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Structural polymorphism of the *Escherichia coli* poly- α -L-glutamate synthetase RimK

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Bacterial RimK is an enzyme that catalyzes the polyglutamylation of the C-terminus of ribosomal protein S6 and the synthesis of poly- α -L-glutamate peptides using L-glutamic acid. In the present study, the crystal structure of the *Escherichia coli* RimK protein complexed with the ATP analogue AMP-PNP was determined at 2.05 Å resolution. Two different conformations of RimK, closed and open forms, were observed in the crystals. The structural polymorphism revealed in this study provided important information to understand the mechanism by which RimK catalyzes the synthesis of poly- α -L-glutamate peptides and the polyglutamylation of ribosomal protein S6.

1. Introduction

Ribosomal protein S6 modification protein (RimK: EC 6.3.2.43) is highly conserved from bacteria to mammals and catalyzes the post-translational polyglutamylation of ribosomal protein S6 (RpsF) in bacteria (Kang *et al.*, 1989). *Escherichia coli* RimK also catalyzes the synthesis of poly- α -L-glutamic acid, using L-glutamic acid as a substrate, in an ATP-dependent manner (Kino *et al.*, 2011). Two genes encoding the RimK homologues RIMKLA and RIMKLB have been identified in mammalian genomes (Collard *et al.*, 2010). RIMKLA and RIMKLB catalyze the synthesis of the neuropeptides N-acetylaspartylglutamate and β -citrylglutamate, respectively (Collard *et al.*, 2010).

RimK belongs to the ATP-dependent carboxylate-amine/thiol ligase superfamily (Galperin & Koonin, 1997). This superfamily also includes a lysine-biosynthesis enzyme (LysX) and an arginine-biosynthesis enzyme (ArgX). LysX and ArgX catalyze the post-translational modification of LysW by adding a single α -aminoadipic acid and a single glutamic acid to the C-terminus of LysW, respectively (Horie *et al.*, 2009; Nishiyama *et al.*, 2009; Ouchi *et al.*, 2013; Yoshida *et al.*, 2016). Although LysX and ArgX both possess the activity to conjugate acidic amino acids to the C-terminus of the protein, only RimK has poly- α -L-glutamic acid synthesis activity (Kino *et al.*, 2011). This unique characteristic of bacterial RimK may allow this enzyme to be used as a tool for poly- α -L-amino-acid production on an industrial scale (Kino *et al.*, 2011; Hamano *et al.*, 2013). However, the mechanism by which RimK catalyzes poly- α -L-amino-acid synthesis has not yet been clarified.

In the present study, we determined two crystal structures of *E. coli* RimK, in open and closed forms, in complex with the ATP analogue AMP-PNP at 2.05 Å resolution.

Table 1

Macromolecule-production information.

Source organism	<i>E. coli</i>
DNA source	<i>E. coli</i>
Forward primer	5'-GGGAATTCATATGAAATTGCCATATT GTCC-3'
Reverse primer	5'-GGGGTACCACCCGTTTTCAGGCA-3'
Cloning vector	pET-29
Expression vector	pET-29
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MKIAILSRDGLTLYSCKRLREAAIQRGHLVE ILDPLSCYMNINPAASSIHYKGRKLPFHD AVIPRIGTAITFYGTAAALRQFEMLSYPL NESVAIARARDKLRSMLLARQGLDLPVT GIAHSPDDTSDLIDMVGGAPLVVKLVEGT QGIGVLAETRQAASVIDAFRGLNAHIL VQEIYKEAQGCIDIRCLVVGDEVVAIERR AKEGDFRSNLHRGGAASVASITPQEREIA IKAARTMALDVAGVDILRANRGLVMEVN ASPGLEGIEKTTGIDIAKGMIRWIERHAT TEYCLKTGGGTLVPRGSMASDPNSSVD KLAAALEHHHHHH

Table 2

Crystallization.

