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Glycols modulate terminator stem stability and ligand-dependency of a glycine riboswitch

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ABSTRACT

The *Bacillus subtilis* glycine riboswitch comprises tandem glycine-binding aptamers and a putative terminator stem followed by the *gcvT* operon. Gene expression is regulated *via* the sensing of glycine. However, we found that the riboswitch behaves in a "glycine-independent" manner in the presence of polyethylene glycol (PEG) and ethylene glycol. The effect is related to the formation of a terminator stem within the expression platform under such conditions. The results revealed that increasing PEG stabilized the structure of the terminator stem. By contrast, the addition of ethylene glycol destabilized the terminator stem. PEG and ethylene glycol have opposite effects on transcription as well as on stable terminator stem formation. The glycine-independency of the riboswitch and the effects of such glycols might shed light on the evolution of riboswitches.

1. Introduction

Riboswitches are composed of a *cis*-acting RNA-based genetic control apparatus, which is located within the untranslated regions of the mRNA (Nudler and Mironov, 2004; Roth and Breaker, 2009; Winkler and Breaker, 2005). The binding of small ligands brings about structural changes within the riboswitches; this affects transcriptional or translational efficiency and therefore gene expression (Mandal et al., 2004; Yarnell and Roberts, 1999). The existence of riboswitches instead of protein regulators is important when considering evolutionary pathways, especially the transition from an RNA- to a protein-centric regulatory model. The glycine riboswitch (Mandal et al., 2004) is a naturally occurring RNA aptamer that recognizes the smallest and the most prebiotically abundant amino acid, glycine (Miller, 1953), suggesting that it might have been a remnant of early evolution of the ribonucleoprotein (RNP) world.

Two similar structural regions (I and II) are typically arranged in tandem and connected by a short nucleotide linker in the glycine riboswitch (Mandal et al., 2004). These two regions function as two glycine-binding aptamers, followed by a single expression platform. The results of bioinformatics searches suggest that the glycine riboswitches are localized upstream of the *gcvT* operon, which controls the expression of enzymes involved in the metabolism of glycine (Mandal et al., 2004). In addition, the glycine riboswitch is known to work cooperatively: the binding of glycine to one of the two aptamers assists the subsequent binding of glycine to the second aptamer (Erion and Strobel, 2011; Kwon and Strobel, 2008; Mandal et al., 2004). The results of a small-angle X-ray scattering (SAXS) and hydroxyl radical footprinting study showed that glycine binding affects the arrangement of the tandem riboswitch (Lipfert et al., 2007). A crystal structure of the tandem glycine riboswitch from *Fusobacterium nucleatum* revealed the glycine binding sites (Butler et al., 2011; Huang et al., 2010). On the other hand, in the 5′-elongated glycine riboswitches of *Vibrio cholerae*, the 5′-leader–linker interaction forms a kink-turn motif that abolishes glycine-binding cooperativity in glycine binding (Baird and Ferré-D'Amaré, 2013; Sherman et al., 2012). However, the molecular basis of the ON/OFF regulatory mechanism of glycine riboswitches is still unclear.

To date, more than 20 distinct classes of riboswitches have been identified (Erion and Strobel, 2011). The ability of riboswitches to regulate the efficiency of gene expression is determined by the ligand with which they interact. In the present study, we focused on the behavior of the putative intrinsic terminator stem present in the glycine riboswitch of *Bacillus subtilis* (Mandal et al., 2004). The last 11 nucleotides of aptamer II of the *B. subtilis* glycine riboswitch contains sequences that are complementary to a part of the expression platform, and the binding of glycine is expected to unfold

