Crystallographic analysis of a subcomplex of the transsulfursome with tRNA for Cys-tRNA^{Cys} synthesis

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In most organisms, Cys-tRNA^{Cys} is directly synthesized by cysteinyl-tRNA synthetase (CysRS). Many methanogenic archaea, however, use a two-step, indirect pathway to synthesize Cys-tRNA^{Cys} owing to a lack of CysRS and cysteine-biosynthesis systems. This reaction is catalyzed by *O*-phosphoseryl-tRNA synthetase (SepRS), Sep-tRNA:Cys-tRNA synthase (SepCysS) and SepRS/SepCysS pathway enhancer (SepCysE) as the transsulfursome, in which SepCysE connects both SepRS and SepCysS. On the transsulfursome, SepRS first ligates an *O*-phosphoserine to tRNA^{Cys}, and the mischarged intermediate Sep-tRNA^{Cys} is then transferred to SepCysS, where it is further modified to Cys-tRNA^{Cys}. In this study, a subcomplex of the transsulfursome with tRNA^{Cys} (SepCysS–SepCysE–tRNA^{Cys}), which is involved in the second reaction step of the indirect pathway, was constructed and then crystallized. The crystals diffracted X-rays to a resolution of 2.6 Å and belonged to space group *P*6₅22, with unit-cell parameters *a* = *b* = 107.2, *c* = 551.1 Å. The structure determined by molecular replacement showed that the complex consists of a SepCysS dimer, a SepCysE dimer and one tRNA^{Cys} in the asymmetric unit.

1. Introduction

In the cell, each aminoacyl-tRNA synthetase specifically recognizes its assigned amino acid and cognate tRNA to form aminoacyl-tRNA, enabling accurate protein synthesis at the ribosome (Carter, 1993). In most organisms, cysteinyl-tRNA synthetase (CysRS) is responsible for Cys-tRNA^{Cys} synthesis by pairing a cysteine and its cognate tRNA^{Cys}; however, many methanogenic archaea do not have CysRS and cysteine-biosynthesis systems. These organisms use an indirect pathway in which two enzymes, *O*-phosphoseryl-tRNA synthetase (SepRS) and Sep-tRNA:Cys-tRNA synthase (SepCysS), are used for Cys-tRNA^{Cys} synthesis. Firstly, SepRS ligates *O*-phosphoserine to tRNA^{Cys} to form a mischarged intermediate, Sep-tRNA^{Cys}, which is then captured by SepCysS and converted to Cys-tRNA^{Cys} (Sauerwald *et al.*, 2005). SepRS and SepCysS fail to form a stable binary complex, and the *in vitro* activities of both enzymes are very low (Zhang *et al.*, 2008; Hauenstein *et al.*, 2008; Liu *et al.*, 2014), suggesting that other cofactors are necessary for this pathway. Recently, a third protein, SepRS/SepCysS pathway enhancer (SepCysE), was found to be essential for efficient SepRS and SepCysS activity by bridging the two enzymes to form a stable ternary complex. The three proteins constitute a functional unit in a 4:4:4 ratio with a molecular weight of approximately 540 kDa (Liu *et al.*, 2014). Here, we call this functional unit the transsulfursome because of its function in two-step cysteine addition.

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The previously determined crystal structure of SepRS in complex with tRNA^{Cys} revealed how SepRS recognizes the anticodon of tRNA^{Cys} to ensure the fidelity of the first reaction step (Fukunaga & Yokoyama, 2007). Little is known about the interaction between SepCysS and tRNA^{Cys} in the second reaction step. In addition, the previously reported structure of SepCysS in complex with the N-terminal domain of SepCysE [SepCysS–SepCysE(NTD)] elucidated the interaction between SepCysS and SepCysE (Liu *et al.*, 2014), but the structure and function of the C-terminal domain of SepCysE [SepCysE(CTD)] remained unclear. How is tRNA^{Cys} bound to SepCysS during the second reaction step? Does SepCysS also show certain discriminants for Sep-tRNA^{Cys} to guarantee errorless reactions throughout the pathway? A structure of SepCysS–SepCysE with tRNA^{Cys} or Sep-tRNA^{Cys} is required to answer these questions. Here, we report the crystallographic analysis of a subcomplex of the transsulfurosome with tRNA^{Cys} (SepCysS–SepCysE–tRNA^{Cys}). This complex represents the second reaction step of tRNA^{Cys} synthesis in the indirect pathway involving the transsulfurosome. The results of molecular replacement showed that the complex consists of two heterodimers of SepCysS–SepCysE and one tRNA^{Cys} in an asymmetric unit.

