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Pentatricopeptide repeat motifs in the processing enzyme PRORP1 in *Arabidopsis thaliana* play a crucial role in recognition of nucleotide bases at TψC loop in precursor tRNAs

Takayoshi Imai [a], Takahiro Nakamura [b], Taku Maeda [a], Kaoru Nakayama [a], Xuzhu Gao [c], Takashi Nakashima [a,c], Yoshimitsu Kakuta [a,c], Makoto Kimura [a,c,*]

[a] *Laboratory of Biochemistry, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Science, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan*
[b] *Laboratory of Plant Biosciences, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Science, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan*
[c] *Laboratory of Structural Biology, Graduate School of Systems Life Sciences, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan*

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ABSTRACT

Proteinaceous RNase P (PRORP1) in *Arabidopsis thaliana* is an endoribonuclease that catalyzes hydrolysis to remove the 5′-leader sequence of precursor tRNAs (pre-tRNAs). PRORP1 is composed of pentatricopeptide repeat (PPR) motifs, a central linker region, and a metal nuclease domain, the NYN domain. The PPR motifs are single-stranded RNA-binding motifs that recognize bases in a modular fashion. To obtain insight into the mechanism by which the PPR motifs in PRORP1 recognize a target sequence in catalysis, N-terminal successive deletion mutants were overproduced in *Escherichia coli*, and the resulting proteins were characterized in terms of enzymatic activity using chloroplast pre-tRNA^{Phe} as a substrate. Although Δ89, in which all PPR motifs are present, retained the pre-tRNA cleavage activity, Δ129 devoid of the first PPR motif (PPR1) had significantly reduced cleavage activity. Likewise, deletions of the second (PPR2) or third PPR (PPR3) motif abolished the cleavage activity, suggesting that PPR motifs play a crucial role in catalysis. A proposed recognition code for PPR motifs predicted that PPR2-PPR5 in PRORP1 recognize C, A/U, A, and U, respectively, whose sequence is in good agreement with C56-A57-A58-A59 in the TψC loop in pre-tRNA^{Phe}. Mutational analyses of nucleotide residues in the TψC loop as well as nucleotide-specifying residues (NSRs) in PPR motifs further suggested that PPR2 and PPR3 in PRORP1 favorably recognize nucleotide bases C56 and A57 at the TψC loop in pre-tRNA^{Phe}, respectively. This prediction and previous biochemical data were combined to construct a fitting model of tRNA onto PRORP1, showing that the mechanism by which PRORP1 recognizes pre-tRNAs appears to be distinct from that by bacterial RNase P.

1. Introduction

Ribonuclease P (RNase P) is a ribonucleoprotein that catalyzes the processing of 5′ leader sequences from tRNA precursors (pre-tRNA) and other noncoding RNAs in all living cells [1,2]. Ever since Altman and co-workers discovered that *Escherichia coli* RNase P RNA (M1 RNA) is a ribozyme [3], biochemical and structural studies have focused mainly on eubacterial RNase P RNAs and a large amount of information on structure–function relationships has become available for eubacterial RNase Ps [4,5]. Recently, the X-ray structure of *Thermotoga maritima* RNase P in complex with tRNA was determined, and a structural basis for the catalytic activity of eubacterial RNase P has been proposed [6]. In addition, information on the structure–function relationships of archaeal and eukaryotic RNase Ps has begun to emerge [7,8]. Despite rigorous investigations, the presence of RNase P in plant cells and organelles in eukaryotic cells had remained largely unknown until the discovery of a proteinaceous enzyme in human mitochondria in 2008 [9].

Abbreviations: NSRs, nucleotide-specifying residues; PPR, pentatricopeptide repeat; PRORP, proteinaceous ribonuclease P; RNase P, ribonuclease P; pre-tRNA, precursor tRNA.

* Corresponding author at: Laboratory of Biochemistry, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Science, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Fax: +81 92 642 2853.
E-mail address: mkimura@agr.kyushu-u.ac.jp (M. Kimura).

