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Crystallization and preliminary X-ray diffraction analysis of a redox-sensing repressor Rex from *Thermotoga maritima*

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The redox-sensing repressor Rex is a homodimeric transcriptional regulator involved in expression of respiratory genes. Because nicotinamide adenine dinucleotide (NAD) exists oxidized or reduced form by catabolic metabolism, intracellular NAD^+/NADH ratio can be a key signal indicating the cellular redox state. The Rex from hyperthermophilic bacterium, *Thermotoga maritima* (TmRex), was cloned and overexpressed in *Escherichia coli*. The TmRex is composed of 208 amino-acid residues with a molecular mass of 22,954 Da. The TmRex crystals were obtained by the sitting-drop vapour-diffusion method and diffracted to 1.95 Å resolution. The crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 53.54 Å, b = 88.34 Å, c = 87.84 Å, and β = 96.74°. Two dimeric molecules of TmRex were present in an asymmetric unit, giving a solvent contents of 45.17%.

INTRODUCTION

Bacteria have adapted and responded to changes of environmental oxygen level for their survival through transcriptional regulation of genes involved in respiratory pathways (Giaccia et al., 2004). Nicotinamide adenine dinucleotides (NAD) is metabolically involved in a variety redox reaction, which is a coenzyme found in all living cells (Belenky et al., 2007). The NAD exists in both oxidized and reduced forms as NAD^+ and NADH, respectively. The NADH is a major source of ATP through re-oxidizing to NAD^+ by reduction of oxygen acting as an electron acceptor in electron transport chain (Rich, 2003). When the NADH level is elevated from low environmental oxygen level or inhibition of electron transport chain, the intracellular NAD^+/NADH ratio is changed (Williamson et al., 1967). Therefore, the aspect of NAD^+/NADH ratio can be a critical indicator for the recognition of intracellular redox state.

The Rex family of transcription factors are well conserved to control respiratory pathway, which sense changes of redox state according to intracellular NAD^+/NADH balance (Brekasis and Paget, 2003; Gyan et al., 2006; McLaughlin et al., 2010). The Rex proteins negatively regulate expression of the genes involved in catabolic metabolism such as cytochrome *bd* oxidase (*cydABCD*), proton-translocating NADH dehydrogenase (*nuoA-N*), and NADH-linked fermentative lactate dehydrogenase (*lctP-ldh*) in the high level of NAD^+ (Brekasis and Paget, 2003; Gyan et al., 2006). The Rex homologs show an affinity to both oxidized NAD^+ and reduced NADH but have more preference to the reduced NADH (Brekasis and Paget, 2003; Wang et al., 2008). When the reduced NADH level is elevated, the Rex protein makes a complex with NADH and is dissociated with the Rex operator (ROP) (McLaughlin et al., 2010; Wang et al., 2008).

The Rex from *Thermotoga maritima* (TM0169, TmRex) consists of 208 amino-acid residues with 44% sequence identity to *Thermus aquaticus* Rex, 43% sequence identity to *Streptomyces coelicolor* Rex, and 38% sequence identity to *Bacillus subtilis* Rex based on the BLAST server (https://blast.ncbi.nlm.nih.gov/). Here, as a first step in structure determination we report the cloning, purification, crystallization, and preliminary X-ray diffraction data of TmRex.

