active site has already been described (Cavarelli *et al.*, 1998).

2. Results and discussion

2.1. Purification of the macromolecules

Gene expression and purification of yArgRS followed a previously published protocol (Cavarelli *et al.*, 1998). Overexpression of eukaryotic tRNAs in a prokaryotic system usually fails and represents a real problem which has not yet been solved. This may arise from the complexity of the eukaryotic tRNA promoters and the specific machinery that acts on them (Sprague, 1995). In this work, we have used native yeast $tRNA^{Arg}$ which was purified

Table 1
Crystallization and crystallographic data of yeast arginyl-tRNA synthetase–yeast $tRNA^{Arg}_{ICG}$ complexes.

Numbers in parentheses indicated for crystallographic statistics correspond to those in the last resolution shell.

Crystal form	Form I	Form II	Form III
Crystallization conditions			
Temperature (K)	290	277	277
Composition of the crystallization drop†	5 mg ml^{-1} ArgRS, 2 mg ml^{-1} tRNA, 10 m*M* ATP, 30 m*M* $MgSO_4$	5 mg ml^{-1} ArgRS, 5 m*M* L-Arg, 2 mg ml^{-1} tRNA, 10 m*M* ATP, 20 m*M* $MgSO_4$	8 mg ml^{-1} ArgRS, 3.8 mg ml^{-1} tRNA, 10 m*M* ATP, 15 m*M* $MgSO_4$
Reservoir	2.3 *M* $(NH_4)_2SO_4$, 100 m*M* Na cacodylate pH 6.5	2.0 *M* $(NH_4)_2SO_4$, 5% 1,6-hexanediol, 100 m*M* Na cacodylate pH 7.5	2.1 *M* $(NH_4)_2SO_4$, 3.5% 1,6-hexanediol, 100 m*M* Na cacodylate pH 6.5
Data collection			
Beamline (ESRF)	ID14-4	ID14-4	ID14-3
Wavelength (Å)	0.9315	0.9322	0.9310
Space group	P21212	P21212	I222
Unit-cell parameters (Å)	a = 160.7, b = 106.5, c = 61.3	a = 129.6, b = 107.5, c = 71.4	a = 107.7, b = 129.6, c = 184.0
Resolution (Å)	25–3.0	25–2.2	25–2.9
Number of observations	52102	358386	200845
Number of unique reflections	17346	51493	28892
Completeness (%)	81.0 (81.0)‡	99.8 (99.3)§	100.0 (100.0)¶
R_{sym}†† (%)	6.6 (22.1)	8.9 (21.0)	5.6 (26.2)
Molecular replacement			
Resolution range (Å)	7–3.5	7–3.5	7–3.5
Molecular probe	yArgRS	yArgRS	yArgRS
Best solution	C‡‡ = 0.35, R§§ = 0.46	C = 0.17, R = 0.49	C = 0.20, R = 0.51
Second-best solution	C = 0.24, R = 0.48	C = 0.10, R = 0.53	C = 0.13, R = 0.52
Resolution range (Å)		7–3.5	7–3.5
Molecular probe		Complex form I	Complex form II
Best solution		C = 0.25, R = 0.49	C = 0.63, R = 0.34
Second-best solution		C = 0.14, R = 0.52	C = 0.35, R = 0.44

† After vapour equilibration in the drops. Crystallization drops were made by mixing 2 µl of macromolecule solution with 2 µl of reservoir solution. 50 m*M* Tris–HCl pH 7.5 buffer was used for yArgRS, while $tRNA^{Arg}_{ICG}$ was in 5 m*M* sodium cacodylate buffer (Na cacodylate) at pH 6.5 containing 2 m*M* $MgCl_2$, 0.2 m*M* EDTA. ‡ Last resolution shell 3.1–3.0 Å. § Last resolution shell 2.3–2.2 Å. ¶ Last resolution shell 3.0–2.9 Å. †† R_{sym} indicates agreement of individual reflections over the set of unique averaged reflections, $R_{sym} = \sum_h \sum_i |\langle I_h\rangle - I_{h,i}|$, where $I_{h,i}$ is the ith observed intensity of a measured reflection of Miller index h and $\langle I_h\rangle$ is the average intensity for this unique reflection. ‡‡ $C = \sum_h (|F_{obs}|^2 - \langle|F_{obs}|^2\rangle)(|F_{calc}|^2 - \langle|F_{calc}|^2\rangle) / [(\sum_h(|F_{obs}|^2 - \langle|F_{obs}|^2\rangle)^2 \times \sum_h (|F_{calc}|^2 - \langle|F_{calc}|^2\rangle)^2]^{1/2}$, where $|F_{obs}|$ and $|F_{calc}|$ are the observed and calculated structure-factor amplitudes, respectively, of a measured reflection of Miller index h and $\langle|x|\rangle$ represents the mean value of the variable x. §§ $R = \sum_h ||F_{obs}| - |F_{calc}|| / \sum_h |F_{obs}|$, where $|F_{obs}|$ and $|F_{calc}|$ are the observed and calculated structure-factor amplitudes, respectively, of a measured reflection of Miller index h.