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Holzmann et al., using a combinatorial purification/proteomics approach, revealed that the protein complex comprising MRPP1, MRPP2, and MRPP3 can process the 5′-leader sequence of pre-tRNA in human mitochondria [9]. This finding demonstrated that human mitochondrial RNase P is a proteinaceous enzyme that does not require a trans-acting RNA component for catalysis. MRPP3 is predicted to be the enzyme's metallonuclease moiety, while MRPP1 and MRPP2 are supposed to act as cofactors, belonging to the tRNA methyltransferase and short-chain dehydrogenase/reductase families, respectively [9,10]. Subsequently, Gobert et al., found that *Arabidopsis thaliana* has three homologues, PRORP1 PRORP2, and PRORP3, of MRPP3, and that PRORP1 is localized in mitochondria and chloroplasts, while PRORP2 and PRORP3 are in the nucleus [11,12]. It was further found that PRORP1 could perform the endonucleolytic maturation of pre-tRNA and could complement RNase P activity in *E. coli* [11]. Recently, the crystal structure of PRORP1 was determined at a resolution of 1.75 Å [13]. PRORP1 comprises five tandem pentatricopeptide repeat (PPR) motifs, a central linker domain, and a metallonuclease domain belonging to the NYN family [14]. Furthermore, cross-linking and mutational analyses suggested that G19 and C56 in tRNA are involved in the interaction with PRORP1 [15,16].

PPR motif-containing proteins are eukaryote-specific and widely distributed in RNA-binding proteins in plants, which are involved in organelle transcript processing and stability in a gene-specific manner [17–19]. They are often found in tandem and are composed of a helix-turn-helix fold of ~35 amino acids [13,20]. Computational and biochemical analyses have proposed the putative RNA recognition code by PPR proteins [21–23]. Recently, the crystal structure of the maize chloroplast protein PPR10 in complex with single-stranded RNA fully supported the proposed recognition code and also revealed the molecular basis for the specific and modular recognition of bases [24].

Although structural information has become available for PRORP1, the molecular mechanism by which the PPR motifs in PRORP1 recognize substrates has remained unclear. To address this issue, we prepared N-terminal successive deletion mutants of PRORP1 and characterized them in terms of pre-tRNA cleavage activity. Furthermore, we predicted on the basis of the proposed recognition code that the PPR motifs PPR2, PPR3, PPR4, and PPR5 in PRORP1 recognize C, A/U, A, and U, respectively. This prediction, biochemical data, and existing crystallographic structural information were combined to generate a fitting model of tRNA onto PRORP1. The model structure suggests that PPR2 and PPR3 in PRORP1 favorably recognize nucleotides C56 and A57 at the TψC loop in pre-tRNAs, respectively.

2. Materials and methods

2.1. Materials

A full-length cDNA (Accession ID, AT2G32230) encoding PRORP1 from *A. thaliana* was purchased from the Rice Genome Resource Center (Tsukuba, Japan). Oligonucleotide primers used in the study were purchased from Operon. Restriction enzymes and DNA-modifying enzymes were purchased from MBI Fermentas. *E. coli* expression vector pET-15b was obtained from Novagen. All other chemicals were of analytical grade for biochemical use.

2.2. Preparation and purification of PRORP1 and its mutants

The cDNA fragment encoding mature PRORP1 (henceforth referred to as PRORP1), in which a transit peptide consisting of 38 amino acids was deleted, was obtained by PCR with specific primers. In addition, the cDNA fragments encoding the N-terminal deletion mutants were PCR amplified. The resulting PCR products were ligated into expression vector pET-15b, and their expression was induced in *E. coli* BL21 (DE3) Codon Plus RIL in a conventional manner. The overproduced proteins were purified to homogeneity using COSMOGEL His Accept (Nacalai Tesque) column followed by gel filtration on a Superdex 200 column. Site-specific mutants of PRORP1 were prepared in the same way as those described above, after PCR amplification with individual mutations.

2.3. Preparation of substrates

The DNA fragment encoding chloroplast pre-tRNA^{Phe} in *A. thaliana* was amplified by PCR with an upstream primer containing the T7 promoter and a sequence-specific downstream primer, and then pre-tRNA^{Phe} with a 20-nt leader sequence at the 5′-end was transcribed *in vitro* using the amplified gene fragment as a template. The resulting RNA was purified on a column of DEAE-Sepharose, as described by Easton et al. [25]. Mutant pre-tRNA^{Phe}s were prepared in the same way as described above after PCR amplification with individual mutations.