RESULT AND DISCUSSION

The recombinant TmRex containing hexahistidine tag at the N-terminus was overexpressed in *Escherichia coli* and purified to give a final yield of ~10 mg per litre of culture (Table 1). The TmRex was successfully purified by two steps of chromatography, immobilized-metal-affinity-chromatography (IMAC) and size exclusion chromatography (Figure 1). The TmRex crystals were obtained reproducibly by the sitting-drop vapour diffusion at 296 K and grew in optimized reservoir solution containing 20% (*w/v*) PEG 3350 and 200 mM KCl, which grew up approximately 0.2 mm x 0.16 mm x 0.15 mm within a month (Figure 2 and Table 2). The best crystal was transferred into cryoprotectant solution consisting of the reservoir solution with 15% (*v/v*) glycerol and 25% (*v/w*) PEG 3350, and flash-cooled in liquid nitrogen. The merged data set was 99.6% complete to 1.95 Å resolution and gives an R_{merge} of 8.1% (Figure 3). The crystal belonged to a monoclinic space group $P2_1$, with unit-cell parameters a = 53.54 Å, b = 88.34 Å, c = 87.84 Å, and β = 96.74°. The asymmetric unit contained four molecules of TmRex, giving a crystal volume per mass (V_M) of 2.24 $Å^3$ Da^{-1} and

TABLE 1 | Rex production information

Source organism	*Thermotoga maritima*
DNA source	Chromosomal DNA
Forward primer	GGAATTCCATATGGCGGAAAAGATACCGAAG
Reverse primer	CCGCTCGAGTCAAGAATTCCTCCTCAC
Cloning vector	pET28b(+)
Expression host	*E. coli* BL21 Star (DE3) pLysS
Complete amino acid sequence of the construct produced	MAEKIPKPVSKRLVSYYMCLERLLDEGVEVVSSEELARRLDLKASQIRKDLSYFGEFGKR GVGYNVEHLYDAIGEILGVKKEWKLVVVGAGNIGRAVANYTVMKEKGFRIIGIFDSDPSK IGKEAAPGLTVSDVSELEKFVEEHGVEIGVIAVPAEHAQEIAERLEKAGIKGILNFAPVK IKVSVPVENIDITASLRVLTFEIVRRNS

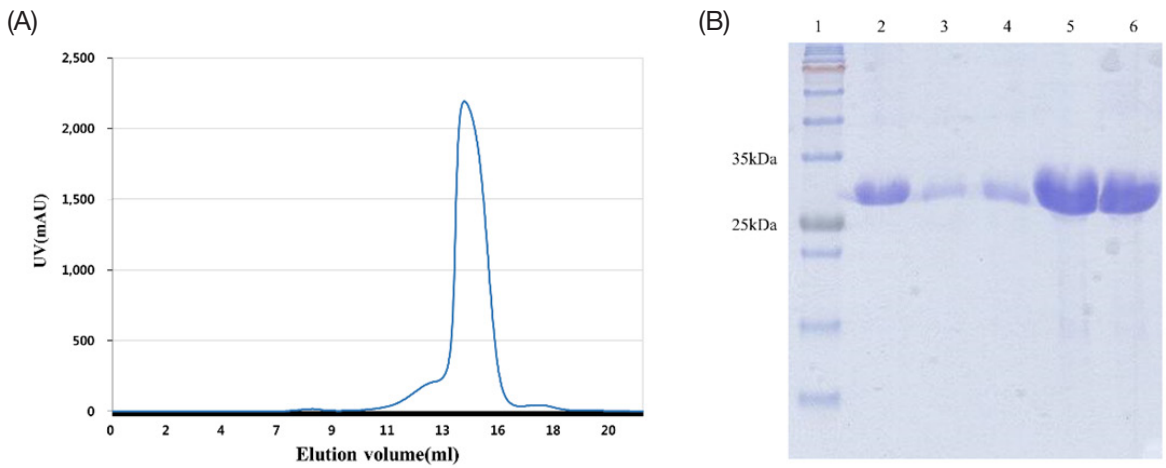

FIGURE 1 | **Purification of the *T. maritima* Rex.** (A) The Rex protein was isolated by size-exclusion chromatography using a Superdex-200 gel filtration column. (B) The purified Rex was identified by SDS-PAGE; lane 1, protein marker; lane 2~4, eluted Rex from Ni-NTA column; lane 5 and 6, eluted Rex from gel filtration column