Method	Vapour diffusion
Plate type	VDX plate (Hampton Research)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	3.3
Buffer composition of protein solution	100 mM Tris-HCl pH 8.2, 150 mM NaCl, 1 mM dithiothreitol, 7.7 mM AMP-PNP, 7.7 mM L-glutamic acid, 7.7 mM magnesium sulfate
Composition of reservoir solution	100 mM bis-tris propane pH 7.0, 210 mM sodium sulfate, 16% PEG 3350
Volume and ratio of drop	2 µl, 1:1 ratio
Volume of reservoir (µl)	500

2. Materials and methods

2.1. Protein expression and purification

E. coli RimK was expressed as a C-terminally His₆-tagged protein in an *E. coli* BL21(DE3) strain harbouring pET-29b(+)-RimK. The His₆-tagged RimK was purified by Ni²⁺-affinity chromatography using a HisTrap HP column (GE Healthcare). The His₆-tagged RimK that eluted from the HisTrap HP column was desalted on a PD-10 column (GE Healthcare). The desalted His₆-tagged RimK was treated with thrombin protease (Wako) and was again subjected to HisTrap HP column chromatography to remove the resulting His₆-tag peptide. The sample was further purified by chromatography on a HiLoad Superdex 200 pg 16/60 column (GE Healthcare). The purified RimK was stored in 100 mM Tris-HCl pH 8.2 buffer containing 150 mM NaCl and 1 mM dithiothreitol at -80°C. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

The crystallization of RimK was performed by the hanging-drop vapour-diffusion method. RimK solution (1 µl at 3.3 mg ml⁻¹) containing 100 mM Tris-HCl pH 8.2, 150 mM NaCl, 1 mM dithiothreitol, 7.7 mM AMP-PNP, 7.7 mM L-glutamic acid and 7.7 mM magnesium sulfate was mixed

Table 3

Data collection and processing.

Diffraction source	BL17A, Photon Factory
Wavelength (Å)	0.98000
Temperature (K)	100
Detector	PILATUS3 S 6M
Crystal-to-detector distance (mm)	415.92
Rotation range per image (°)	0.2
Total rotation range (°)	0-360
Exposure time per image (s)	0.2
Space group	C2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	212.62, 121.63, 119.74
α , β , γ (°)	90, 118.74, 90
Resolution range (Å)	50.000-2.050 (2.120-2.050)
Total No. of reflections	875207
No. of unique reflections	164792
Completeness (%)	98.700 (98.800)
Multiplicity	5.300 (4.100)
$\langle I/\sigma(I) \rangle$	21.2 (1.67)
<i>R</i> _{r.i.m.}	0.080 (0.629)
<i>R</i> _{p.i.m.}	0.033
Overall <i>B</i> factor from Wilson plot (Å ²)	34.650

Table 4

Structure solution and refinement.

Resolution range (Å)	48.2130-2.0500 (2.0733-2.0500)
Completeness (%)	98.2
σ Cutoff	$F > 1.370\sigma(F)$
No. of reflections, working set	155710 (4957)
No. of reflections, test set	8436 (272)
Final <i>R</i> _{cryst}	0.196 (0.2871)
Final <i>R</i> _{free} †	0.240 (0.3305)
No. of non-H atoms	
Protein	17697
AMP-PNP	248
Ion	78
Solvent	1186
Total	19209
R.m.s. deviations	
Bonds (Å)	0.008
Angles (°)	1.067
Average <i>B</i> factors (Å ²)	
Protein	42.2
AMP-PNP	49.2
Ion	43.0
Solvent	56.9
Ramachandran plot	
Most favoured (%)	96.59
Allowed (%)	3.06
Outliers (%)	0.35

† The free *R* factor was calculated using a randomly selected 5% of reflections omitted from the refinement.

with 1 µl reservoir solution (100 mM bis-tris propane pH 7.0, 210 mM sodium sulfate, 16% PEG 3350) and the sample was incubated with 500 µl reservoir solution at 20°C for four weeks (Table 2). The resulting RimK crystals were soaked in a cryoprotectant solution (90 mM Tris-HCl pH 7.5, 90 mM bis-tris propane pH 7.5, 135 mM NaCl, 6.9 mM magnesium sulfate, 6.9 mM AMP-PNP, 6.9 mM L-glutamic acid, 190 mM sodium sulfate, 14% PEG 3350, 15% ethylene glycol) at 4°C. The RimK crystals were flash-cooled with liquid nitrogen.