2. Materials and methods

2.1. Macromolecule production

Methods for the expression and purification of SepCysS–SepCysE were derived from previous research (Liu *et al.*, 2014). Macromolecule-production information is summarized in Table 1. Two plasmids carrying *Methanococcus jannaschii* SepCysS and SepCysE, respectively, were co-transformed into *Escherichia coli* strain B834(DE3) plus pRARE2 (Novagen) by electroporation. The cells were grown in 3 l Luria broth medium at 310 K until OD₆₀₀ reached 0.6 and co-expression was then induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 16 h of cultivation at 298 K, the cells were harvested by centrifugation at 4500g for 30 min at 283 K and were resuspended in buffer A (50 mM HEPES–NaOH pH 8.0, 300 mM NaCl, 5 mM MgCl₂). The cells were disrupted by sonication in the presence of 0.5 mg ml^{−1} lysozyme, 0.1 mg ml^{−1} DNase I and a tablet of protease-inhibitor cocktail (cOmplete EDTA-free; Roche) followed by heat-treatment at 348 K for 30 min. The cell lysate was then centrifuged at 40 000g for 30 min at 298 K. The supernatant was applied onto a HisTrap HP column (GE Healthcare), and after washing out impurities with 20 mM imidazole the target protein complex was obtained by elution with a linear 20–500 mM imidazole gradient in buffer A. The collected fractions were then loaded onto a HiTrap Heparin HP column (GE Healthcare) to eliminate nucleic acids and were eluted by increasing the NaCl gradient to 1 M in buffer B (50 mM HEPES–NaOH pH 8.0, 5 mM MgCl₂). The eluted proteins were further purified using a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare) equilibrated with buffer C (50 mM HEPES–NaOH pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol). The purified protein was pooled,

Table 1

Macromolecule-production information.

Source organism	<i>M. jannaschii</i>
DNA source	<i>M. jannaschii</i> genome
SepCysS	
Cloning site	NdeI–XhoI
Expression vector	pET-15b (Novagen)
Expression host	<i>E. coli</i> B834(DE3) plus pRARE2 (Novagen)
Complete amino-acid sequence of the construct produced†	MGSSHHHHHSSGLVPRGSHNMELEGPYSKKFEVITLDI–NLDKYNLTRLSTREFINLNP IQRGGILPKEAKKAVY–EYWDGYSVCDYCHGRLEDEVTCPIKDFLEDIAKFLNMDCARPTHGAREGKFIVMHAICKEGDYVVLKNAHYTS–YVAERAKLNVAEVGYEEYPTKYINLEGYKEVIDNL–EDKGKNGVILLTHVDGEYGNLNDAKKVGKIAKEKGI–PFLNLCAYTVGRMPVNGKEVKADFIASGHKSMAASA–PCGILAFSEEFSDKITKTSEKFPVKEIEMLGCTSRGL–PIVTLMASFPHVVERVVKWDEELKKTRYVVDLEKIG–FKQLGIKPKHELDIKFETPVLDIAKKDKRRGFFFYD–ELKKRGIGGIRAGVTKEIKMSVYGLEWEQVEYVVAI–KEIVESCK
SepCysE	
Cloning site	NdeI–XhoI
Expression vector	Modified pCDF-Duet-1
Additional residues	Downstream box‡
Expression host	<i>E. coli</i> B834(DE3) plus pRARE2 (Novagen)
Complete amino-acid sequence of the construct produced†	MNHRVEYSKDLIRKAGISTISQLKAKIRVEKDKKISY–KDAKPGKIDVNEFKKAIYLLIEADDFLYKKAPKHELN–EEEAKFECKLIICKQEHNLKILANFGFEFEEKEIDEG–ALYTVSNKKLFKLLKNKNPNLKVCTEGMLDIEDMRA–IGVPEKALEGLKKKVEIARKNVERFIEKYKPEKIFVV–VEDDKDELLYLRAKNLYNAEKLDADEILD
Synthesis of DNA template for tRNA ^{Cys}	
Forward primer§	5′-TAATACGACTCACTATAGCCGGGTAGTCTAGGGGCTAGGCAGCGGACT-3′
Middle primer	5′-CTAGGCAGCGGACTGCAGATCCGCCTACGTGGGT-3′
Reverse primer¶	5′-TGGAGCCGGGGTGGGATTGAACCCACGTAAGGC-3′

† Amino acids from the vector are underlined. ‡ The downstream box is a sequence element that enhances translation (Sprengart *et al.*, 1996). § The T7 promoter is underlined. ¶ The underlined base G is modified by 2′-O-methylation.

concentrated using an Amicon Ultra 30 kDa MWCO centrifugal concentrator (Millipore) and finally stored at 193 K.