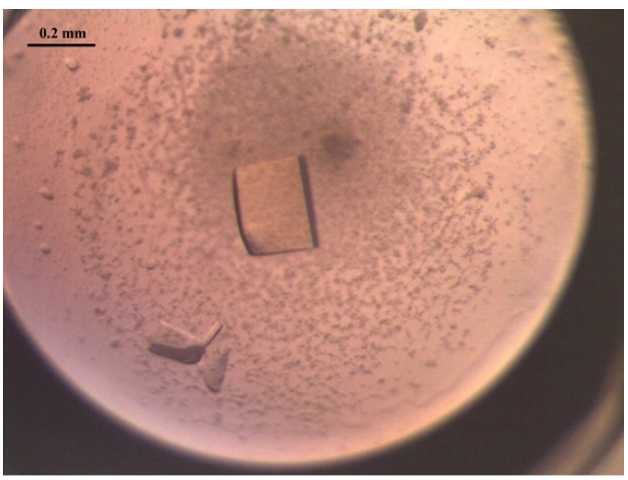

FIGURE 2 | **Monoclinic crystals of the *T. maritima* Rex.** The crystals with the best quality were obtained in 20% (w/v) PEG 3350 and 200 *mM* potassium chloride using the sitting-drop vapour-diffusion method at 296 K. Their approximate dimensions are 0.2 mm x 0.16 mm x 0.15 mm.

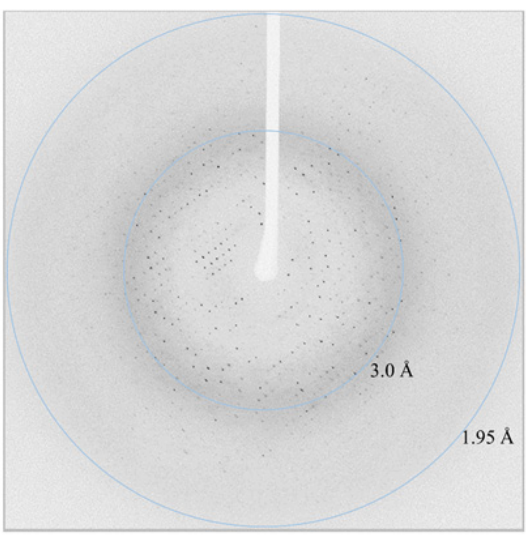

FIGURE 3 | **X-ray diffraction image of the *T. maritima* Rex crystal.** The crystal was diffracted 1.95 Å resolution on beamline BL44XU of the SPring-8, Japan.

TABLE 2 | Crystallization

Method	Sitting drop vapor diffusion
Plate type	96-Well Sitting-Drop Crystallography Plate
Temperature (K)	296
Protein concentration (mg ml^{-1})	30
Buffer composition of protein solution	20 m*M* Tris pH 8.5, 200 m*M* NaCl
Composition of reservoir solution	20 % (*w*/*v*) PEG 3,350, 200 m*M* KCl
Volume and ratio of drop	2μl; 1:1 ratio of protein and reservoir solution
Volume of reservoir (μl)	80

TABLE 3 | Data collection and processing statistics

Diffraction source	BL44XU beamline of the SPring-8, Japan
Wavelength (Å)	0.90000
Temperature (K)	100
Detector	MAR300HE CCD image plate
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1
Total rotation range (°)	195
Exposure time per image (s)	1
Space group	$P2_1$
a, b, c (Å)	53.54, 88.34, 87.84
α, β, γ (°)	90.00, 96.74, 90.00
Resolution range (Å)	50.0 – 1.95
Total reflections/unique reflections	243328 / 59055
Completeness (%)	99.3 (89.7)
R_{merge} (%)	8.4 (51.9)
Averge I/σ(I)	11.6 (2.6)

a solvent content of 45.17%. The statistics of data collection are summarized in Table 3.

Molecular replacement was attempted using the structures of several bacterial homologs [*T. aquaticus* Rex, PDB entry 3IKT, 44% sequence identity (McLaughlin et al., 2010); *S. coelicolor* Rex, PDB entry 3KEO, 43% sequence identity] with the program *Phaser* (McCoy et al., 2007), but it was not successful. To determine the crystal structure, selenomethionine-substituted TmRex is being purified and crystallized.

