Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Evolution of archaeal Rib7 and eubacterial RibG reductases in riboflavin biosynthesis: Substrate specificity and cofactor preference

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ARTICLE INFO

Article history:
Received 22 May 2018
Accepted 1 June 2018
Available online xxx

Keywords:
Riboflavin biosynthesis
Rib7 reductase
RibG
Substrate specificity
Cofactor preference
Structural stabilization

ABSTRACT

Archaeal/fungal Rib7 and eubacterial RibG possess a reductase domain for ribosyl reduction in the second and third steps, respectively, of riboflavin biosynthesis. These enzymes are specific for an amino and a carbonyl group of the pyrimidine ring, respectively. Here, several crystal structures of *Methanosarcina mazei* Rib7 are reported at 2.27–1.95 Å resolution, which are the first archaeal dimeric Rib7 structures. Mutational analysis displayed that no detectable activity was observed for the *Bacillus subtilis* RibG K151A, K151D, and K151E mutants, and the *M. mazei* Rib7 D33N, D33K, and E156Q variants, while 0.1–0.6% of the activity was detected for the *M. mazei* Rib7 N9A, S29A, D33A, and D57N variants. Our results suggest that Lys151 in *B. subtilis* RibG, while Asp33 together with Arg36 in *M. mazei* Rib7, ensure the specific substrate recognition. Unexpectedly, an endogenous NADPH cofactor is observed in *M. mazei* Rib7, in which the 2′-phosphate group interacts with Ser88, and Arg91. Replacement of Ser88 with glutamate eliminates the endogenous NADPH binding and switches preference to NADH. The lower melting temperature of ~10 °C for the S88E and R91A mutants suggests that nature had evolved a tightly bound NADPH to greatly enhance the structural stability of archaeal Rib7.

1. Introduction

The FAD/FMN coenzymes derived from riboflavin, are involved in a wide variety of essential physiological processes and hence are ubiquitously found in all organisms [1]. The precursor riboflavin is biosynthesized in most prokaryotes, fungi, and plants [2,3]. In contrast, animals lack this biosynthetic pathway and hence must obtain this vitamin from nutritional sources. Some pathogenic microorganisms lack an efficient uptake system and can only obtain this vitamin by biosynthesis. Therefore, the enzymes of the riboflavin biosynthesis appear to become antimicrobial targets, particularly for the development of new agents against antibiotic-resistant pathogens [2,4,5].

Most commercial riboflavin is currently produced by *Bacillus subtilis*, *Candida famata*, and *Ashbya gossypii* [6–8]. During the biosynthetic pathways [2,3], the imidazole ring of GTP is first hydrolytically opened by GTP cyclohydrolase II (GCH II) to yield formate, pyrophosphate, and 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (DAROPP) [9]. In archaea and fungi, DAROPP is reduced by Rib7 into 2, 5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (DARIPP) and subsequently deaminated by Rib2 into 5-amino-6-ribitylamino-2,4 (1H, 3H)-pyrimidinedione 5′-phosphate (ARIPP) (Fig. 1) [10,11]. In contrast, in eubacteria and plants, DAROPP is deaminated into 5-diamino-6-ribosylamino-2,4(1H, 3H)-pyrimidinedione 5′-phosphate (AROPP) and then reduced into ARIPP. In most eubacteria, the responsible enzyme is a bifunctional protein RibG (or RibD), which is composed of an N-terminal deaminase domain and a C-terminal reductase domain [12]. Recently, we have solved the tetrameric structures of *Bacillus subtilis* RibG (BsRibG) in complex with the NADPH cofactor and substrate [13–15]. The deaminase domain belongs to the cytidine deaminase superfamily, which includes several mononucleotide deaminases involved in nucleotide metabolism, and RNA/DNA-editing deaminases associated with gene diversity and

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https://doi.org/10.1016/j.bbrc.2018.06.002
0006-291X/