M. jannaschii tRNA^{Cys} was transcribed *in vitro* as described previously, with modification (Nakamura *et al.*, 2006). Firstly, PCR was performed to synthesize template DNA for tRNA^{Cys} transcription. The sequences of the DNA oligonucleotides used in PCR are listed in Table 1. After 5 h transcription at 310 K, the reaction was terminated and the product was purified by loading the sample onto 10% denaturing urea polyacrylamide gel electrophoresis (urea PAGE). The indicated tRNA band was excised under UV light and extracted from the gel using an Elutrap electroelution system (Whatman; Suzuki *et al.*, 2015). The pooled tRNA^{Cys} was precipitated with ethanol and dissolved in a solution consisting of 20 mM HEPES–KOH pH 8.0, 5 mM MgCl₂; the purity was examined by urea PAGE prior to storage at 243 K.

2.2. Crystallization

SepCysS–SepCysE was mixed with tRNA^{Cys} in different ratios and incubated at room temperature for 1 h. Crystallization drops were then set up with commercially available kits (The JCSG Core Suites I–IV, Qiagen) by mixing a 0.75 µl aliquot of the sample with an equivalent volume of reservoir solution. Using a sample consisting of SepCysS–SepCysE and tRNA^{Cys} mixed in a 1:1.2 ratio, with a final protein

concentration of 16 mg ml^{-1} , initial crystals were obtained in several reservoir conditions as follows: (i) 0.1 M MES pH 6.0, 0.2 M Li_2SO_4 , 35% MPD, (ii) 0.1 M Tris-HCl pH 7.0, 0.5 M ammonium sulfate, 10% glycerol, 30% PEG 600, (iii) 0.1 M MES pH 5.0, 2.4 M ammonium sulfate. After optimization of the buffer pH and precipitant concentration, high-quality crystals were obtained for data collection (Fig. 1). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

For data collection, crystals of SepCysS-SepCysE-tRNA^{Cys} were soaked in cryoprotectant solution consisting of reservoir solution with a final concentration of 20% glycerol for several seconds before flash-cooling under a stream of nitrogen gas. The X-ray diffraction experiment was performed on beamline BL41XU at SPring-8, Harima, Japan (proposal Nos. 2014B1033 and 2015B1024). Diffraction data were collected

Figure 1
Crystals of SepCysS-SepCysE-tRNA^{Cys} (the crystals diffracted to $2.6 \text{ \AA}$ resolution).

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	96-well
Temperature (K)	293
Protein concentration (mg ml^{-1})	16
Buffer composition of protein solution	20 mM HEPES-KOH pH 8.0, 100 mM KCl, 5 mM MgCl_2 , 5% glycerol
Composition of reservoir solution	0.1 M MES pH 5.3, 2.37 M ammonium sulfate
Volume and ratio of drop	$0.75 \text{ \mu l}$, 1:1
Volume of reservoir ($\text{\mu l}$)	75

with 0.01° oscillation and 0.01 s exposure time per image, with a total rotation of 180° . The data were processed using the CCP4 and XDS packages (Winn *et al.*, 2011; Kabsch, 2010), and statistical information is summarized in Table 3.

3. Results and discussion

The binding affinity of SepCysS-SepCysE to tRNA^{Cys} was investigated using an electrophoretic mobility shift assay (EMSA). The results showed that in solution, SepCysS-SepCysE bound to tRNA^{Cys} in a 2:1 ratio (Fig. 2a), although the crystals were obtained at a 1:1.2 ratio of SepCysS-SepCysE to tRNA^{Cys}.