2.3. Data collection and processing

X-ray diffraction data were collected on the BL17A beamline at the Photon Factory, Tsukuba, Japan. The data sets were processed and scaled at 2.05 Å resolution using the

HKL-2000 and *CCP4* program suites (Otwinowski & Minor, 1997; Winn *et al.*, 2011). Data-collection and processing statistics are provided in Table 3.

2.4. Structure solution and refinement

The structure of RimK was determined by the molecular-replacement method with *Phaser* (McCoy *et al.*, 2007) using the atomic coordinates of the RimK–ADP complex structure (PDB entry 4iwx; Zhao *et al.*, 2013) as the search model. The model was refined using the *PHENIX* suite and *Coot* (Adams *et al.*, 2010; Emsley *et al.* (2010)). All structural figures were produced using *PyMOL* (<http://www.pymol.org>). Structure-solution and refinement statistics are provided in Table 4. The atomic coordinates of RimK have been deposited in the Protein Data Bank (PDB entry 5zct).

2.5. R.m.s.d. and *B*-factor calculations

The atomic coordinates of the closed form (chain *A*) and open form (chain *D*) of RimK were superimposed and root-

mean-square deviation (r.m.s.d.) values for the C α atoms of the amino-acid pairs were calculated using *PyMOL*. The *B* factors were calculated by *phenix.refine* (Adams *et al.*, 2010). The *B* factors of the C α atoms of the amino-acid residues of the closed form (chain *A*) and open form (chain *D*) of RimK were extracted and plotted.

3. Results and discussion

3.1. The crystal structure of *E. coli* RimK

We determined the crystal structure of the *E. coli* RimK protein with a nonhydrolyzable ATP analogue (AMP–PNP) at 2.05 Å resolution (Table 1). Since RimK requires ATP hydrolysis to catalyze polyglutamic acid synthesis (Kino *et al.*, 2011), the RimK–AMP–PNP complex structure presented here may reflect a snapshot of the structure before the poly- α -L-glutamic acid synthesis reaction. Two RimK tetramers were found in the asymmetric unit of the crystal (Fig. 1*a*). Tetramer formation by RimK is consistent with the previous

Figure 1

Crystal structure of the RimK protein. (*a*) Two RimK tetramers in the asymmetric unit of the crystal. The open and closed forms are coloured yellow and green, respectively. (*b*) The structure of the RimK tetramer (left). The protomers of the RimK tetramer are shown on the right. (*c*) The structures of the two different forms of the RimK protomer. The right and left panels present the open and closed forms of the RimK protomer, respectively.

gel-filtration and crystallographic analyses (Zhao *et al.*, 2013). Interestingly, two different RimK conformations, an open form (coloured yellow) and a closed form (coloured green), were observed in a single RimK tetramer (Figs. 1*b* and 1*c*).

