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Crystallization of leucyl-tRNA synthetase complexed with $tRNA^{Leu}$ from the archaeon *Pyrococcus horikoshii*

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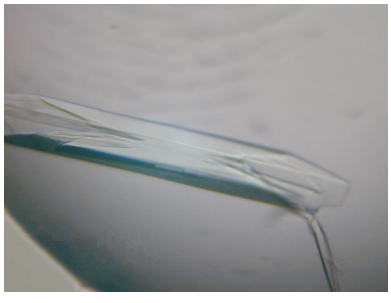

All five $tRNA^{Leu}$ isoacceptors from the archaeon *Pyrococcus horikoshii* have been transcribed *in vitro* and purified. The leucyl-tRNA synthetase (LeuRS) from *P. horikoshii* was overexpressed in *Escherichia coli* and purified, and cocrystallizations with each of the $tRNA^{Leu}$ isoacceptors were attempted. Cocrystals were obtained by the hanging-drop vapour-diffusion method, but only when the $tRNA^{Leu}$ isoacceptor with the anticodon CAA was used. Electrophoretic analyses revealed that the crystals contain both LeuRS and $tRNA^{Leu}$, suggesting that they are LeuRS–$tRNA^{Leu}$ complex crystals. A data set diffracting to 3.3 Å resolution was collected from a single crystal at 100 K. The crystal belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 118.18, b = 120.55, c = 231.13 Å. The asymmetric unit is expected to contain two complexes of LeuRS–$tRNA^{Leu}$, with a corresponding crystal volume per protein weight of 2.9 $Å^3\,Da^{-1}$ and a solvent content of 57.3%.

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) specifically attach each cognate amino acid to the 3′-end of its cognate tRNA. The high specificity of aaRSs for both the amino acid and tRNA is crucial for accurate protein synthesis. A precise understanding of the high selectivity of aaRSs requires their three-dimensional structures. Leucyl-tRNA synthetase (LeuRS) is a large monomeric class Ia synthetase (its molecular weight is more than 100 kDa). $tRNA^{Leu}$ has a characteristic long variable loop. The LeuRS–$tRNA^{Leu}$ complex has one of the largest molecular weights of all of the aaRS–tRNA complexes. A common feature of the LeuRSs from bacteria, archaea and eukarya is that they use the discriminator base A73 as one of the identity elements through a base-specific interaction, but do not use anticodon nucleotides as recognition determinants, except for the case of the lower eukarya (Asahara *et al.*, 1993; Breitschopf *et al.*, 1995; Soma *et al.*, 1996, 1999). In addition to these common mechanisms, the bacterial, archaeal and eukaryal LeuRSs also use different $tRNA^{Leu}$-recognition mechanisms: the archaeal and higher eukaryal LeuRSs use the long variable loop of $tRNA^{Leu}$ as a recognition determinant (Small *et al.*, 1992; Breitschopf *et al.*, 1995; Soma *et al.*, 1999), while the bacterial and lower eukaryal LeuRSs do not (Asahara *et al.*, 1993; Soma *et al.*, 1996). Since most of the LeuRSs do not recognize the anticodon and their identity elements are different among species, they are considered to be good engineering targets for genetic code expansion (Anderson & Schultz, 2003).

In addition to LeuRS, the closely related isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS) also belong to the class Ia family of aaRSs. These three enzymes have high sequence and structural homology and may have evolved from a common ancestral enzyme (Brown & Doolittle, 1995). These three aaRSs have an editing domain (the CP1 domain), which is inserted into the catalytic Rossmann-fold domain and hydrolyzes misactivated (the pre-transfer editing) and mischarged (the post-transfer editing) non-cognate amino acids (Baldwin & Berg, 1966; Jakubowski & Goldman, 1992; Schmidt & Schimmel, 1995; Lin *et al.*, 1996; Hale *et al.*, 1997; Nureki *et al.*, 1998; Silvian *et al.*, 1999; Chen *et al.*, 2000; Fukai *et al.*, 2000; Lincecum *et al.*, 2003; Fukunaga *et al.*, 2004). For pre-transfer editing, the tRNA is necessary, although the reason is still unknown (Baldwin

