STRUCTURAL BIOLOGY COMMUNICATIONS
ISSN 2053-230X

Crystallographic study of the 2-thioribothymidine-synthetic complex TtuA–TtuB from *Thermus thermophilus*

Minghao Chen,[a] Shun Narai,[a] Naoki Omura,[a] Naoki Shigi,[b] Sarin Chimnaronk,[c] Yoshikazu Tanaka[a,c,d]* and Min Yao[a,c]

[a]Graduate School of Life Science, Hokkaido University, Kita 10, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan, [b]Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan, [c]Faculty of Advanced Life Science, Hokkaido University, Kita 10, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan, and [d]PRESTO, Japan Science and Technology Agency, Kita 10, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan. *Correspondence e-mail: tanaka@sci.hokudai.ac.jp

Received 10 August 2016
Accepted 7 September 2016

Edited by T. C. Terwilliger, Los Alamos National Laboratory, USA

Keywords: post-transcriptional modification; sulfur transfer; ubiquitin-like protein; Zn-MAD; TtuA; TtuB.

The ubiquitin-like protein TtuB is a sulfur carrier for the biosynthesis of 2-thioribothymidine (s^2T) at position 54 in some thermophilic bacterial tRNAs. TtuB captures a S atom at its C-terminus as a thiocarboxylate and transfers it to tRNA by the transferase activity of TtuA. TtuB also functions to suppress s^2T formation by forming a covalent bond with TtuA. To explore how TtuB interacts with TtuA and switches between these two different functions, high-resolution structure analysis of the TtuA–TtuB complex is required. In this study, the TtuA–TtuB complex from *Thermus thermophilus* was expressed, purified and crystallized. To mimic the thiocarboxylated TtuB, the C-terminal Gly residue was replaced with Cys (G65C) to obtain crystals of the TtuA–TtuB complex. A Zn-MAD data set was collected to a resolution of 2.5 Å. MAD analysis successfully determined eight Zn sites, and a partial structure model composed of four TtuA–TtuB complexes in the asymmetric unit was constructed.

1. Introduction

Thiolation is a general transfer RNA (tRNA) post-transcriptional modification that is widely conserved in all three kingdoms of life (Machnicka *et al.*, 2013). Thiolation is related to various important cellular functions such as maintenance of translation (Krüger *et al.*, 1998; Yarian *et al.*, 2002), recognition of aminoacyl-tRNA synthases (Krüger & Sørensen, 1998), sensing ultraviolet (UV) radiation stress (Caldeira de Araujo & Favre, 1985) and stabilization of the ternary structure of tRNA (Watanabe *et al.*, 1976; Shigi, Suzuki *et al.*, 2006; Alings *et al.*, 2015). A total of four tRNA thiolation modification groups have been found to date: 2-thiouridine (s^2U), 4-thiouridine (s^4U), 2-thiocytidine (s^2C) and 2-methylthio-N^6-alkyladenodine (ms^2x^6A) (Boschi-Muller & Motorin, 2013). Each modification is introduced *via* distinct sulfur-transfer pathways using specific sulfur transferases (Shigi, 2014), although the S atoms for these thiolation modifications are commonly supplied by the cysteine desulfurase IcsS. Although the pathways for introducing these thiolation modifications have been fully described, mechanistic knowledge of the pathways remains limited.

TtuB and TtuA are the sulfur carrier and transferase, respectively, for the biosynthesis of 2-thioribothymidine (s^2T)

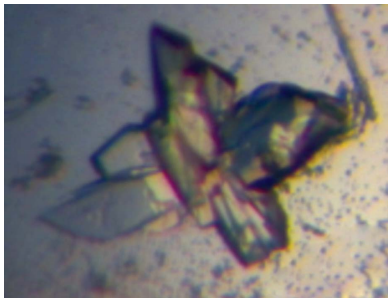


Table 1
Macromolecule-production information.

