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Crystallization and preliminary X-ray analysis of the archaeosine tRNA-guanine transglycosylase from *Pyrococcus horikoshii*

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The archaeosine tRNA-guanine transglycosylase from the hyperthermophilic archaeon *Pyrococcus horikoshii* was crystallized and preliminary X-ray characterization was performed. Single crystals were grown by the hanging-drop vapour-diffusion method, using sodium/potassium phosphate and sodium acetate as precipitants. The space group is $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 99.28\,(14)$, $c = 363.74\,(56)$ Å. The cryocooled crystals diffracted X-rays beyond 2.2 Å resolution using synchrotron radiation from station BL44XU at SPring-8 (Harima). Selenomethionine-substituted protein crystals were prepared in order to solve the structure by the MAD phasing method.

1. Introduction

Various modified nucleosides have been found in tRNA (Sprinzl *et al.*, 1998; Limbach *et al.*, 1994; McCloskey & Crain, 1998); they are thought to play an important role in the translation of genetic information (Persson, 1993; Björk *et al.*, 1987). Many tRNA-modification enzymes corresponding to the modified nucleosides have also been found; however, their characteristics, including the higher order structures of these enzymes, are still largely unknown (Björk, 1995). In particular, the mechanism of tRNA recognition by these enzymes remains to be elucidated. The tRNA-guanine transglycosylase (TGT) introduces the modified nucleoside into tRNA by a base-exchange reaction without cleaving the phosphodiester bonds. TGT exists widely in eukarya, bacteria and archaea. In bacteria and eukaryotes (with the exception of some organisms such as yeast), TGT is involved in the biosynthesis of the hypermodified nucleoside queuosine (Q; 7-{[(4,5-*cis*-dihydroxy-2-cyclopenten-1-yl)-amino]methyl}-7-deazaguanosine; Nishimura, 1983), whereas in archaea, TGT is involved in the biosynthesis of the nucleoside archaeosine (G*; 7-formamidino-7-deazaguanosine; Watanabe *et al.*, 1997) (Fig. 1).

The queuosine nucleoside exists in position 34 of the bacterial and eukaryotic tRNAs corresponding to Tyr, His, Asp and Asn (Nishimura, 1983). The roles of queuosine are not fully understood, but it is involved not only in the fine tuning of translation (Meier *et al.*, 1985; Durand *et al.*, 1994) but also in cellular events such as development, differentiation, aging and cancer in eukaryotes (Dirheimer *et al.*, 1995). In bacteria, the queuosine is synthesized *de novo*. The precursor, 7-aminomethyl-7-deazaguanine ($preQ_1$), is synthesized from GTP (Kuchino *et al.*, 1976), is incorporated in tRNA by queuosine TGT (QueTGT; Okada & Nishimura, 1979; Okada *et al.*, 1979) and is further modified to queuosine within the tRNA (Slany & Kersten, 1994; Frey *et al.*, 1988; Kinzie *et al.*, 2000) (Fig. 1). In contrast, in eukaryotes Q base is a nutrient and the eukaryotic QueTGT directly incorporates the Q base into tRNA (Katze *et al.*, 1984; Shindo-Okada *et al.*, 1980; Slany & Muller, 1995). The bacterial QueTGT recognizes the minihelix composed of the anticodon arm as a substrate *in vitro* and is therefore thought to bind the anticodon stem and to recognize the $U^{33}G^{34}U^{35}$ sequence of the anticodon loop (Curnow *et al.*, 1993; Nakanishi *et al.*, 1994; Curnow & Garcia, 1995). The crystal structure of the *Zymomonas mobilis* QueTGT suggests that the positively charged zinc-binding subdomain binds to the tRNA anticodon arm and the negatively charged catalytic pocket recognizes the $U^{33}G^{34}U^{35}$ sequence of the anticodon loop (Romier *et al.*, 1996). On the other hand, *in vivo* biochemical experiments have suggested that the eukaryotic QueTGT recognizes the overall structure of the correctly folded tRNA (Carbon *et al.*, 1983; Grosjean *et al.*, 1996).