3.2. Structural differences between the RimK protomers

The open and closed forms of the RimK structure were superimposed and the r.m.s.d. values for amino-acid residue pairs were plotted (Figs. 2*a* and 2*b*). We found dramatic

Figure 2

Structural comparison of the open and closed forms of the RimK protomer. (a) The structures of the open and closed forms of RimK were superimposed and the r.m.s.d. value for each C α -atom pair was plotted. (b) Superimposition of the structures of the open form (yellow) and closed form (green) of RimK. Secondary structures of the RimK region containing amino-acid residues 135–225 are presented at the top. Helical cylinders and arrows represent α -helices and β -strands, respectively. (c, d, e) Structural comparisons of the open and closed forms of RimK in region 1 (amino-acid residues 136–154) (c), region 2 (amino-acid residues 155–178) (d) and region 3 (amino-acid residues 202–222) (e). (f) The B factors for each C α atom of the open and closed forms of RimK are plotted in yellow and green, respectively.

structural differences between the open and closed forms of the RimK protomer in the region of amino-acid residues 138–154 (region 1; Fig. 2*a*). Region 1 is buried in the tetramer unit and does not directly contact the neighbouring tetramer in the crystal. Therefore, these structural differences may not be a consequence of crystal-packing effects. In the open form, region 1 forms an antiparallel β -sheet structure (Fig. 2*c*, yellow). Intriguingly, in the closed form, the region including residues 142–151 of this β -sheet structure forms a loop structure (Fig. 2*c*, green). The shortened β -sheet structure of the closed form is conserved among RimK homologues such as LysX (PDB entry 3vpd), ArgX (PDB entry 3vpc) and D-alanyl-D-alanine ligase (PDB entry 2dln) (Fan *et al.*, 1994; Ouchi *et al.*, 2013). However, the extended β -sheet structure observed in the open form, which was previously described in the ADP-bound RimK structure (PDB entry 4iwx), has not been reported in the structures of other RimK homologue. In addition, the region containing amino-acid residues 157–174 (region 2) differs structurally between the open and closed

forms of RimK (Fig. 2*a*). The region 2 α -helix in the open form is clearly shorter than that in the closed form (Fig. 2*d*). Additionally, the region 2 α -helix positions were shifted by approximately 2 Å between the open and closed forms (Figs. 2*b* and 2*d*).

The region 3 loop (amino-acid residues 204–218) in the open form contains a short helix-like structure which is absent in the closed form (Fig. 2*e*). Interestingly, the *B* factors of the region 3 amino-acid residues are quite high in the open form but not in the closed form (Fig. 2*f*). This finding suggests that the region 3 loop is flexible in the open form. In the closed form the region 3 loop directly interacts with the region 1 loop, which only exists in the closed form; the region 1 loop forms an extended β -sheet structure in the open form (Fig. 2*c*). Therefore, the interaction between the region 1 and 3 loops may stabilize region 3 in the closed form but not in the open form.

The open form of RimK contains an extended β -sheet structure in region 1, which expands the ATP- and substrate-

Figure 3

The structures around the ATP- and substrate-binding groove in the open and closed forms of the RimK protomer. (*a*) The surface structure of the closed-form RimK protomer. (*b*) The surface structure of the open-form RimK protomer.

binding groove compared with that in the closed form (Figs. 3*a* and 3*b*). The structural difference in the substrate-binding groove between the open and closed forms of RimK may regulate the substrate-binding and catalytic activities.

4. Conclusion

In the present study, we determined the crystal structure of the RimK protein. The RimK tetramer contained two structurally different RimK protomers in open and closed forms. The open-form structure has not been reported in the structures of other RimK homologues. RimK is a unique enzyme in the ATP-dependent carboxylate-amine/thiol ligase superfamily, as its substrates are large molecules such as the RpsF protein and poly- α -L-glutamic acid (Galperin & Koonin, 1997; Kino *et al.*, 2011). RimK is the only enzyme that can catalyze a chain reaction to perform the polyglutamylation of RpsF and poly- α -L-glutamate peptide synthesis among the ATP-dependent carboxylate-amine/thiol ligase superfamily enzymes (Kino *et al.*, 2011). The expanded ATP- and substrate-binding groove in the open-form RimK protomer may be suitable for incorporating large substrate molecules and may function in the specific enzyme reaction of RimK. The detailed structural information reported here will be useful for the design of RimK mutants which may be suitable for industrial applications.

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