Macromolecule	*Tth*TtuA	*Tth*TtuB (G65C)
Source organism	*T. thermophilus*	
DNA source	UniProt Q72LF3	UniProt Q72LF4
Expression vector	pCDFDuet-1	Modified pET-28a
Expression host	*E. coli* B834 (DE3), co-expression	
Complete amino-acid sequence of the construct produced†	MVCKVCGQKAQVEMRSRGLALCREHYLDWFVKETERAIRRHR- MLLPGERVLVAVSGGKDSLALWDVLSRLGYQAVGLHIELG- IGEYSKRSLEVTQAFARERGLELLVVDLKEAYGFGVPELA- RLSGRVACSACGLSKRYIINQVAVEEGFRVVATGHNLDDE- AAVLFGNLLNPQEETLSRQGPVLPEKPGLAARVKPFYRFS- EREVLSYTLLRGIRYLHEECPNAKGAKSLLYKEALNLVER- SMPGAKLRFLDGFLEKIRPRLDVGEEVALRECERCGYPTT- GAVCAFCRMWDAVYRRAKKRKLLPEEVSFRPRVKPLRAG	MGSSHHHHHHSSGLVPRGSHMRVVLRLPERKEVEVKGNRPLR- EVLEELGLNPETVVAVRGEELLTLEDEVREEDTLEVLSAI- SGC

† The artifactual sequences, *i.e.* His_6 tag, TEV protease recognition site (LVPRGS) and point mutation G65C, are underlined.

at position 54 in some thermophilic bacterial tRNAs (Shigi, Sakaguchi *et al.*, 2006), which is important for survival in a high-temperature environment (Watanabe *et al.*, 1976; Shigi, Suzuki *et al.*, 2006). TtuB, composed of approximately 65 amino-acid residues, is a homologue of eukaryotic ubiquitin (sharing about 35% identity). TtuB contains a characteristic Gly-Gly sequence at the C-terminus, which is used for holding an S atom on the C-terminus as a thiocarboxylate group (Shigi *et al.*, 2008). This S atom is transferred to tRNA by the enzymatic activity of a sulfur transferase, TtuA. It has been reported that TtuB and TtuA form a stable complex during sulfur transfer to tRNA (Shigi, Sakaguchi *et al.*, 2006; Shigi *et al.*, 2008). Similar sulfur carriers are also found in other biosynthetic pathways of sulfur-containing biomolecules, such as ThiS in thiamin biosynthesis and MoaD in molybdenum-cofactor biosynthesis (Begley *et al.*, 2012; Iobbi-Nivol & Leimkühler, 2013).

Furthermore, TtuB has been reported to be a bifunctional ubiquitin-like protein in eubacteria (Shigi, 2012). It forms a covalent linkage with Lys137, Lys226 and Lys229 of TtuA in a ubiquitin-like manner. These covalent linkages cause inhibition of the sulfur-transfer activity of TtuA; TtuB acts not only as a sulfur carrier, but also as a post-translational modifier. Interestingly, ubiquitin-related modifier-1 (Urm1), a similar bifunctional ubiquitin-like protein that acts both as a sulfur carrier and as a post-translational modifier, has similarly been found in eukaryotic cells (Leidel *et al.*, 2009). These observations suggest that the eukaryotic ubiquitin system evolved from the prokaryotic sulfur-transfer system.

Although the structure of TtuA from *Pyrococcus horikoshii* has previously been reported (Nakagawa *et al.*, 2013), the mechanism by which TtuB interacts with TtuA and switches between two distinct functions remains poorly understood. To address these questions, elucidation of the three-dimensional structure of the complex between TtuA and TtuB is required. In the present study, we expressed, purified and crystallized the TtuA–TtuB complex from *Thermus thermophilus* (hereafter referred to as *Tth*TtuA–TtuB). Zn-MAD data were collected to a resolution of 2.5 Å, and multiple-wavelength anomalous diffraction (MAD) analysis successfully identified eight Zn sites.