from counter-current fractions (Dirheimer & Ebel, 1967). In the yeast genome, there are 19 genes which encode five different isoacceptors of $tRNA^{Arg}$ whose sequences display few conserved nucleotides. Our initial counter-current fractions contained two $tRNA^{Arg}$ isoacceptors: $tRNA^{Arg}_{ICG}$ and $tRNA^{Arg}_{mcm5UCU}$. $tRNA^{Arg}_{ICG}$, where ICG represents the three bases of the anticodon of the tRNA, reads three arginine codons, while $tRNA^{Arg}_{mcm5UCU}$ constitutes the major $tRNA^{Arg}$ in yeast. Inosine (I) and 5-methoxycarbonylmethyl uridine (mcm5U) are two post-transcriptionally modified nucleosides (see Limbach, Crain, Pomerantz *et al.*, 1995, for a recent review).

Each isoacceptor has been purified with a similar three-step protocol involving different chromatographic supports starting from counter-current fractions which were slightly enriched (about 15%) in $tRNA^{Arg}$.

The first column used the hydrophobic interactions (TosoHaas: Toyopearl TSK HW65 Butyl column, size 24 × 60). The tRNAs were eluted by an ammonium sulfate gradient (2.4–1.2 *M*) at pH 4.5 with a flow rate of 2.5 ml min^{-1}. The active fractions, identified by an aminoacylation activity, were pooled and the ammonium sulfate was removed by buffer exchange in a concentration procedure using an Amicon dialysis concentrator at 277 K. The tRNAs were concentrated in 5 m*M* sodium cacodylate buffer pH 6.5 containing 2 m*M* $MgCl_2$, 0.2 m*M* EDTA (buffer *A*).

In the second step, the tRNAs were loaded onto a hydroxyapatite column (BioRad, CHT5-1, size 10 × 64 cm). They were eluted by a linear gradient of potassium phosphate buffer pH 6.5 (10–500 m*M*) with a flow rate of 1 ml min^{-1}. The active fractions were pooled and the phosphate buffer was removed by buffer exchange as described above. The tRNAs were concentrated at about 1 mg ml^{-1} in 5 m*M* sodium cacodylate buffer pH 6.5 containing 22 m*M* $MgCl_2$, 0.2 m*M* EDTA at 277 K. The tRNAs were then refolded by heating at 343 K for 8 min and slow-cooled to 277 K for 12 h.

The last step involved an anion-exchange column (MonoQ, Pharmacia, size 10 × 10 cm), where the tRNAs were eluted by a linear NaCl gradient (300–700 m*M*) at pH 5 with a flow rate of 4 ml min^{-1}. The active and pure fractions were precipitated with two volumes of ethanol, concentrated by buffer exchange (buffer *A*) and stored at 253 K.

After purification, the purity and integrity of $tRNA^{Arg}$ were checked using three different methods: electrophoresis in 15%(*w*/*v*) polyacrylamide gel in the presence of 7 *M* urea (12 h at 10 W, 40 cm gel), aminoacylation activity and mass spectroscopy (Limbach, Crain & McCloskey, 1995). All these experiments confirmed that both isoacceptors of $tRNA^{Arg}$ were pure and fully active.

2.2. Crystallization

Initial crystallization conditions were found using the hanging-drop method by screening several precipitant agents such as ammonium sulfate, polyethylene glycol (PEG) and 2-methyl-2,4-pentanediol (MPD) at different concentrations within the pH range 6.5–8.5 and at two temperatures (277 and 290 K). The stoichiometry tested for the two macromolecules (ArgRS, $tRNA^{Arg}$) in the crystallization drop ranged from 1.1 to 1.9 for both the binary complex (ArgRS, $tRNA^{Arg}$) and for other complexes which also contained the small substrates of the aminoacylation reaction (ATP, L-Arg, magnesium ions). Refinements of the promising initial crystallization conditions were performed by screening several additives. This process led to the formation of three different crystal forms (I, II, III) using ammonium sulfate as precipitant (see Table 1 for details). A few crystals of form I appeared and reached maximum dimensions of 0.05 × 0.05 × 0.02 mm after one month. However, it was not possible to reproduce this crystal form. A few crystals of form II or III grew after 10–21 d as thin plates (typical dimensions 0.3 × 0.2 × 0.01 mm).