2.4. Assay for enzymatic activity

Cleavage reactions of pre-tRNA^{Phe} were performed using PRORP1 or its mutants in 30 mM Tris–HCl, pH 7.5, containing 40 mM NaCl, 6 mM $MgCl_2$, 2 mM DTT, 20 μg/ml BSA, and 100 units/ml RNase inhibitor (Promega) for 60 min at 37 °C. The resulting RNA fragments were separated on 15% polyacrylamide denaturing gels in TBE buffer (900 mM Tris–borate containing 10 mM EDTA) at 150 V for 1 h. After electrophoresis, the reaction products were visualized by staining in a 0.1% toluidine blue solution, and the resulting image was used to obtain values for the processing activity. The cleavage activity was calculated as follows: the quantity of (matured RNA) × 100/the quantity of (precursor RNA + matured RNA), and the cleavage activities of the mutants are expressed relative to that of PRORP1 as 100%.

2.5. Prediction of a target site for PPR motifs in PRORP1

The nucleotide-specifying residues (NSRs; residues 1, 4, and ii) were extracted from the PPR motifs of PRORP1, PRORP2, and PRORP3, according to the tertiary structure of PRORP1 [13]. The NSRs were converted into a probability matrix that indicated the decoding nucleotide frequency according to the PPR code, as described by Yagi et al. [22,23]. The probability matrix was also shown using Logo (http://weblogo.threeplusone.com/create.cgi).

2.6. A fitting model of PRORP1 onto tRNA

A fitting model of tRNA onto PRORP1 was constructed using MOE (2014.03; Chemical Computing Group Inc., Montreal, Canada). MOE's inbuilt tools and functions were used to incorporate features extracted from the coordinates of PRORP1 (PDB: 4G23), tRNA (PDB: 3Q1Q), and pseudouridine synthase in complex with the TψC loop (PDB: 1K8W) [26]. The coordinates of the PRORP1 model in complex with tRNA are available from the corresponding author.

3. Results

3.1. Preparation and characterization of the N-terminal deletion mutants of PRORP1

During purification of the PRORP1 protein (Fig. 1A), we found that it was highly sensitive to proteolysis, giving rise to a fragment