2. Materials and methods

2.1. Cloning, expression and purification of TtuA–TtuB

A DNA fragment encoding *Tth*TtuA was cloned into the NcoI and HindIII sites of the pCDFDuet-1 vector (Novagen). A DNA fragment encoding *Tth*TtuB was cloned into the NdeI and XhoI sites of a modified pET-28a vector (Novagen), in which a *Tobacco etch virus* (TEV) protease recognition site was introduced between the His_6 tag and the N-terminus of the target protein (Asano *et al.*, 2015). *Tth*TtuA and *Tth*TtuB were co-expressed in *Escherichia coli* strain B834 (DE3). Macromolecule-production information is summarized in Table 1. *E. coli* strain B834 (DE3) cells harbouring expression vectors for both *Tth*TtuA and *Tth*TtuB were incubated on lysogeny broth (LB)–agar plates containing 50 mg l^{-1} streptomycin and 25 mg l^{-1} kanamycin overnight at 310 K. A single colony was inoculated into 100 ml LB preculture containing 50 mg l^{-1} streptomycin and 25 mg l^{-1} kanamycin and was incubated overnight at 310 K with shaking at 150 rev min^{-1}. A 100 ml aliquot of the preculture was added to 900 ml LB culture containing 50 mg l^{-1} streptomycin and 25 mg l^{-1} kanamycin and incubated at 310 K. When the absorption at 600 nm reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 m*M*. The culture was further incubated at 298 K for an additional 20 h. The *E. coli* cells were collected by centrifugation at 4000*g* for 10 min. Further procedures were conducted under anaerobic conditions consisting of 5% hydrogen and 95% nitrogen gas (Vinyl Anaerobic Chamber, Coy). The collected cells were sonicated for 20 min on ice in a sonication buffer consisting of 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.6, 200 m*M* ammonium sulfate, 50 m*M* ammonium acetate, 1 m*M* magnesium acetate, 12% glycerol, 0.1% Triton X-100, followed by heat treatment at 343 K for 20 min. The precipitation was then removed by centrifugation at 7000*g* for 30 min. The supernatant was loaded onto a HisTrap HP column (GE Healthcare, Waukesha, Wisconsin, USA) pre-equilibrated with buffer *A* (25 m*M* HEPES pH 7.6, 200 m*M* ammonium sulfate, 50 m*M* ammonium acetate, 1 m*M* magnesium acetate, 12% glycerol). After washing with 15 ml buffer *A*, the adsorbed protein was eluted with a 0–0.5 *M*

gradient of imidazole in buffer *A*. Fractions containing the *Tth*TtuA–TtuB complex were desalted on a PD-10 desalting column (GE Healthcare) pre-equilibrated with buffer *A* and were then further purified on a HiLoad 16/60 Superdex 200 column (GE Healthcare). Purified *Tth*TtuA–TtuB complex was concentrated to 10 mg ml^{-1} and used for crystallization. *Tth*TtuA in complex with the *Tth*TtuB (G65C) mutant was prepared by the same procedure as used for wild-type (WT) *Tth*TtuA–TtuB.

2.2. Crystallization

The initial screening of crystallization conditions for each sample was performed with The JCSG I–IV, Classics, Classics II, MPD, PEGs, PEGs II, Protein Complex, Anions and Cations Suites (Qiagen) crystallization kits using the sitting-drop vapour-diffusion method at 293 K under anaerobic conditions. Although WT *Tth*TtuA–TtuB did not crystallize, crystals of the *Tth*TtuA–TtuB (G65C) mutant were successfully obtained. Crystals of the *Tth*TtuA–TtuB (G65C) mutant suitable for further experiments grew from a buffer consisting of 0.1 *M* HEPES pH 7.6, 6%(*w*/*v*) PEG 8000, 4%(*w*/*v*) ethylene glycol. Crystallization information is provided in Table 2.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	MRC 2 96-well crystallization plate
Temperature (K)	293
Protein concentration (mg ml^{-1})	10
Buffer composition of protein solution	25 m*M* HEPES pH 7.6, 200 m*M* ammonium sulfate, 50 m*M* ammonium acetate, 1 m*M* magnesium acetate, 12% glycerol
Composition of reservoir solution	0.1 *M* HEPES pH 7.6, 6%(*w*/*v*) PEG 8000, 4%(*w*/*v*) ethylene glycol
Volume and ratio of drop	1 µl, 1:1
Volume of reservoir (µl)	70

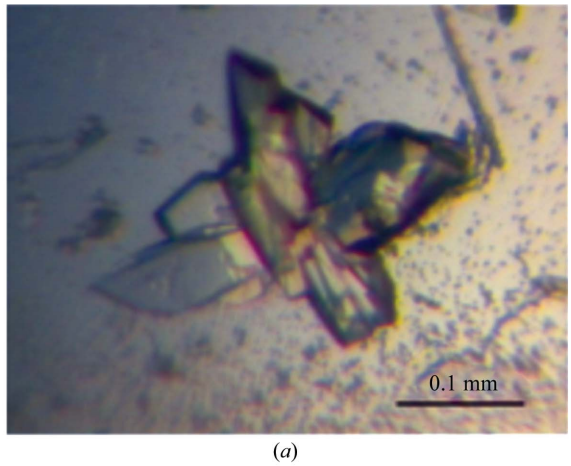
2.3. Data collection and processing

An X-ray diffraction experiment was conducted at 100 K on the NE3A beamline at Photon Factory, Tsukuba, Japan. Crystals were mounted on the diffractometer after soaking in a buffer consisting of 0.1 *M* HEPES pH 7.6, 25%(*w*/*v*) PEG 6000, 4%(*w*/*v*) ethylene glycol. Three wavelengths were chosen for the MAD data on the basis of the fluorescence spectrum of the Zn *K* absorption edge: peak, 1.28251 Å; edge, 1.28311 Å; remote, 1.00000 Å. The MAD data set was collected to a resolution of 2.5 Å. The data were indexed, integrated and scaled with *XDS* (Kabsch, 2010).