2.3. Data collection and processing

For crystal forms I and II, X-ray diffraction data were collected on a ADSC Quantum 4CCD detector at beamline ID14-4 at ESRF, Grenoble. For crystal form

III, X-ray diffraction data were collected on a MAR 132 mm CCD detector system at beamline ID14-3 at ESRF, Grenoble. Each data set was collected from a single frozen crystal. The crystals were prepared for cryocooling by transferring them for 2 min into solutions of mother liquor containing 20% glycerol and were then plunged into liquid ethane. They were inserted into a nitrogen-gas flow at 100 K just prior to data collection.

Crystals of forms I and II belong to the orthorhombic space group $P2_12_12$ (see Table 1 for details). Crystals of form I diffracted to only 2.9 Å at ID14-4. A data set has been recorded between 25 and 3.0 Å from 400 frames of 0.25° oscillations (exposure time of 3 s per oscillation, crystal-to-detector distance of 200 mm). Crystals of form II diffracted beyond 2.2 Å at ID14-4 and a complete data set was obtained between 25 and 2.2 Å from 400 frames of 0.5° oscillations (exposure time of 3 s per oscillation with an attenuated beam, crystal-to-detector distance of 200 mm). A crystal of form III belonged to the orthorhombic space group $I222$ and diffracted beyond 2.9 Å at ID14-3; a complete data set was obtained between 25 and 2.9 Å from 340 frames of 0.5° oscillations (exposure time of 30 s per oscillation, crystal-to-detector distance of 190 mm). All data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997); other crystallographic calculations were carried out with the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Data-collection statistics for each crystal form are presented in Table 1.

2.4. Structure determination

For all crystal forms, structure determination was performed by the molecular-replacement method with the program *AMoRe* (Navaza, 1994).

2.4.1. Crystal form I. The structure of yArgRS alone (607 residues; Cavarelli *et al.*, 1998) was taken as a rigid-body search model between 7 and 3.5 Å. The estimated V_m of 2.8 Å^3 Da^{-1} indicated one molecule of complex in the asymmetric unit; therefore, one monomer of yArgRS was used as a probe. The first solution given by the molecular-replacement procedure (see Table 1 for details) was further refined, using data in the resolution range 15–3.0 Å, by rigid-body and torsion-angle molecular-dynamics refinement with a cross-validated maximum-likelihood crystallographic target as implemented in the program *CNS* (Brunger *et al.*, 1998). Examination of a cross-validated σ_A-weighted map with coefficients $3F_{\rm obs} - 2F_{\rm calc}$ (Kleywegt & Brünger, 1996; Kleywegt & Jones, 1994), using the program *O* (Jones *et al.*, 1991) showed extra electron density outside the protein domains with characteristic features of RNA helical regions. Model building and refinement have been completed and will be published elsewhere (Delagoutte *et al.*, 2000).

2.4.2. Crystal form II. The estimated V_m of 2.6 Å^3 Da^{-1} indicated one molecule of complex in the asymmetric unit. Using the structure of yArgRS alone, the above procedure did not lead to any interpretable map in the expected tRNA regions. Several initial conditions have been tested without any success by varying the resolution range of the data, the initial B factors of the atoms of the probe model or the molecular-replacement method used (*AMoRe* or *CNS* direct-space rotation function).

However, the use of an initial model of the form I crystal structure where less than half of the tRNA molecule was built (nucleotides 6–18, nucleotides 22–28 and nucleotides 45–68) leads to a better correlation coefficient with *AMoRe* (see Table 1 for details). This initial complex model solution was therefore refined between 15 and 3 Å with the same procedure as described above. Examination of a cross-validated σ_A-weighted map with coefficients $3F_{\rm obs} - 2F_{\rm calc}$ enabled the building of the full tRNA molecule (76 nucleotides). Model building and refinement have been completed and will be published elsewhere. This indicated that the phasing power of the protein alone was not sufficient in this case for the molecular-replacement method. This can now be explained by several severe conformational changes, which will be described elsewhere (Delagoutte *et al.*, 2000), induced by the binding of the small substrates to the enzyme.

2.4.3. Crystal form III. The estimated V_m of 3.4 Å^3 Da^{-1} indicated one molecule of complex in the asymmetric unit. This crystal form was solved without any difficulties using the refined structure of crystal form II as a probe. Model building and refinement have been completed and will be published elsewhere (Delagoutte *et al.*, 2000).

We thank Sean McSweeney and the staff of the ESRF beamline ID14-4 and Ed Mitchell and the staff of the ESRF beamline ID14-3 for use of their synchrotron instrumentation and help during data collection. We thank Dr Anne-Catherine Dock-Bregeon for sharing her expertise in tRNA purification procedures. We are indebted to Dr Noelle Potier for the mass-spectroscopy experiments. We also thank Dr B. Rees for careful reading of the manuscript. This work was supported by grants from CNRS and by EEC contracts.

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