& Berg, 1966; Hale *et al.*, 1997). The crystal structure of LeuRS without tRNA from the bacterium *Thermus thermophilus* has been determined (Cusack *et al.*, 2000; Lincecum *et al.*, 2003). The structure revealed the substrate amino-acid recognition mechanism in the aminoacylation and editing active sites of this eubacterial LeuRS. However, its tRNA recognition and aminoacylation mechanisms, and the role of tRNA in the editing reactions still need to be elucidated. In the present research, we have carried out a crystallization and preliminary X-ray crystallographic analysis of LeuRS from the archaeon *Pyrococcus horikoshii*, complexed with *in vitro*-transcribed *P. horikoshii* $tRNA^{Leu}$.

2. Methods and results

2.1. Purification of *in vitro*-transcribed *P. horikoshii* $tRNA^{Leu}$

All five *P. horikoshii* $tRNA^{Leu}$ isoacceptors were transcribed *in vitro* with T7 RNA polymerase. The transcribed $tRNA^{Leu}$ was purified by phenol/chloroform treatment followed by urea–PAGE. The $tRNA^{Leu}$ was further purified by anion-exchange chromatography with a ResourceQ column using 20 m*M* Tris–HCl buffer pH 8.0 containing 8 m*M* $MgCl_2$ as a starting buffer with a linear gradient of 0–1.0 *M* NaCl. The $tRNA^{Leu}$-containing fractions were pooled, ethanol-precipitated and dried. The $tRNA^{Leu}$ was dissolved in 20 m*M* Tris–HCl buffer pH 8.0 containing 10 m*M* $MgCl_2$.

2.2. Overexpression and purification of the native *P. horikoshii* LeuRS

The full-length *P. horikoshii* LeuRS is composed of 976 amino-acid residues with a molecular weight of 113 kDa. The recombinant LeuRS was overexpressed in *Escherichia coli* and purified using the methods used for the C-terminally truncated form of *P. horikoshii* LeuRS, using a phenyl-Toyopearl column (Tosoh), a ResourceQ column (Amersham Biosiences) and a HiTrap Heparin column (Amersham Biosiences) (Fukunaga & Yokoyama, 2004). Purified LeuRS was dialyzed against 10 m*M* Tris–HCl buffer pH 8.0 containing 5 m*M* $MgCl_2$, 200 µ*M* zinc acetate and 5 m*M* β-mercaptoethanol and was concentrated to a final concentration of 12 mg ml^{-1} with a Centricon YM-30 filter (Millipore).

2.3. Crystallization and X-ray data collection

Before crystallization, the $tRNA^{Leu}$ was heated at 353 K for 5 min and was gradually cooled to room temperature for refolding. 100 m*M* AMPPNP (a non-hydrolyzable analogue of ATP) solution was added to the LeuRS to give a final concentration of 1 m*M*. LeuRS and $tRNA^{Leu}$ were mixed in a molar ratio of 1:1.1 with a final LeuRS concentration of 10 mg ml^{-1}, heated at 348 K for 10 min and gradually cooled to room temperature. Combinations of LeuRS and each of the $tRNA^{Leu}$ isoacceptors were assessed for cocrystallization. Crystals were obtained in only one crystallization condition from the more than 400 tried for each of the tRNA isoacceptors. Crystals were generated only when the $tRNA^{Leu}$ isoacceptor with the anticodon CAA was used. The initial crystallization conditions were refined by optimizing the sample and precipitant concentrations, as well as the molecular ratio between LeuRS and $tRNA^{Leu}$, the pH, temperature, additive reagents and sample/reservoir solution volumes. Thus, crystals suitable for X-ray analysis were obtained in 1 d with the hanging-drop vapour-diffusion method by mixing 1 µl sample solution (in a molar ratio of 1:1.2 for LeuRS:$tRNA^{Leu}$ with a final LeuRS concentration of 10 mg ml^{-1}) and 1 µl reservoir solution consisting of 30% 2-propanol, 200 m*M* trisodium citrate and 100 m*M* sodium cacodylate buffer pH 6.5 at 293 K (Fig. 1). The mixed sample was equilibrated against 500 µl reservoir solution. To determine whether these were actually LeuRS–$tRNA^{Leu}$ complex crystals, they were harvested, washed well, dissolved and examined by SDS–PAGE and urea–PAGE. The results showed that the crystals contained both LeuRS and $tRNA^{Leu}$ (data not shown), suggesting that they are LeuRS–$tRNA^{Leu}$ complex crystals.