The archaeosine nucleoside occurs only in archaeal tRNA at position 15 in the D loop, a site that is not modified in the other two phylogenic domains (Gregson *et al.*, 1993). Archaeosine has been found in the tRNAs corresponding to 15 amino acids (Gupta, 1984). The role of the archaeosine is not clear, but it is thought to contribute to the structual stability of the tRNA (Gregson *et al.*, 1993).

The archaeosine nucleoside is synthesized *de novo* as follows (Watanabe *et al.*, 1997) (Fig. 1). Firstly, the archaeosine TGT (ArcTGT) incorporates 7-cyano-7-deazaguanine ($preQ_0$) into position 15 of the tRNA, which is then further modified to archaeosine. The ArcTGT has a catalytic domain which shows high sequence similarity to that of QueTGT and therefore ArcTGT is considered to catalyze the base-exchange reaction by a mechanism similar to that of QueTGT (Romier *et al.*, 1997).

In contrast, the tRNA-recognition mechanism of ArcTGT is considered to be quite different from that of QueTGT, since the target position in tRNA is completely different. In a folded tRNA, the D loop provides tertiary base pairs with the TΨC loop and forms the core of the L-shaped tRNA. Therefore, ArcTGT must disrupt these base pairs and pull the D loop from the core of the tRNA, although ArcTGT consumes no energy source such as ATP in the catalysis. A previous *in vitro* analysis (Watanabe *et al.*, 2000) suggested that guanine 15 of the tRNA is strictly recognized by ArcTGT, while no residue other than guanine 15 is specifically recognized. Moreover, ArcTGT does not require the correctly folded L-shape of tRNA for the recognition. Compared with QueTGT, ArcTGT has an extra C-terminal region composed of about 300 amino-acid residues which shows no sequence similarity with any proteins with known structures. Recent progress in the genome analyses of various organisms has revealed that this C-terminal region contains a putative RNA-recognition domain, the so-called PUA domain, which is often observed in archaeal and eukaryotic RNA-modification enzymes (Aravind & Koonin, 1999). This suggests that this C-terminal region is involved in tRNA recognition.

To elucidate the tRNA-recognition mechanism of ArcTGT, we purified and crystallized ArcTGT, a 66 kDa protein, from the hyperthermophilic archaeon *P. horikoshii* and performed an X-ray analysis of these crystals. The selenomethionine-substituted protein was purified and crystallized for MAD phasing.

2. Experimental and results

2.1. Crystallization and crystal characterization

The *P. horikoshii* archaeosine TGT (ArcTGT) protein was overexpressed and purified as previously described in Watanabe *et al.* (2000). Prior to crystallization screening, the protein solution was dialyzed against 10 m*M* Tris–HCl buffer pH 7.5 containing 410 m*M* NaCl, 5 m*M* $MgCl_2$ and 10 m*M* 2-mercaptoethanol at 277 K and was concentrated to 10 mg ml^{-1} using a Centricon 10 ultrafiltration device (Amicon) at 277 K. Initial crystallization screening experiments were carried out at 293 K by the hanging-drop vapour-diffusion method using the commercially available Crystal Screen I, Crystal Screen II and Natrix kits (Hampton Research). Each experiment utilized a 2 µl drop containing equal volumes of concentrated protein solution and screening solution and each hanging drop was equilibrated against a 500 µl reservoir of screening solution. Polycrystals were observed with the screening condition containing ammonium sulfate, lithium sulfate or sodium/potassium phosphate as the precipitant after several weeks. The thin plate-like polycrystals (form *A*; Fig. 2) were obtained from the screening condition containing 0.1 *M* Na HEPES pH 7.5, 0.8 *M* KH_2PO_4 and 0.8 *M* NaH_2PO_4. Optimizations around this condition involved variation of pH, the concentration of precipitant and temperature, and the inclusion of additives. The addition of 0.2%(*w*/*v*) benzamidine hydrochloride to the drop changed the thin polycrystals to thick plate-like single crystals. This condition was further improved by the addition of sodium acetate (70 m*M* to the drop and 140 m*M* to the reservoir). Under this refined condition, single crystals grew to maximum dimensions of 0.3 × 0.3 × 0.1 mm in one week (form *B*; Fig. 2). As the form *B* crystals diffracted X-rays weakly at room temperature, mesurements under cryoconditions were required. We soaked the form *B* crystals in a mother liquor containing glycerol, ethyleneglycol or trehalose as cryoprotectants, but the crystals dissolved under all conditions tested. Although the stabilization of the crystals required an increase in the ionic strength of the harvest solution, we could not increase the concentration of the KH_2PO_4 solution to more than 1.5 *M*. Finally, by replacing the potassium salt (KH_2PO_4) with sodium salt (NaH_2PO_4) and adjusting the pH with K_2HPO_4, we found that the form *B* crystals were relatively stable in a cryoprotectant solution containing 0.15 *M* Na HEPES pH 7.5, 3.2 *M* NaH_2PO_4, 0.9 *M* K_2HPO_4 and 30%(*v*/*v*) glycerol.