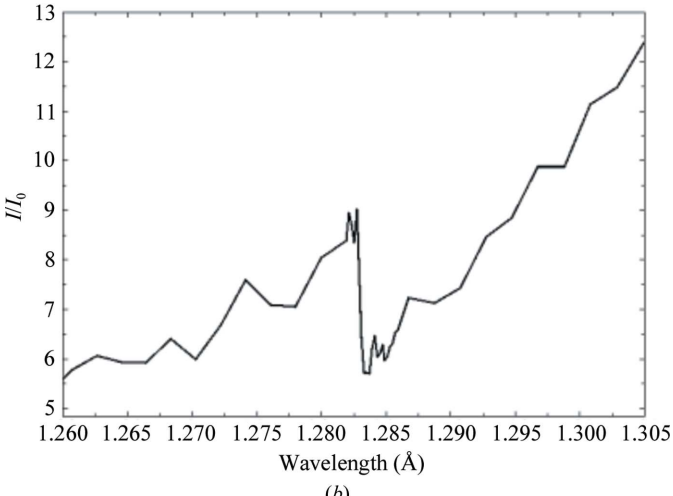
3. Results and discussion

*Tth*TtuA and *Tth*TtuB are reported to form a stable complex *in vivo* (Shigi, Sakaguchi *et al.*, 2006). Therefore, in the present study we attempted to express *Tth*TtuA and *Tth*TtuB together in *E. coli* cells and to purify the *Tth*TtuA–TtuB complex spontaneously formed in the cell using a His_6 tag attached to *Tth*TtuB. SDS–PAGE of the fractions eluted from nickel-affinity chromatography clearly showed that TtuA is co-purified with TtuB, indicating that the *Tth*TtuA–TtuB complex forms spontaneously in the *E. coli* cells. Although an excess of TtuB was present, further purification using size-exclusion chromatography yielded pure *Tth*TtuA–TtuB complex (see Supporting Information). *Tth*TtuA–TtuB had a strong tendency to aggregate, which hampered further experiments. Since we expected TtuA to be a sulfur transferase that transfers S atoms using Cys residue(s) and that the redox state of Cys residue may influence the protein stability, we tried to prepare the *Tth*TtuA sample under anaerobic conditions. We finally determined that *Tth*TtuA is unstable under aerobic conditions but stable under anaerobic conditions. Furthermore, a high concentration (200 m*M*) of ammonium sulfate is effective in enhancing the solubility of TtuA–TtuB. Finally, the *Tth*TtuA–TtuB complex was successfully obtained with high yield and with a purity suitable for crystallization (Supplementary Fig. S1).

Next, we screened crystallization conditions for the purified *Tth*TtuA–TtuB complex using commercially available crystallization kits. Unfortunately, no crystals of the WT *Tth*TtuA–TtuB complex were obtained. Previous biochemical data indicated that *Tth*TtuB carries an S atom using its thio-



(*a*)



(*b*)

Figure 1
(*a*) A crystal of the *Tth*TtuA–TtuB complex obtained by the sitting-drop vapour-diffusion method. (*b*) X-ray absorption spectrum of the *Tth*TtuA–TtuB complex crystal near the Zn *K* absorption edge.

Table 3
Data-collection and processing statistics for the *Tth*TtuA–TtuB (G65C) complex.

Values in parentheses are for the outer shell.