For the diffraction data collection, the crystals were directly soaked in a cryoprotectant solution consisting of 30% 2-propanol, 1 m*M* AMPPNP, 33 m*M* trisodium citrate, 100 m*M* sodium cacodylate buffer pH 6.5 and 20% MPD. Native X-ray diffraction data sets were collected using cryocooled (100 K) crystals at BL26B1, SPring-8 (Harima, Japan). The diffraction data were collected using 0.3° oscillations with a crystal-to-detector distance of 320 mm. A data set diffracting to 3.3 Å resolution was collected from a single crystal at 100 K (Fig. 2). The data were indexed and scaled with *HKL*2000 (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1. The crystals belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 118.18, b = 120.55,

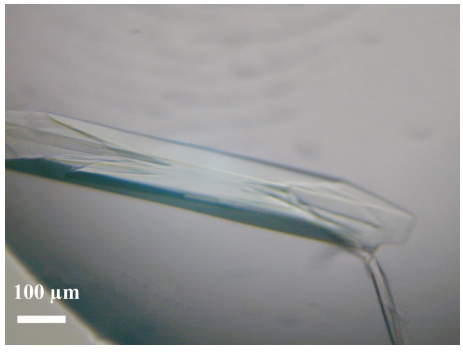

Figure 1
A native crystal of the *P. horikoshii* LeuRS–$tRNA^{Leu}$ complex.

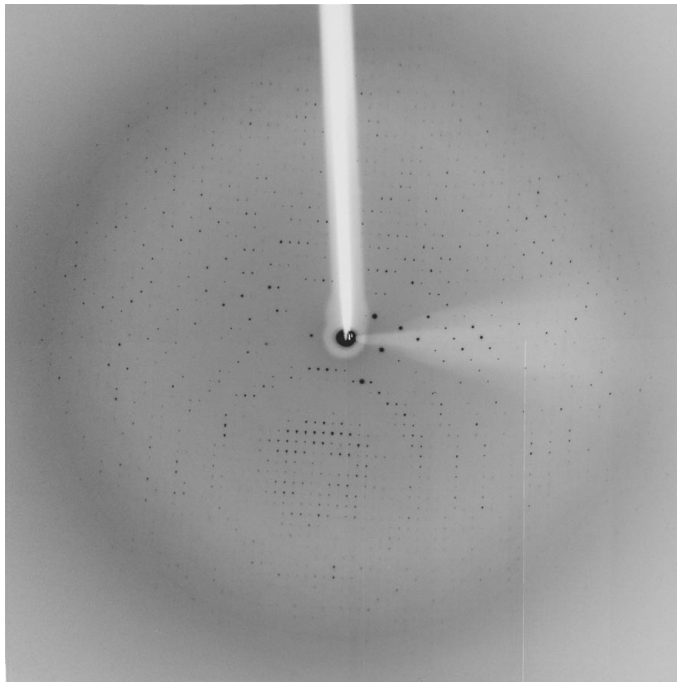

Figure 2
X-ray diffraction image from a native *P. horikoshii* LeuRS–$tRNA^{Leu}$ crystal. The edge of the detector corresponds to a resolution of 3.2 Å.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.020
Resolution range (Å)	50–3.3 (3.42–3.3)
Measured reflections	219343
Unique reflections	46168
Completeness (%)	92.2 (81.9)
Mean $I/\sigma(I)$	18.0 (3.0)
$R_{\rm merge}$† (%)	5.1 (18.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_{hkli} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i \langle I\rangle$.

c = 231.13 Å. The asymmetric unit is estimated to contain two complexes of LeuRS–tRNA$^{\rm Leu}$, with a corresponding crystal volume per protein weight of 2.9 Å^3 Da^{-1} and a solvent content of 57.3%.

Attempts to solve the structure of the *P. horikoshii* LeuRS–tRNA$^{\rm Leu}$ complex by either molecular-replacement procedures or the MAD method using selenomethionine-labelled LeuRS are in progress.

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