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Crystallization and preliminary X-ray diffraction study of mammalian mitochondrial seryl-tRNA synthetase

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The mitochondrial seryl-tRNA synthetase (mt SerRS) from *Bos taurus* was overexpressed in *Escherichia coli* and crystallized using the sitting-drop vapour-diffusion method. Crystals grew in a very narrow range of conditions using PEG 8000 as precipitant at room temperature. An appropriate concentration of lithium sulfate was critical for crystal nucleation. Crystals diffracted well beyond a resolution of 1.6 Å and were found to belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 79.89, b = 230.42, c = 135.60 Å. There is one dimer ($M_r \simeq 113$ kDa) in the asymmetric unit, with a solvent content of 55%. Efforts to solve the phase problem by molecular replacement are under way.

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are a diverse family of enzymes that catalyze the specific attachment of amino acids to the 3′-ends of their cognate tRNAs. This reaction (aminoacylation) occurs in a two-step process in which an enzyme-bound amino acid is first activated by adenosine triphosphate (ATP) to form an aminoacyl adenylate as a high-energy intermediate and is subsequently transferred to the 3′-terminal adenosine of tRNA. The ability of aaRSs to discriminate their cognate tRNAs from structurally similar non-cognate species is thus essential for maintaining the high fidelity of the translation (Ibba & Söll, 1999, 2000). This specificity is strictly governed by the identity elements embedded in each cognate tRNA, which are composed of a defined set of nucleotides or structural characteristics (Giegé *et al.*, 1998). These tRNA identities are frequently located in the anticodon loop and in the acceptor stem of the tRNAs (Fig. 1). However, in the case of tRNA for serine ($tRNA^{Ser}$), it has been reported that the long variable arm of $tRNA^{Ser}$ is a major determinant for seryl-tRNA synthetase (SerRS; Himeno *et al.*, 1990; Shimizu *et al.*, 1992; Sampson & Saks, 1993).

SerRS is a homodimeric enzyme belonging to the class II aminoacyl-tRNA synthetases, characterized by a catalytic domain constructed from the antiparallel β-sheet architecture with three short conserved motifs: 1, 2 and 3 (Eriani *et al.*, 1990; Cusack *et al.*, 1990). Structural studies have revealed that bacterial SerRS consists of two domains: the globular C-terminal catalytic domain and the unique N-terminal antiparallel coiled-coil helical arm (Cusack *et al.*, 1990; Fujinaga *et al.*, 1993). The crystal structures of *Thermus thermophilus* SerRS complexed with the cognate $tRNA^{Ser}$ showed that the specific recognition of $tRNA^{Ser}$ is performed by the mutual interaction between the long α-helical arm of SerRS and the elongated variable arm of $tRNA^{Ser}$ (Fig. 1; Biou *et al.*, 1994; Cusack *et al.*, 1996). The helical arm of SerRS principally recognizes not the specific bases but rather the orientation of the variable arm of $tRNA^{Ser}$. Furthermore, recent biochemical studies have revealed that yeast and archaeal SerRSs employ a similar mechanism to discriminate their cognate tRNAs (Lenhard *et al.*, 1999; Bilokapic *et al.*, 2004), thus suggesting that the idiosyncratic recognition of the variable arm of $tRNA^{Ser}$ by SerRS is evolutionarily conserved in all three kingdoms of life.

On the other hand, in the case of the cellular organelles, the mammalian mitochondrial (mt) translation system utilizes two $tRNA^{Ser}$ isoacceptors, the structures of which deviate significantly from those of cytoplasmic tRNAs (Fig. 1; Helm *et al.*, 2000). Mammalian mt $tRNA^{Ser}_{GCU}$, responsible for codons AG*Y* (*Y* = C or U), lacks the entire D arm (Steinberg *et al.*, 1994), whereas mt $tRNA^{Ser}_{UGA}$, for codons UC*N* (*N* = A, G, C or U), has an unusual cloverleaf configuration with an extended anticodon stem (Yokogawa *et al.*, 1991). Notably, neither possesses the elongated variable arm that is the identity determinant of cytoplasmic $tRNAs^{Ser}$ (Fig. 1). We have previously reported the identification and characterization of mt SerRS from *Bos taurus* (484 amino-acid residues, M_r = 54 635) and demonstrated that the single enzyme can efficiently charge serine to each of the two isoacceptors with equal activity (Yokogawa *et al.*, 2000). Furthermore, mt SerRS specifically recognizes the TΨC loop of each isoacceptor, but the D-loop–TΨC-loop interaction is additionally required for