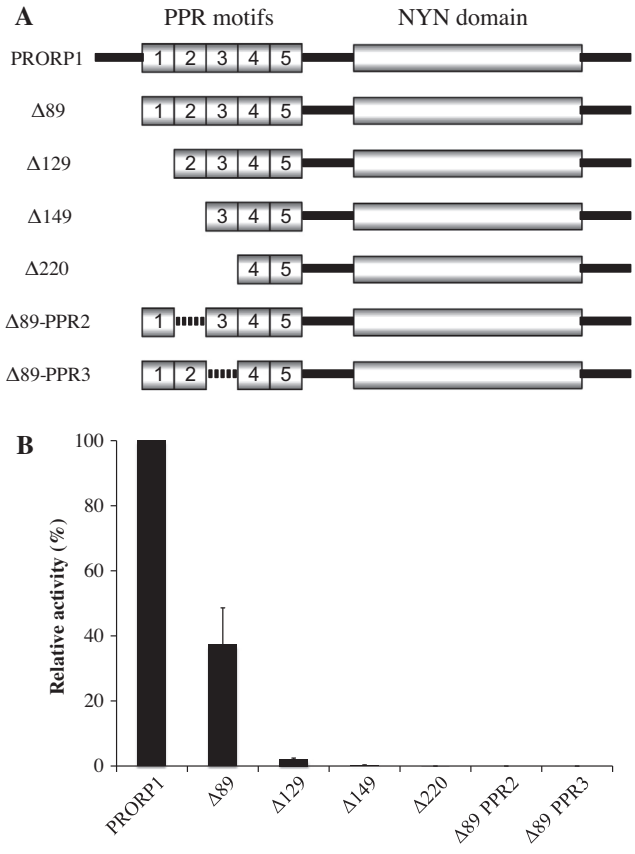

Fig. 1. The pre-tRNA cleavage activity of PRORP1 and its mutants. (A) Schematic diagram of the modular architecture of PRORP1 with approximate domain boundaries indicated. The N-terminal deletion mutants used in this study are shown. (B) The pre-tRNA cleavage activity of PRORP1 or its deletion mutants. Pre-tRNA cleavage activities of the enzymes were assayed at 37 °C using *A. thaliana* pre-tRNA^{Phe}, as described in Section 2. The cleavage activities of the mutants are expressed relative to that of PRORP1 at 60 min as 100%. The experiments were carried out in triplicate, and the mean values are presented.

with a molecular mass of 50 kDa. N-terminal amino acid sequencing analysis identified a scissile peptide bond as Lys-Gly at positions 89 and 90, being consistent with the crystal structure that the N-terminal residues up to position 90 are highly disordered [13]. Hence, PRORP1 as well as the N-terminal truncated fragment designated Δ89 were purified, and the resulting proteins were characterized in terms of enzymatic activity using chloroplast pre-tRNA^{Phe}. The Δ89 protein retained the cleavage activity of pre-tRNA, although it did so less efficiently than PRORP1 (Fig. 1B). This result indicated that the 89 N-terminal residues are slightly involved in the cleavage activity.

To obtain more insight into the function of the PPR motifs in PRORP1, we prepared three mutants, Δ129, Δ149, and Δ220, in which the 129, 149, and 220 N-terminal residues comprising PPR1, PPR1-PPR2, and PPR1-PPR3 were deleted, respectively (Fig. 1A). The mutant proteins were purified in the same manner as described for PRORP1 and characterized with respect to pre-tRNA cleavage activity (Fig. 1B). The truncation of the first PPR motif (PPR1) and further successive deletions (Δ149 and Δ220) abolished the pre-tRNA cleavage activity. This result suggested that PPR1 plays a crucial role in substrate recognition or stabilization of PRORP1 for the pre-tRNA cleavage activity.

In general, the polypeptide immediately preceding or following an α-helix is known as an Nt- or Ct-cap, respectively, and plays a crucial role in the stabilization of α-helices due to lack of intrahelical hydrogen bonds in the first and last turns [27,28]. Moreover, CAPS-DB (http://www.bioinsilico.org/CAPSDB), a database for searching for an Nt- or Ct-capping structure, predicted that PPR1 forms a capping helix. It is thus likely that PPR1 plays an important role in stabilization of the other PPR motifs, serving as the capping motif in PRORP1.

To examine further the involvement of PPR motifs in catalysis, we prepared two mutants, Δ89-PPR2 and Δ89-PPR3, in which residues 130–169 and 175–204 comprising PPR2 and PPR3, respectively, were deleted from Δ89. The resulting mutants were characterized them with respect to the pre-tRNA cleavage activity. Both mutants exhibited little pre-tRNA^{Phe} cleavage activity (Fig. 1B), indicating that PPR2 and PPR3 play a crucial role in the recognition of pre-tRNAs in catalysis.

3.2. Prediction of a target sequence of PPR motifs in PRORP1

On the basis of the proposed recognition code for PPR motifs [22,23], we attempted to predict the target sequence by PPR motifs in PRORP1. Since we expected that the target sequence for PPR motifs in PRORP1 should be identical to those for its isozymes, PRORP2 and PRORP3, their five PPR motifs were first modeled using the crystal structure of PRORP1 as a template. Next, NSRs that encode the PPR-RNA recognition code were extracted from the PPR motifs (Fig. 2A). A polar amino acid located at the 4th position is supposed to be the most important determinant for purine/pyrimidine discrimination. Although the NSRs were divergent among PRORP1-3, PPR2 and PPR3 displayed conserved Asn/Gln and Thr at residue 4, suggesting the importance of PPR2 and PPR3 for *Arabidopsis* PRORP's function (Fig. 2A). The NSRs were converted into a probability matrix that predicts the target bases for PPR motifs in PRORP1. As shown in Fig. 2B, the possible nucleotides for each determinant for PPR2, PPR3, PPR4, and PPR5 of PRORP1 were predicted to be C, A/G, A/U and U, respectively. In contrast, no nucleotide was assigned for PPR1, which may support the assumption that PPR1 plays a distinct role from the other PPR