METHODS

Protein expression and purification

The *rex* gene (TM0169) was amplified by polymerase chain reaction using genomic DNA of *T. maritima*. The forward and reverse oligonucleotide primers used were: 5' - GGAATTCCATATGGCGGAAAAGATACCGAAG - 3' and 5' - CCGCTCGAGTCAAGAATTCCTCCTCAC - 3', respectively. The amplified DNA was digested with NdeI/XhoI, and then inserted into the NdeI/XhoI-digested expression vector pET28b(+) (Novagen, Germany) containing a hexahistidine tag (Table 1). The recombinant plasmid was introduced into the *E. coli* strain BL21 Star (DE3) pLysS (Invitrogen, USA) for protein expression. The transformed cells were grown up to OD_{600} ~0.5 at 310 K in Luria-Bertani medium containing 30 μg/ml chloramphenicol and kanamycin, respectively. Protein expression was induced with 1.0 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were continually incubated at 303 K for 4 h after IPTG induction, and then harvested by centrifugation at 2,300 g (Hanil Supra 22K A500S-6 rotor) for 15 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (20 m*M* Tris-HCl pH 8.0, 10% (*v*/*v*) glycerol, 500 m*M* NaCl and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)) and homogenized with an ultrasonic processor (Sonics™ Vibra Cell VCX750, USA) at 277 K. The lysate was centrifuged at 36,000 g (Hanil Supra 22K A50S-8 rotor) for 1 h at 277 K and the insoluble fraction including cellular debris was removed. The supernatant was applied to a Nickel-charged-His trap immobilized-metal-affinity-chromatography (IMAC) column (GE Healthcare, UK). The recom-binant protein was eluted with elution buffer (20 m*M* Tris-HCl pH 8.0, 10% (*v*/*v*) glycerol, 500 m*M* NaCl, and 300 m*M* imidazole). Further purification was performed by size-exclusion chromatography on a Superdex-200 gel Filtration column (GE Healthcare, UK), which was previously equilibrated with 10 mM Tris-HCl pH 8.0, 5% (*v*/*v*) glycerol, 200 m*M* NaCl, 2 m*M* $MgCl_2$, and 1 m*M* Dithiothreitol (DTT). Homogeneity of the purified protein was assessed by polyacrylamide gel electrophoresis in the presence of 0.1% (*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970). The protein solution was concentrated using a Centrifugal Filter (Amicon R Ultra-4, Ireland) to approximately 30 mg/ml. The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated molar extinction coefficient of 14,440 $M^{-1} \cdot cm^{-1}$ (SWISS-PROT; http://www.expasy.ch/).

Crystallization and X-ray data collection

Crystallization experiments were carried out using the sitting-drop vapour diffusion method at 296 K using 96-well CrystalQuick plates (Greiner Bio-one, Germany). A sitting drop was prepared by mixing equal volume (1 ml each) of the protein and reservoir solutions. The sitting drop was placed over 80 μl of the reservoir solution. Initial crystallization condition were established using screening kits from Qiagen (The Nucleix Suite, The Protein Complex Suite), AXYGEN (CP-CUSTOM-102, 103), and HAMPTON (PEG/Ion I, II Screen™, Natrix™). Crystals of TmRex proteins obtained using PEG 3350 as a precipitant were further optimized.

Crystals were flash-frozen in a liquid nitrogen stream with 15% (*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data of TmRex protein were collected at 100 K on an MAR300HE CCD image plate detector using synchrotron radiation on beamline BL44XU of the SPring-8, Japan. The wavelength of synchrotron X-ray was 0.90000 Å. The crystal was rotated through a total of 280° with 1.0° oscillation range per image with a crystal-to-detector distance of 300 mm. The crystal was exposed to 1 sec

exposure time. The raw data were processed and scaled using HKL-2000 software (Otwinowski and Minor, 1997).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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