$tRNA^{Ser}_{UGA}$ recognition, implying a distinct mechanism for the two substrate $tRNAs^{Ser}$ (*i.e.* a dual mode recognition; Shimada *et al.*, 2001). The fact that the sequence similarity among the bacterial and eukaryotic counterparts is significantly low in the N-terminal domain involved in tRNA recognition indicates that mt SerRS could have an entirely unique structure in the N-terminal domain and could thereby recognize mt $tRNAs^{Ser}$ in a different manner. To verify our hypothesis, we report here the purification, crystallization and preliminary X-ray diffraction analysis of *B. taurus* mt SerRS. To our knowledge, this is the first reported crystal of an aminoacyl-tRNA synthetase from organelles, with the highest resolution of the known structures of full-length aaRSs.

2. Experimental and results

2.1. Protein expression and purification

The *B. taurus* mitochondrial SerRS gene was cloned into the expression vector pET-19b (Novagen) with a hexahistidine tag followed by a thrombin-cleavage site at the N-terminus, as described by Shimada *et al.* (2001). The protein was overexpressed in *Escherichia coli* Rosetta (DE3) cells (Novagen) grown in Luria–Bertani broth containing ampicillin and chloramphenicol (100 and 50 µg ml^{-1}, respectively) at 310 K. Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 10 µ*M* at A_{600} = 0.7 and proceeded overnight at 298 K. Harvested cells were disrupted by sonication or French press and cell debris was removed by ultracentrifugation (100 000*g*, 90 min) at 277 K. All the following purification processes were carried out at 277 K because of the labile nature of the mitochondrial enzyme. In the first step, the clear supernatant was loaded directly onto a nickel-ion-charged HiTrap chelating column (Amersham Biosciences) pre-equilibrated with buffer *A* (50 m*M* HEPES–NaOH pH 7.6, 10 m*M* $MgCl_2$, 100 m*M* KCl, 10% glycerol, 7 m*M* β-mercaptoethanol). After washing the column with buffer *A* containing 50 m*M* imidazole, bound proteins were eluted from the resin with a linear gradient of 50–350 m*M* imidazole in buffer *A*. The fractions containing His_6-tagged protein were batched together and dialyzed against buffer *A* containing 0.5 m*M* EDTA to ensure elimination of nickel ions, as we found that the protein tends to be aggregated in the presence of nickel ions. The purified fractions were subsequently subjected to a 10 ml Source Q anion-exchange column (Amersham Biosciences) equilibrated with buffer *B* [20 m*M* HEPES–NaOH pH 7.6, 5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1 m*M* EDTA, 5% glycerol, 1 m*M* dithiothreitol (DTT)] and eluted with a linear gradient of 50–300 m*M* KCl in buffer *B*. His_6-tagged mt SerRS, eluted at approximately 150 m*M* KCl, was collected and concentrated using a Centricon YM-30 centrifugal filter device (Amicon). The final step of purification was performed using a Superdex 200 gel-filtration column (Amersham Biosciences) in crystallization buffer (50 m*M* HEPES–NaOH pH 7.6, 10 m*M* $MgCl_2$, 150 m*M* KCl, 8% glycerol, 1 m*M* DTT). The elution profile of the gel filtration strongly indicated the dimeric nature of mt SerRS in solution, as observed for the bacterial counterparts (data not shown). The homogeneity of the purified protein was assessed by SDS–PAGE. Despite the low-temperature purification procedure and careful handling of the protein, we detected a small trace of proteolytic fragments on SDS–PAGE after the gel-filtration purification step. Attempts to improve the purity of the protein by modifying the purification protocol were not successful. The purified recombinant protein was then divided into small aliquots, flash-frozen with liquid nitrogen and stored at 193 K. The final yield was approximately 4 mg of pure protein per litre of culture.