B

PPR	1	2	3	4	5
NSR	AKS	YNN	ATR	RGE	PAS
code	***	*NN	*T*	*G*	*AS
A	ND	0.15	0.45	0.32	0.31
C	ND	0.45	0.09	0.13	0.08
G	ND	0.14	0.31	0.26	0.06
U	ND	0.27	0.15	0.30	0.56

Fig. 2. Prediction of a target site in tRNA for PPR motifs in PRORP1. (A) The conservation of the amino acids that determine target RNA specificity for PRORPs. The nucleotide-specifying residues (NSRs; 1, 4, and ii) of PRORP1, 2 and 3 are shown for each PPR motif (PPR1-5) by logo representation using Weblogo3 (http://weblogo.threeplusone.com/create.cgi). The amino acids are colored by the hydrophobicity: blue for hydrophilic amino acids (R, K, D, E, N, or Q), green for neutral amino acids (S, G, H, T, A, or P), and black for hydrophobic amino acids (Y, V, M, C, L, F, I, or W). (B) The binding RNA specificity of PPR motifs in PRORP1. The extracted NSRs were converted to a probability matrix that indicates the target RNA sequences indicated as "code" [22,23]. The nucleotide occurrence frequency is also represented by the logo. The asterisks indicate any amino acid. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

motifs, as described above. When the predicted target nucleotides C, A/G, A/U, and U were compared with those in pre-tRNA^{Phe}, they were in good agreement with the sequence C56-A57-A58-U59 at the TψC loop in pre-tRNA^{Phe}. It is known that the nucleotide sequence (T-ψ-C-A/G-A) at positions 54–58 on the TψC loop in tRNAs is completely conserved. It was thus predicted that PPR motifs may recognize the highly conserved nucleotide bases on the TψC loop in a modular fashion.

3.3. Mutational analyses of pre-tRNA^{Phe} and PPR motifs in PRORP1

To evaluate our prediction, we prepared pre-tRNA^{Phe} mutants in which C56, A57, A58, and U59 at the TψC loop in pre-tRNA^{Phe} were in turn replaced with three other nucleotides, and the resulting mutant substrates were characterized with respect to their susceptibility to PRORP1 in the same manner as those described above (Fig. 3A). Replacements of C56 and A58 with the three other nucleotides significantly reduced the susceptibility to PRORP1, ranging from 15% to 40% of that of the wild-type pre-tRNA^{Phe}. In addition, replacing A57 with C or U reduced their susceptibility to PRORP1 (~35%), whereas that with G had little influence on the susceptibility (62%), consistent with the fact that the base at position 57 is semi-invariant as a purine. In contrast, the pre-tRNA^{Phe} mutants of A59 retained their susceptibility to PRORP1. Since it is known that the base at position 59 is variable, these results suggested that the invariant bases C56, A57, and A58 at the TψC loop participate in the recognition by PRORP1. However, it should be noted that a Watson–Crick base pairing of A58 with T54 is involved in stabilization of the TψC loop. Hence, we cannot rule out the possibility that the replacement of A58 causes a conformational change that results in the insusceptibility to PRORP1.