Preliminary characterization of the form *B* crystals was carried out using an R-AXIS IV area detector (Rigaku) mounted on an UltraX18 rotating Cu anode X-ray generator (Rigaku) operated at 40 kV, 100 mA. The form *B* crystal was flash-cooled in a nitrogen-gas Cryostream at 100 K and diffracted to about 4.0 Å. The data set from this crystal was collected to 3.0 Å resolution at station BL45PX at SPring-8 (Harima, Japan) using an R-AXIS IV area detector. These crystals belong to the space group *C*2, with unit-cell parameters a = 139.8 (8), b = 227.7 (21), c = 133.2 (21) Å, β = 117.8 (3)°. However, these crystals showed extremely high mosaicity (about 2.0°) and were not considered to be suitable for a structure determination.

Further refinement of the crystallization conditions was carried out by partly blending in other types of precipitant.

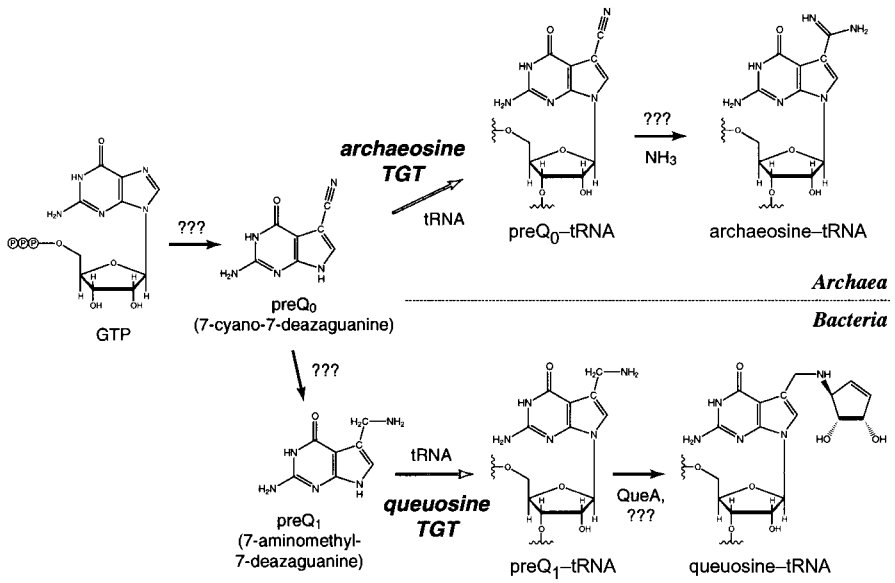

Figure 1
Biosynthetic pathway of 7-deazaguanine derivatives in archaea and bacteria.

Table 1
Data-reduction statistics of a native and a selenomethionine-labelled form *C* crystals.

Values in parentheses are for the highest resolution shell.