After obtaining crystals of SepCysS-SepCysE-tRNA^{Cys}, we checked the components of the crystals using SDS-PAGE and urea PAGE. Bands corresponding to the size of SepCysS and SepCysE in SDS-PAGE and of tRNA^{Cys} in urea PAGE were observed (Figs. 2b and 2c). SDS-PAGE showed degradation of SepCysS and SepCysE (Fig. 2b). To clarify which parts of SepCysS and SepCysE were likely to degrade, we analyzed degraded SepCysS and SepCysE by N-terminal sequencing. The results showed that SepCysS and SepCysE degraded from the 16th and 18th residues at the N-terminus, respectively, which is consistent with the published structure of SepCysS-SepCysE(NTD), which has invisible N-termini for SepCysS

Figure 2
Analysis of SepCysS-SepCysE-tRNA^{Cys}. (a) tRNA^{Cys}-binding analysis of SepCysS-SepCysE by EMSA. The molar ratios of SepCysS-SepCysE to tRNA^{Cys} in the samples in lanes 1, 2, 3 and 4 are 0:1, 1:1, 2:1 and 4:1, respectively. (b) SDS-PAGE analysis of crystals. A dissolved crystal was analyzed on a 15% SDS-PAGE gel stained with Coomassie Brilliant Blue. Purified SepCysS-SepCysE was used as a control. (c) The presence of tRNA^{Cys} in the crystals was confirmed by 10% urea PAGE stained with ethidium bromide. *In vitro*-transcribed tRNA^{Cys} was used as a control.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL41XU, SPring-8
Wavelength (Å)	0.979
Temperature (K)	100
Detector	PILATUS3 6M
Crystal-to-detector distance (mm)	500
Rotation range per image (°)	0.01
Total rotation range (°)	180
Exposure time per image (s)	0.01
Space group	<i>P</i> ₆ ₅ ₂₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	107.2, 107.2, 551.1
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.043
Resolution range (Å)	50–2.60 (2.75–2.60)
Total No. of reflections	448086 (72295)
No. of unique reflections	59262 (9258)
Completeness (%)	99.5 (98.6)
Multiplicity	7.56 (7.81)
$\langle I/\sigma(I) \rangle$	15.97 (2.01)
$R_{\text{meas}}^{\dagger}$ (%)	9.7 (85.7)
Overall <i>B</i> factor from Wilson plot (Å ²)	64.2

$\dagger R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively.

and SepCysE. The dissolved crystals were further analyzed by TOF mass spectrometry, which confirmed the existence of SepCysS, SepCysE and tRNA^{Cys} with the corresponding sizes.

The diffraction pattern of snapshot images showed that the unit cell is very large, and the subsequent analysis of reflections with *iMosflm* (Battye *et al.*, 2011) indicated that the length of one axis is over 500 Å. Thus, 0.01° oscillations were used to collect the data set in order to avoid reflection overlap (Fig. 3). The space group and unit cell were first estimated using *POINTLESS* from the *CCP4* package (Evans, 2006).

Figure 3

X-ray diffraction image of a SepCysS–SepCysE–tRNA^{Cys} crystal. The resolution limit of 2.6 Å is marked with a dashed circle.

Based on the results from *POINTLESS*, diffraction data were successfully indexed, integrated, scaled and merged to a resolution of 2.6 Å using the *XDS* package. The space group of the SepCysS–SepCysE–tRNA^{Cys} crystals was defined as *P*₆₁₂₂ or *P*₆₅₂₂, with unit-cell parameters *a* = *b* = 107.2, *c* = 551.1 Å.

The structure of SepCysS–SepCysE–tRNA^{Cys} was resolved by molecular replacement using *AutoMR* from the *PHENIX* package (Adams *et al.*, 2010; McCoy *et al.*, 2007). The structures of SepCysS–SepCysE(NTD) from *M. jannaschii* (PDB entry 3wkr; Liu *et al.*, 2014) and of tRNA^{Cys} from *E. coli* (PDB entry 1b23; Nissen *et al.*, 1999) were used as search models. The SepCysS–SepCysE(NTD) and tRNA^{Cys} solutions were successfully found with a TFZ score of 93.4 in space group *P*₆₅₂₂. As a result, two heterodimers of a SepCysS dimer, a SepCysE dimer and one tRNA^{Cys} were observed in the asymmetric unit (Matthews coefficient *V*_M of 3.19 Å³ Da^{−1} with 61% solvent content) in accordance with the binding ratio determined by EMSA. Model building of the missing C-terminal domain of SepCysE and refinement of the complex structure are currently being performed.

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