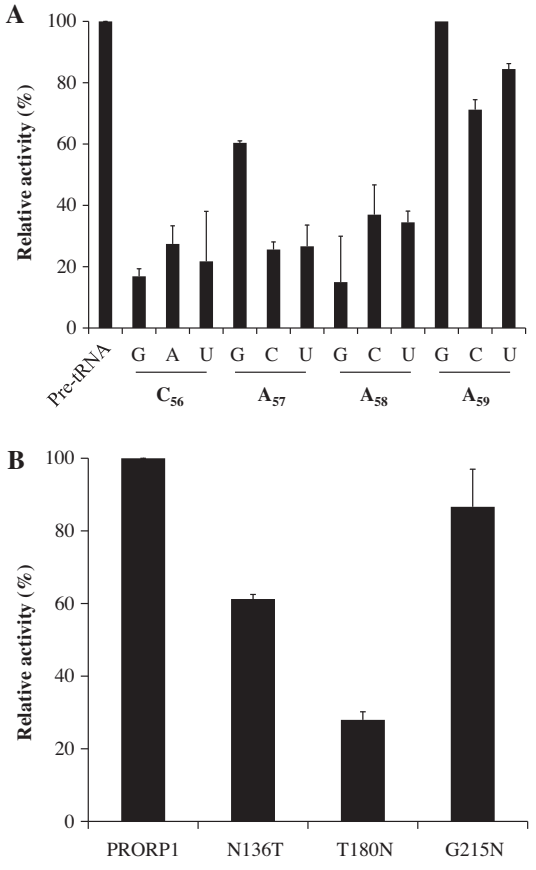

Fig. 3. Assay for pre-tRNA cleavage activity. (A) The susceptibility of pre-tRNA^{Phe} or its mutants to PRORP1 digestion. Pre-tRNA cleavage reactions were carried out, and the cleavage products were resolved, as described in Fig. 1. The susceptibility of the pre-tRNA mutants is expressed relative to that of the wild type at 60 min as 100%. The experiments were carried out in triplicates, and the mean values are presented. (B) The pre-tRNA cleavage activity of PRORP1 or its site-specific mutants, in which amino acids at the 4th position in PPR2, PPR3, and PPR4 were in turn replaced with other amino acids. The cleavage activities of the mutants are expressed relative to that of PRORP1 at 60 min as 100%.

Recently, structural analysis of the maize chloroplast protein PPR10 in complex with its target RNA has revealed that the side chain of the 4th residue in each repeat plays an essential role in recognition, namely, hydrogen bonding to the bases [24]. To validate our prediction further, we prepared PRORP1 mutants, in which amino acids Asn136, Thr180, and Gly215 at the 4th position in PPR2, PPR3, and PPR4, respectively, were in turn replaced by other amino acids; the resulting PRORP1 mutants were again characterized with respect to the pre-tRNA cleavage activity. As shown in Fig. 3B, mutations of Asn136 in PPR2 and Thr180 in PPR3 moderately reduced the pre-tRNA^{Phe} cleavage activity, while that of Gly215 in PPR4 had little influence on the cleavage activity. This result, together with the prediction, suggested that PPR motifs PPR2 and PPR3 recognize the highly conserved nucleotide bases C56 and A57 at the TψC loop in a modular fashion.

4. Discussion

Since the discovery of ribozymes, it has been proposed that biological catalysts have been evolved from RNA-based enzymes to polypeptide catalysts [29]. However, this fascinating hypothesis has not yet been verified because of the lack of suitable ribozymes and enzymes that catalyze an identical reaction. RNase P and PRORP1 catalyze hydrolysis to remove the 5′-leader sequence of pre-tRNA. Thus, detailed comparison of RNase P and PRORP1 may shed light on fundamental issues in the evolution of biological catalysis. The crystal structure of *T. maritima* RNase P in complex with tRNA showed that A112 and G147 in the S-domain in RNase P RNA were arranged adjacent to G19 and C56 in the D and TψC loops, respectively [6]. Previously, cross-linking and mutational analyses suggested that G19 and C56 in tRNA^{Phe} are involved in the interaction with PRORP1 [15,16]. It was therefore proposed that PRORP1 recognizes pre-tRNA in a similar manner to bacterial RNase Ps. This recognition mode is, however, unlikely because it is known that each PPR motif recognizes a base on single-stranded RNA [21–24]. In the present study, computational data and mutational analysis suggest that PRORP1 recognizes the highly conserved nucleotides C56 and A57 at the TψC loop in pre-tRNA^{Phe}.