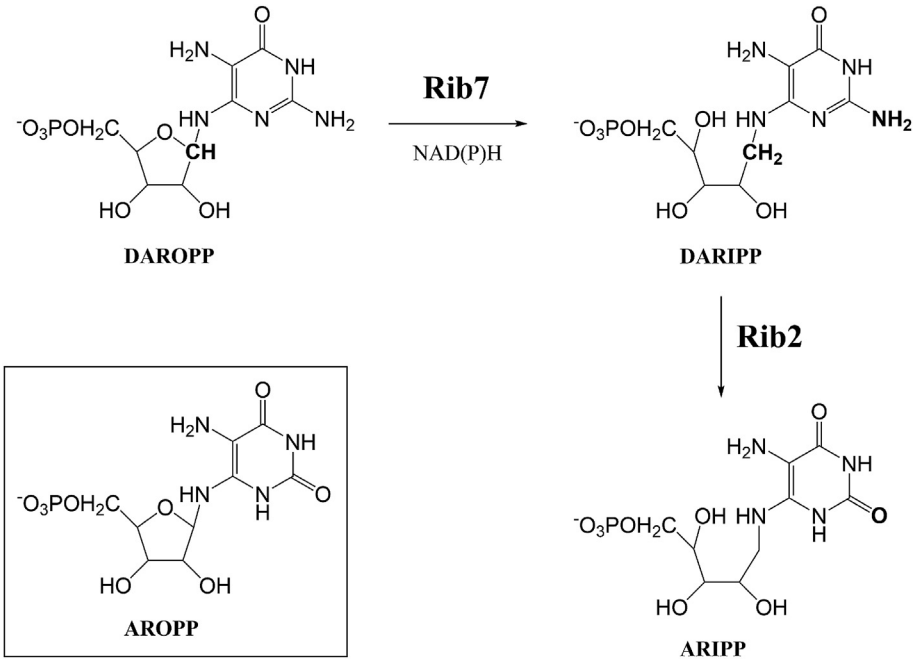

Fig. 1. Ribosyl reduction and pyrimidine deamination in vitamin B2 biosynthesis.

antivirus defence [16,17]. On the other hand, the reductase domain and the pharmaceutically important enzyme, dihydrofolate reductase, share a conserved core structure, NADPH-binding site, and catalytic mechanism [14].

The open reading frame MM0826 of *Methanosarcina mazei* encodes a 228-residues protein, which shares 45%, 25%, and 28% sequence identity to *Methanocaldococcus jannaschii* Rib7 (MjRib7), *Saccharomyces cerevisiae* Rib7 (ScRib7), and the reductase domain of BsRibG (BsRibG-R) respectively. Here we first expressed and isolated the recombinant protein of MM0826, which like MjRib7 [18], is able to catalyze the conversion of DAROPP into DARIPP and hence named as MmRib7. In contrast to ScRib7 displaying a comparable activity toward DAROPP and AROPP [19], MmRib7 cannot use AROPP as substrate. Several crystal structures of MmRib7 were also determined, which revealed an endogenous NADPH cofactor. A mutational analysis was carried out to illustrate the divergences between archaeal Rib7, and eubacterial RibG, particularly the specific substrate recognition and the NAD(P)H cofactor preference.

2. Materials and methods

2.1. Protein preparation and characterization

Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene). The mm0826 gene was amplified from *M. mazei* N2M9705 genomic DNA, which was kindly provided by Dr. Mei-Chin Lai [20]. The recombinant *E. coli* GCH II, BsRibG, and MmRib7 were expressed in *E. coli* using the pQE30 vector (Qiagen). Protein purification, preparation of the DAROPP, DARIPP, and AROPP compounds, and enzyme activity assay were carried out as previously described [14,15]. The reductase activity was monitored by either the absorbance decrease at 340 nm due to the NAD(P)H consumption, or the diacetyl treatment followed by the fluorescence intensity with the corresponding excitation wavelengths. Recombinant proteins were exposed to high temperature, and the unfolding process was monitored by circular dichroism spectral changes using an Aviv 202 spectrometer.

2.2. Structure analysis

Initial crystallization screening of MmRib7 was carried out with screening kits using the hanging-drop vapor-diffusion method at 22 °C. Crystals were grown in 3–7 days with a protein concentration of 15–20 mg/ml. For the soaking trials, the reaction solution was added to the crystal drops for 30–60 min with a final concentration of ~5–10 mM DAROPP [15]. Co-crystallization experiments were also performed in the presence of DAROPP. X-ray diffraction data were collected and processed on beamlines BL15A and BL13C1 at NSRRC, Hsinchu, Taiwan. The structures were determined by molecular replacement using the MjRib7 structure (PDB entry 2AZN) [18] as the search model and were refined using REFMAC [21]. The statistics of data collection and refinement are summarized in Table 1. Fig. 2A–B, 3, and 4A were generated by *PyMOL* [22], and Fig. 4B by *LigPlot* + [23].

3. Results and discussion

3.1. The MmRib7 structures

The wild-type and D33A mutant crystals were grown in 20–25% Tacsimate (pH 7.0), 2% PEG 3350, and 100 mM HEPES (pH 7.0), while the D33N mutant crystals were in 10% Tacsimate (pH 7.0), 10% PEG 5000, and 100 mM HEPES (pH 7.0). The D33A mutant crystals belong to the $P6_422$ space group with one molecule in an asymmetric unit and the atomic model containing residues 2–225. However, the wild-type and D33N crystals belong to the R3 space group with six subunits in an asymmetric unit. Perhaps due to crystal contacts, clear electron density was observed for the additional histidine-tagged vector residues ($MRGSH_6GS$) at the N terminus and residues 1–228 in protomers A and F. Particularly, these vector residues interact with the cofactor NADPH and Arg^{135} of protomers B and E in the adjacent asymmetric unit. The current models include residues (−9)-228, 1–224, 1–224, 2–224, 2–224, and (−10)-228 for protomers A to F, respectively.

The individual MmRib7 protomers in various crystal forms did

Please cite this article in press as: S.-C. Chen, et al., Evolution of archaeal Rib7 and eubacterial RibG reductases in riboflavin biosynthesis: Substrate specificity and cofactor preference, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.06.002