2.2. Crystallization

For initial crystallization screening, the hanging-drop vapour-diffusion method was used with commercially available screening kits (Hampton Research and Emerald BioStructures). Each drop was prepared by mixing 1 µl of protein solution with an equal volume of reservoir solution and was vapour-equilibrated against 250 µl of reservoir solution at 277 K. 534 conditions were tested with two different protein concentrations (20 and 10 mg ml^{-1}). Crystals only appeared in the presence of ammonium sulfate or lithium sulfate as precipitants, but grew as bundles of very thin needles. For further optimization of the crystallization conditions, the sitting-drop method was used as an alternative and the volume of reservoir was increased to 500 µl. Despite extensive

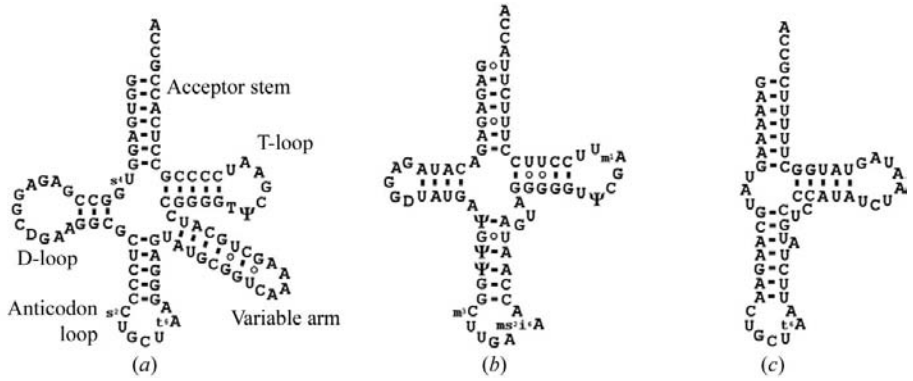

Figure 1
The cloverleaf secondary structure of three different $tRNAs^{Ser}$: (*a*) $tRNA^{Ser}_{GCU}$ from *E. coli*, (*b*) $tRNA^{Ser}_{UGA}$ and (*c*) $tRNA^{Ser}_{GCU}$ from *B. taurus* mitochondria. Note that the identity element for recognition by SerRS is the long variable arm, which is missing in both of the mitochondrial $tRNAs^{Ser}$. Furthermore, mt $tRNA^{Ser}_{GCU}$ also entirely lacks the D-arm, which is replaced by a short loop.

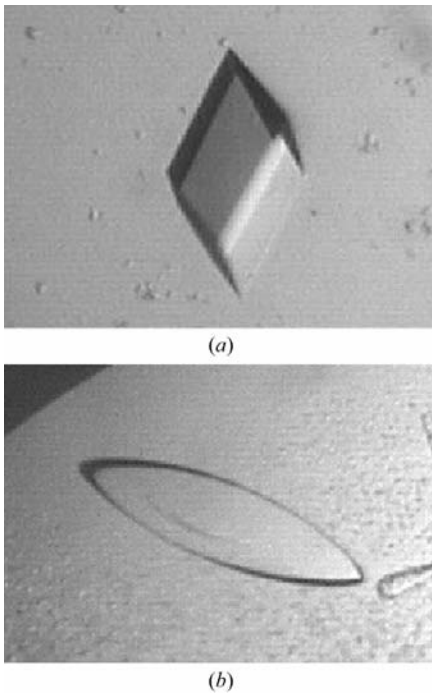

Figure 2
Crystals of the mitochondrial seryl-tRNA synthetase from *B. taurus*. (*a*) A trigonal crystal grown from ammonium sulfate with 1%(*w*/*v*) PEG 35 000 as additive. The crystal dimensions are approximately 0.25 × 0.25 × 0.25 mm. (*b*) The high-resolution diffracting crystal grown against a reservoir containing 50 m*M* MES pH 5.8, 23%(*w*/*v*) PEG 8000, 250 m*M* $LiSO_4$ and 1 m*M* DTT to dimensions of 0.50 × 0.10 × 0.50 mm.