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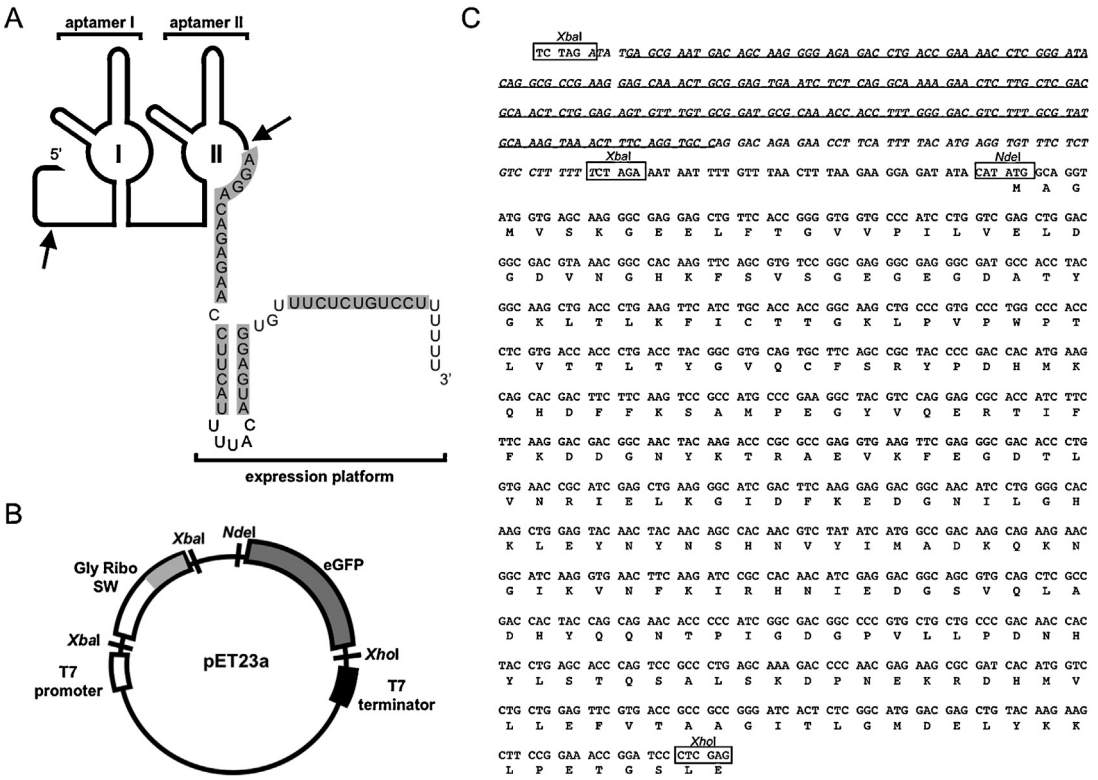

Fig. 1. (A) Schematic representation of the secondary structure of the *B. subtilis* glycine riboswitch composed of tandem aptamers and expression platform. The nucleotides in the putative intrinsic terminator stem are explicitly indicated. Shading shows complementary sequences. Arrows indicate the positions for preparing the aptamer-deleted (I and II) mutant. (B) Schematic representation of the plasmid construct. The glycine riboswitch region was ligated into *Xba*I site of eGFP vector (pET23a). (C) The sequence of the *B. subtilis* glycine riboswitch and the eGFP gene. The original plasmid for eGFP expression was prepared between the *Nde*I and *Xho*I sites. A region of the glycine riboswitch (shown in italics) was ligated into the *Xba*I site of the eGFP vector (pET23a). The translated sequence and corresponding amino acids are shown. Underlined areas indicate the regions corresponding to aptamers I and II, which were deleted for further experiments.

the terminator stem, thereby accelerating the transcription by RNA polymerase. In the present study, we analyzed the formation of the terminator stem under various conditions and discussed the effect on RNA stability and its relationship with evolution of riboswitches.

2. Materials and methods

2.1. Plasmid construction and preparation of oligonucleotides

Unlabeled deoxyribonucleotides were synthesized by Operon Biotechnologies Inc. (Tokyo, Japan). HPLC-purified unlabeled or dye-labeled oligonucleotides with fluorescein and DABCYL (where fluorescein is a fluorophore, and DABCYL, a quencher) were prepared by Japan Bio Services Co. Ltd. (Saitama, Japan). The concentrations of the oligonucleotide solutions were determined from the absorbance at a wavelength of 260 nm. A PCR-amplified glycine riboswitch region from *B. subtilis* genomic DNA (a kind gift from Professor Koichi Ito, University of Tokyo) was ligated into the *Xba*I-site of the eGFP vector (pET23a, a kind gift from Professor Shigetoshi Miura, Tokyo University of Science) (Fig. 1). The plasmids of the deletion and substitution mutants were prepared using the PrimeSTAR Mutagenesis Basal Kit according to the manufacturer's protocol (TaKaRa). All the plasmid DNA sequences were confirmed by Operon Biotechnologies Inc. (Tokyo, Japan). PCR-amplified related regions of these plasmids were used as templates for the RNA transcription assays.