	Native	SeM edge	SeM peak	SeM remote H	SeM remote L
Wavelength (Å)	0.900000	0.979311	0.979155	0.973923	0.982025
Resolution (Å)	50–2.2 (2.24–2.2)	50–3.2 (3.26–3.2)	50–3.2 (3.26–3.2)	50–3.2 (3.26–3.2)	50–3.2 (3.26–3.2)
Total reflections	530165	383410	385326	383965	381037
Unique reflections	88967	31708	31624	31670	31757
Redundancy	6.0	12.1	12.2	12.1	12.0
Completeness (%)	95.3 (93.0)	99.8 (99.9)	99.8 (99.9)	99.8 (99.9)	99.8 (99.8)
$\overline{I/\sigma(I)}$	34.0 (7.10)	27.6 (8.24)	29.7 (9.68)	28.9 (9.00)	24.2 (6.56)
$R_{\rm sym}$† (%)	9.2 (25.6)	8.4 (20.2)	9.2 (18.7)	8.7 (19.5)	8.9 (23.5)

† $R_{\rm sym} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i |I_{h,i}|$, where h refers to unique reflection indices and i indicates symmetry-equivalent indices.

Needle-like polycrystals were grown by partly replacing the phosphate with acetate. Furthermore, changing the crystallization templature from 293 to 277 K significantly improved the polycrystals to large stick-like single crystals (form *C*; Fig. 2). Finally, the form *C* single crystals grew to maximum dimensions of 0.1 × 0.1 × 0.5 mm in a few months with a solution containing 60 m*M* Na HEPES pH 7.5, 60 m*M* sodium cacodylate pH 6.5, 480 m*M* KH_2PO_4, 480 m*M* NaH_2PO_4 and 840 m*M* sodium acetate.

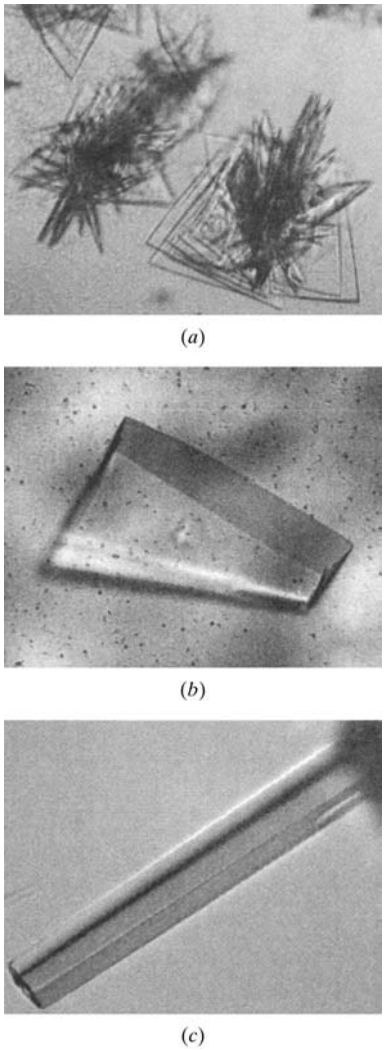

Figure 2
Native crystals of *P. horikoshii* ArcTGT. (*a*) form *A*, (*b*) form *B*, (*c*) form *C*.

The form *C* crystals only diffracted X-rays to 10 Å at room temperature, suggesting severe X-ray damage. The crystals were stable for several weeks in harvesting solution (1.4× reservoir solution) containing 15%(*v*/*v*) glycerol as a cryoprotectant. The crystals were flash-cooled in a nitrogen-gas Cryostream at 100 K and diffracted X-rays to about 5 Å using an R-AXIS IV area detector mounted on an UltraX18 X-ray generator. The data set was collected on station BL44XU at SPring-8 (Harima, Japan) using an array CCD detector PX210 (Oxford Instruments). With a strong undulator X-ray source, the crystals diffracted to 2.0 Å at the maximum. The resulting data set was processed and scaled using the *HKL*2000 program suite (Otwinowski & Minor, 1997). The crystals belong to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 99.28$ (14), $c = 363.74$ (56) Å (Table 1). Therefore, if the asymmetric unit contains two and three molecules, then the $V_{\rm M}$ value should be 3.4 and 2.3 Å^3 Da^{-1}, respectively.

The N-terminul half of ArcTGT shows sequence similarity to QueTGT (26% identity over 356 residues). We tried to determine the initial phases by the molecular-replacement method using the QueTGT structure (Romier *et al.*, 1996) as a search model with the program *AMoRe* (Navaza, 1994). However, we could obtain no interpretable solution, which can be ascribed to the fact that the C-terminal half of ArcTGT is completely absent in QueTGT.