efforts to improve the quality of the crystal involving variation of pH, temperature, concentration of precipitant and protein and addition of additives, as well as the use of seeding techniques, we failed to obtain crystals that were suitable for X-ray diffraction experiments. Therefore, the next strategy we employed was to mix the reservoir with a one-ninth volume of each solution from Crystal Screens I and II (Hampton Research), yielding 98 new conditions around the first condition. With this practice, we noticed that high-molecular-weight PEG might improve or change the morphology of the crystals and large single crystals were finally obtained at 293 K with 1.4 *M* ammonium sulfate at pH 6.9–7.3 containing 1%(*w*/*v*) PEG 35 000 as an additive (Fig. 2*a*). Unfortunately, these well shaped trigonal crystals only diffracted X-rays to about 4 Å on a synchrotron source. Attempts to improve the resolution by optimizing the cryoprotectants or by proteolytic removal of the His_6 tag from the N-terminus of the recombinant protein were not successful. At this stage, we concluded that salts such as ammonium sulfate or lithium sulfate were indispensable for nucleation, but high-molecular-weight PEG was also necessary to obtain single crystals. Thus, we decided to try reversing the effect of the salt and PEG in the crystallization, as reported by Majeed *et al.* (2003) and a new crystal form was produced 12 h after equilibration against a reservoir containing 20%(*w*/*v*) PEG 35 000, 50 m*M* MES–NaOH pH 5.5 and 250 m*M* lithium sulfate or ammonium sulfate. Refinement around this condition, including variation of PEGs, pH and salts, gave leaf-shaped crystals of dimensions 0.05 × 0.1 × 0.5 mm over a period of one week (Fig. 2*b*). The best crystals were obtained in a drop containing 10 mg ml^{-1} mt SerRS, 5 m*M* serine and 2 m*M* ATP equilibrated against reservoir solution [22–24%(*w*/*v*) PEG 8000, 50 m*M* MES–NaOH pH 5.5–5.8, 250 m*M* lithium sulfate, 1 m*M* DTT] at 293 K. The presence of the full intact protein in the crystals was verified by SDS–PAGE analysis (data not shown).

2.3. Data collection and processing

Crystals were mounted in nylon loops (Hampton Research) and rapidly soaked (∼5 s) in a reservoir solution containing 15% glycerol as a cryoprotectant. A complete diffraction data set was collected on beamline X11 at DESY (Hamburg, Germany) from one flash-cooled crystal in a nitrogen-gas stream at 100 K, using a MAR CCD detector at a wavelength of 0.8123 Å in high- and low-resolution swipes. High-resolution data collection was carried out with an oscillation angle of 0.5° and a crystal-to-detector distance of 140 mm. The low-resolution data set was subsequently collected using a 2.0° frame at a distance of 300 mm. Data were processed separately with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). The crystals diffracted well to 1.6 Å and belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 79.86, b = 230.35, c = 135.59 Å. The presence of two monomers (*i.e.* one dimer) per asymmetric unit gave a Matthews (1968) coefficient of 2.8 Å^3 Da^{-1} and a solvent content of 55.1%. Although the diffraction data were collected to a resolution limit of 1.57 Å, we decided to use the good quality data ($R_{\rm merge}$ < 0.40) only to a resolution of 1.65 Å for further analysis. Details of the data-collection statistics are summarized in Table 1. Structure determination using the molecular-replacement method is currently in progress.

Table 1
X-ray data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.69–1.65 Å).

Wavelength (Å)	0.8123
Resolution (Å)	99–1.65
Space group	$C222_1$
Unit-cell parameters (Å)	a = 79.864, b = 230.350, c = 135.590
Observed reflections	917392
Unique reflections	145408
Data completeness (%)	96.6 (96.9)
$R_{\rm merge}$† (%)	5.0 (37.2)
I/σ (I)	30.7 (1.8)
Redundancy	6.3 (4.3)

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$.

3. Discussion

Crystals of mt SerRS that diffracted to high resolution occasionally grew within a quite narrow range of crystallization conditions. Crystals were obtained only in the pH range 5.5–5.8 with 21–25%(*w*/*v*) PEG 8000 in the presence of 250 m*M* lithium sulfate. This may explain why we could not obtain any crystals from PEG in spite of the extensive initial screening with commercial kits. In order to solve the phase problem by the MAD method, we have tried to produce the selenomethionine (SeMet) substituted protein in the *E. coli* methionine-auxotroph B834 (DE3) (Novagen) strain but found that the SeMet protein easily turned into an insoluble form after overexpression. Since there are ten methionine sites in mt SerRS, some of them may affect or alter the property and the activity of the protein. Therefore, we are now focusing on the molecular-replacement method using the highly conserved C-terminal globular domain from *T. thermophilus* SerRS (36% identity to that of mt SerRS) as the initial search model. We hope that our atomic structure will shed light on the co-evolution of the RNA-protein in the mitochondrial translation system and may render some hints as to structure and function of the mitochondrial D-armless $tRNA^{Ser}_{GCU}$.

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