2.2. In vitro transcription

RNA preparation using T7 RNA polymerase was performed in a reaction mixture containing 40 mM Tris–HCl (pH 7.5), 10 mM dithiothreitol, 2 mM spermidine, 6 mM magnesium chloride, 9 mM each NTP, ~0.2 mg/ml template DNA, and 5 mg/ml T7 RNA polymerase. The transcripts were purified *via* 8% polyacrylamide gel electrophoresis. For the preparation of T7 RNA polymerase, pT7-911Q plasmid (a kind gift from Dr. Subray S. Hegde, Albert Einstein College of Medicine) was transformed into *E. coli* BL21-Codon Plus (DE3)-RIL strain (Stratagene). The enzyme was expressed and purified by using Ni-NTA agarose (Qiagen).

For the transcription assay, RNA transcription was performed in a reaction mixture containing 40 mM Tris–HCl (pH 8.0), 10 mM dithiothreitol, 8 mM spermidine, 20 mM magnesium chloride, 25 mM sodium chloride, 9 mM each NTP (pH 4.5 (Figs. 2 and 3) or pH 8.0 (Fig. 4)), ~0.04 mg/ml template DNA, and 1.1 mg/ml T7 RNA polymerase with various concentrations (shown in the figures) of glycine, polyethylene glycol (PEG) 4000 (average molecular weight: ~3000), and ethylene glycol. The transcribed RNA was separated using ethidium bromide-containing 2% agarose gel electrophoresis and visualized.

2.3. Electrophoretic mobility shift assay (EMSA)

Briefly, 40 μM of each RNA corresponding to the terminator stem (wild-type or bulgeless) in water was heated at 95 °C for 5 min and chilled on ice for 5 min. Then, an equal volume of Buffer A (80 mM Tris–HCl (pH 8.0), 40 mM magnesium chloride, 50 mM sodium chloride) was added and the solutions were incubated at 37 °C for 30 min. After the addition of loading buffer containing glycerol, the solution was analyzed by electrophoresis through nondenaturing 10% polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA) (Takahashi et al., 2000). Two RNAs (Strand-1 and Strand-2) which have partially complementary sequences were also used as a dimer control. The specific sequences were as follows and the complementary parts are single and double underlined: wild-type terminator stem: 5′-AGGACAGAGAACCUUCAUUUUACAUGAGGUGUUUCUCUGUCCU-3′; bulgeless terminator stem: 5′-AGGACAGAGAACUUCAUUUUACAUGAGGUUCUCUGUCCU-3′; Strand-1: 5′-AGGACAGAGAACUUCAUUUUACCUUCAUAGGACAGAGAA-3′; Strand-2: 5′-UUCUCUGUCCUAUGAGGUUUACAUGAGGUUCUCUGUCCU-3′. $tRNA^{Ala}$ transcript and $minihelix^{Ala}$ were also used as controls (Francklyn and Schimmel, 1989). The nucleotides were separated *via* 10% native PAGE. The gel was stained with 0.04% toluidine blue.

2.4. Fluorescence resonance energy transfer (FRET) analysis

The fluorophore, fluorescein, and the quencher DABCYL were attached to the 5′- and 3′-termini of the terminator stem RNA and its derivatives, respectively. The RNAs were dissolved in water with various concentrations of PEG4000 and ethylene glycol. FRET measurements (Mergny, 1999; Mergny and Maurizot, 2001; Umehara et al., 2012) were carried out using an FP-6200 spectrofluorometer (JASCO Corporation, Tokyo, Japan) at room temperature using a 3-mm × 3-mm quartz cuvette, promptly after the preincubation at 95 °C for 10 min. For the emission spectra, the excitation wavelength was set at 490 nm.