	Peak	Edge	Remote
Diffraction source	NE3A, Photon Factory		
Detector	Quantum 270, ADSC		
Space group	*P*1		
a, *b*, *c* (Å)	54.1, 93.9, 97.5		
α, β, γ (°)	109.2, 104.6, 106.9		
Temperature (K)	100		
Crystal-to-detector distance (mm)	300		
Rotation range per image (°)	1		
Total rotation range (°)	360		
Exposure time per image (s)	1		
Wavelength (Å)	1.28251	1.28311	1.00000
Resolution range (Å)	47.8–2.50 (2.65–2.50)	47.9–2.50 (2.65–2.50)	47.9–2.50 (2.65–2.50)
Mosaicity (°)	0.414	0.420	0.410
Total No. of reflections	207808	208416	210467
No. of unique reflections	105373	105675	106753
Completeness (%)	94.9 (93.2)	94.8 (92.2)	95.8 (94.3)
Multiplicity	1.97 (1.87)	1.97 (1.87)	1.97 (1.87)
$\langle I/\sigma(I)\rangle$	16.24 (2.79)	15.88 (2.39)	14.90 (1.93)
$R_{\rm meas}$† (%)	5.5 (39.6)	5.9 (47.2)	6.7 (59.1)
$R_{\rm merge}$‡ (%)	3.9 (28.0)	4.2 (33.4)	4.7 (41.2)
$CC_{1/2}$§	99.8 (85.8)	99.8 (80.8)	99.8 (73.0)
Overall *B* factor from Wilson plot (Å^2)	51	52	53

† $R_{\rm meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity for all *i* observations of reflection *hkl*. § Percentage correlation between intensities from random half-data sets (Karplus & Diederichs, 2012).

carboxylated C-terminus (Shigi *et al.*, 2008), suggesting that the thiol group located at the C-terminus may play some significant role in the interaction with *Tth*TtuA. We therefore substituted the C-terminal Gly residue with Cys (G65C; Supplementary Fig. S2*c*) to mimic the thiocarboxylated C-terminus (Supplementary Fig. S2*b*). It is expected that the chemical properties of Cys are more similar to those of the thiocarboxylated C-terminus than to Gly in terms of the presence of a free thiol group, and therefore the structure of the G65C mutant represents the binding manner and/or the reaction mechanism of the sulfur transfer more precisely. Using this mutant, we successfully obtained crystals of the *Tth*TtuA–TtuB complex that diffracted well (Fig. 1*a*).

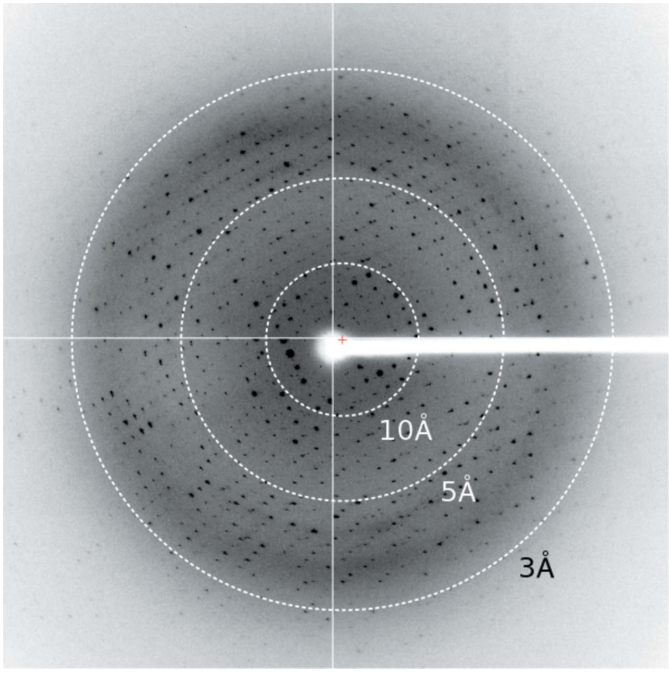


Figure 2
X-ray diffraction image of a crystal of the *Tth*TtuA–TtuB complex.

TtuA contains five characteristic metal ion-binding motifs, C*XX*C/H, suggesting that *Tth*TtuA may contain heavy atoms. We carried out an X-ray absorption spectrum experiment. The result clearly showed the presence of Zn atoms in the *Tth*TtuA–TtuB complex crystal (Fig. 1*b*). Therefore, we attempted to determine the structure using the Zn-MAD method.

Zn-MAD data were collected to a resolution of 2.5 Å (Fig. 2). The crystal of the *Tth*TtuA–TtuB (G65C) mutant complex belonged to space group *P*1, with unit-cell parameters a = 54.1, b = 93.9, c = 97.5 Å, α = 109.2, β = 104.6, γ = 106.9°. Data-collection statistics are summarized in Table 3. Four *Tth*TtuA–TtuB complexes are expected to be located in the asymmetric unit, with a $V_{\rm M}$ value of 2.26 Å^3 Da^{-1} and a solvent content of 45.63%. *SHELXD* (Sheldrick, 2010) successfully determined eight Zn sites. *SOLVE*/*RESOLVE* (Terwilliger & Berendzen, 1999; Terwilliger, 2000, 2003) determined the initial phases with an overall figure of merit of 0.60 and automatically constructed an initial model containing 58% of the residues. Model building and refinement are in progress.