The structural basis of sequence-specific recognition by PPR proteins has been presented by the structural analysis of the maize chloroplast protein PPR10 in complex with its target RNA [24]. It has been further reported that pseudouridine synthase gains access to its substrate by displacing the nucleotide bases ψ55, C56, and A57 from the position they assume within the helical stack of isolated, folded tRNA [26]. On the basis of our prediction, together with these reports, we attempted to construct a model of PRORP1 in complex with tRNA using the molecular modeling program MOE. In this modeling, we took into consideration that the 5′ end of tRNA is located near the two adjacent aspartate residues, Asp474 and Asp475 in PRORP1, which are involved in coordination of Mg^{2+} ions that are important for catalysis [9], and that two arms composed of the metallonuclease domain and the PPR domain in PRORP1 are likely to be highly flexible in solution and to change their relative orientation upon binding to pre-tRNA [13]. This attempt successfully fitted tRNA onto PRORP1, as shown in Fig. 4A. In this model, the side chains of Tyr133, Asn136, and Asn175 on PPR2 recognize C56, and those of Ala177, Thr180, and Arg212 on PPR3 are located in close proximity to A57, where the

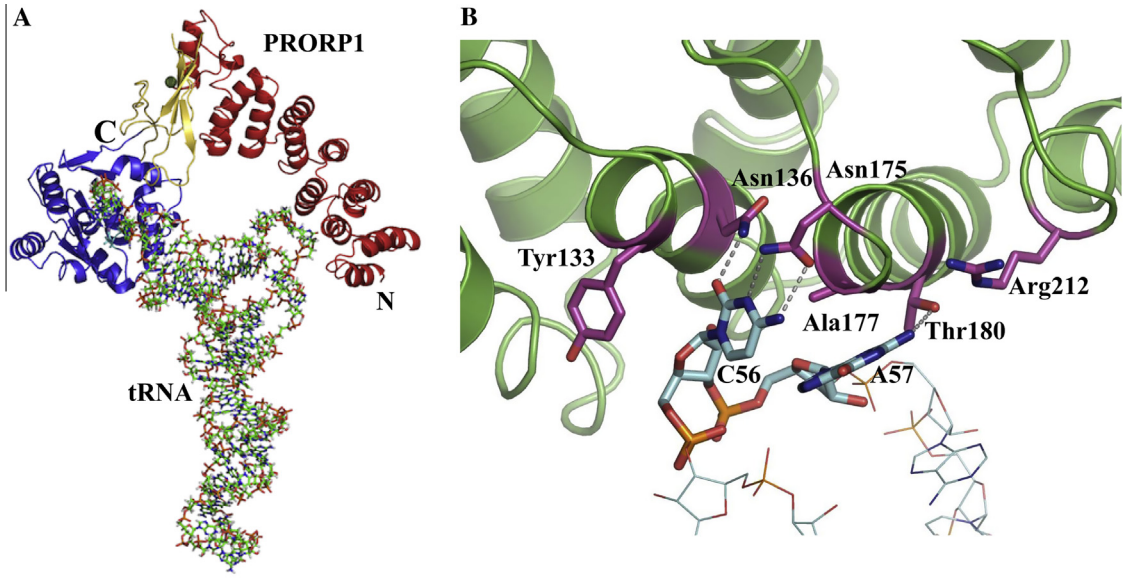

Fig. 4. A model structure of PRORP1 in complex with tRNA. (A) A fitting model of tRNA onto PRORP1. Structures of PRORP1 and tRNA are illustrated as ribbon and sticks representation, respectively. The PPR, central linker, and NYN domains are colored red, yellow, and blue, respectively. (B) Predicted interaction between PPR motifs in PRORP1 and nucleotides on the TψC loop. In this model, Asn136 in PPR2 and Thr180 in PPR3 recognize C56 and A57 by hydrogen bonding at the TψC loop in tRNA, respectively. Figures were drawn with PyMol (http://pymol.sourceforge.net). (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

side chains of Asn136 and Thr180 at the 4th position hydrogen-bond to C56 and A57, respectively (Fig. 4B). This proposed recognition mechanism is slightly distinct from that by bacterial RNase P. It was further described that an *Rp*-phosphothioate modification of the scissile bond has no influence on the cleavage activity by PRORP1, even though RNase P is highly sensitive to the modification [30]. It is therefore likely that PRORP1 catalyzes the pre-tRNA processing in a manner distinct from RNase P. It could thus be assumed that the proteinaceous RNase P, PRORP1, has evolved independently of the molecular mechanism by which RNA-based RNase P cleaves the pre-tRNA in catalysis.

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