2.2. Preparation of heavy-atom derivatives

To search for heavy-atom derivatives, native form *C* crystals were soaked in 20 µl of harvesting solution containing various heavy-atom salts using sitting-drop conditions at 293 K. Almost all mercury compounds produced non-isomorphous derivatives, while other heavy-atom compounds were not incorporated in the crystal. Lanthanoid and actinoid compounds could not be used, because they were insoluble in a harvest solution containing such a high concentration of phosphate salts.

Considering that phosphate ions may disturb the binding of heavy atoms to the protein, we tried to change the precipitant in the harvest solution. By replacing the phosphate and acetate with 20%(*w*/*v*) PEG 1500, the crystals were stable in the harvest solution for several days, while the diffraction pattern became irregular. By replacing the phosphate and acetate with 2.5 *M* Li_2SO_4 or 2.5 *M* $(NH_4)_2SO_4$, the crystals were stable and diffracted X-rays as well as under the original conditions, while the mosaicity became rather high and the diffraction spots were streaked.

2.3. Preparation and analysis of selenomethionine-labelled crystals

Therefore, to determine the phases by the MAD method, selenomethionine-labelled ArcTGT was overexpressed in the methionine auxotrophic *E. coli* strain B834(DE3) (Novagen), which was transformed with the overexpression vector and the transformed strain was cultured in a minimal medium in which methionine was substituted with selenomethionine. The labelled protein was purified in the same way as the native protein. Crystals of the labelled protein were obtained under the same conditions as the native crystals, but they did not grow large enough for data collection. By reducing the precipitant concentration, we could obtain crystals as large as the native crystals.

X-ray fluorescence spectra of the selenomethionine-labelled crystal were measured at station BL44B2 at SPring-8 (Harima, Japan), but we could not detect the selenium fluorescence. The mass spectrum of the selenomethionine-labelled protein revealed a reasonable increase in the mass compared with the native protein (data not shown). It was possible that the arsenic included in the cacodylate buffer might absorb the fluorescence of the selenium and we therefore replaced the cacodylate with HEPES in the crystallization and cryoprotectant solutions. Finally, we were able to measure the X-ray fluorescence of selenium using a crystal grown under arsenic-free crystallization conditions at station BL41XU at SPring-8 (Harima, Japan). Calculation of anomalous scattering factors from the raw fluorescence spectrum was performed using the program

CHOOCH (Evans & Pettifer, 2001), as shown in Fig. 3. The data sets at four wavelengths (peak, edge, and high and low energy remote) were collected from the same station using a marCCD165 detector (mar research). The resulting data sets were processed with the *HKL*2000 program suite (Otwinowski & Minor, 1997), as shown in Table 1. The clearly resolved selenium *K* edge and the quality of the diffraction data indicate that the selenium MAD phasing will be successful.

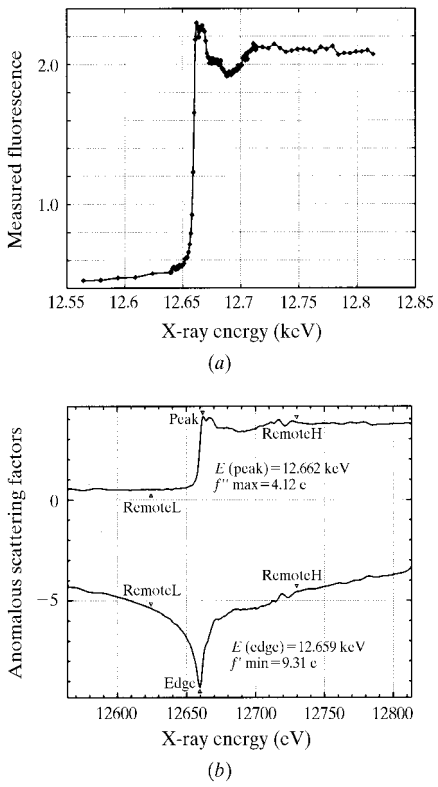

Figure 3
(*a*) X-ray fluorescence spectrum of a single crystal of SeMet *P. horikoshii* ArcTGT; (*b*) anomalous scattering factors determined from the spectrum in (*a*) as a function of X-ray energy.

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