Acknowledgements

The synchrotron-radiation experiments were performed at SPring-8 (proposal No. 2014B1033) and Photon Factory

(proposal No. 2014G080). We thank the beamline staff of SPring-8 and Photon Factory for their assistance with data collection. This work was supported by the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics and Structural Life Science; 11961) from Japan Agency for Medical Research and Development (AMED), JST PRESTO (JPMJPR1517) and Grants-in-Aid for Scientific Research (15J01961, 24000011, 26291008 and 16H00748) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Alings, F., Sarin, L. P., Fufezan, C., Drexler, H. C. A. & Leidel, S. A. (2015). *RNA*, **21**, 202–212.
Asano, N., Kato, K., Nakamura, A., Komoda, K., Tanaka, I. & Yao, M. (2015). *Nucleic Acids Res.* **43**, 4746–4757.
Begley, T. P., Ealick, S. E. & McLafferty, F. W. (2012). *Biochem. Soc. Trans.* **40**, 555–560.
Boschi-Muller, S. & Motorin, Y. (2013). *Biochemistry (Mosc.)*, **78**, 1392–1404.
Caldeira de Araujo, A. & Favre, A. (1985). *Eur. J. Biochem.* **146**, 605–610.
Iobbi-Nivol, C. & Leimkühler, S. (2013). *Biochim. Biophys. Acta*, **1827**, 1086–1101.
Kabsch, W. (2010). *Acta Cryst.* D**66**, 133–144.
Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
Krüger, M. K., Pedersen, S., Hagervall, T. G. & Sørensen, M. A. (1998). *J. Mol. Biol.* **284**, 621–631.
Krüger, M. K. & Sørensen, M. (1998). *J. Mol. Biol.* **284**, 609–620.
Leidel, S., Pedrioli, P. G., Bucher, T., Brost, R., Costanzo, M., Schmidt, A., Aebersold, R., Boone, C., Hofmann, K. & Peter, M. (2009). *Nature (London)*, **458**, 228–232.
Machnicka, M. A., Milanowska, K., Osman Oglou, O., Purta, E., Kurkowska, M., Olchowik, A., Januszewski, W., Kalinowski, S., Dunin-Horkawicz, S., Rother, K. M., Helm, M., Bujnicki, J. M. & Grosjean, H. (2013). *Nucleic Acids Res.* **41**, D262–D267.
Nakagawa, H., Kuratani, M., Goto-Ito, S., Ito, T., Katsura, K., Terada, T., Shirouzu, M., Sekine, S.-I., Shigi, N. & Yokoyama, S. (2013). *Proteins*, **81**, 1232–1244.
Sheldrick, G. M. (2010). *Acta Cryst.* D**66**, 479–485.
Shigi, N. (2012). *J. Biol. Chem.* **287**, 17568–17577.
Shigi, N. (2014). *Front. Genet.* **5**, 67.
Shigi, N., Sakaguchi, Y., Asai, S.-I., Suzuki, T. & Watanabe, K. (2008). *EMBO J.* **27**, 3267–3278.
Shigi, N., Sakaguchi, Y., Suzuki, T. & Watanabe, K. (2006). *J. Biol. Chem.* **281**, 14296–14306.
Shigi, N., Suzuki, T., Terada, T., Shirouzu, M., Yokoyama, S. & Watanabe, K. (2006). *J. Biol. Chem.* **281**, 2104–2113.
Terwilliger, T. C. (2000). *Acta Cryst.* D**56**, 965–972.
Terwilliger, T. C. (2003). *Acta Cryst.* D**59**, 38–44.
Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.
Watanabe, K., Shinma, M., Oshima, T. & Nishimura, S. (1976). *Biochem. Biophys. Res. Commun.* **72**, 1137–1144.
Yarian, C., Townsend, H., Czestkowski, W., Sochacka, E., Malkiewicz, A. J., Guenther, R., Miskiewicz, A. & Agris, P. F. (2002). *J. Biol. Chem.* **277**, 16391–16395.