DNA-rescuable allosteric inhibition of aptamer II ligand affinity by aptamer I element in the shortened *Vibrio cholerae* glycine riboswitch

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Glycine riboswitches contain two aptamers and turn on the expression of downstream genes in bacteria. Although full-length glycine riboswitches were shown to exhibit no glycine-binding cooperativity, the truncated glycine riboswitches were confirmed to bind two glycine molecules cooperatively. Thorough understanding of the ligand-binding cooperativity may shed light on the molecular basis of the cooperativity and help design novel intricate biosensing genetic circuits for application in synthetic biology. A previously proposed sequential model does not readily provide explanation for published data showing a deleterious mutation in the first aptamer inhibiting the glycine binding of the second one. Using the glycine riboswitch from *Vibrio cholerae* as a model system, we have identified a region in the first aptamer that modulates the second aptamer function especially in the shortened glycine riboswitch. Importantly, this modulation can be rescued by the addition of a complementary oligodeoxynucleotide, demonstrating the feasibility of developing this system into novel genetic circuits that sense both glycine and a DNA signal.

Keywords: allosteric control/aptamer/cooperativity/
genetic circuit/glycine riboswitch.

Abbreviations: IPTG, isopropyl β -D-1-thiogalactopyranoside; nt, nucleotide; PCR, polymerase chain reaction; VC, *Vibrio cholerae*.

Synthetic biology utilizes engineered genetic circuits to reprogramme or rewire organisms to achieve diverse functions, such as synthesis of cheaper drugs, generation of renewable fuels, and identification of disease mechanisms and viable drug targets (1, 2). Novel, inexpensive, and versatile genetic control elements are thus essential to the success of synthetic biology (1, 2). Frequently, synthetic biologists turn to natural systems for inspiration of novel genetic control

elements. Riboswitches are one class of naturally occurring genetic control elements, which regulate gene expression by binding to small molecule metabolites independent of proteins (3, 4). Upon binding of the biologically important metabolites, riboswitches undergo conformational changes which may affect the structure of the associated expression platforms and control gene expression through transcriptional termination, translational initiation, or alternative splicing (3–6). With increasing understanding of the gene control mechanism of riboswitches, researchers have designed and engineered several riboswitch-based genetic circuits (7, 8).

Glycine riboswitches are of special interest as potential genetic control elements. Containing two tandem ligand-sensing domains, glycine riboswitches turn on downstream gene expression upon binding glycine molecules (9–14). The glycine riboswitch from *Bacillus subtilis* has been employed to express recombinant proteins using inexpensive glycine as an inducer with yields comparable with that in xylose- and IPTG-driven expression systems (15). Glycine riboswitches may offer more than just a model of a simple metabolite-sensing genetic control gate. Two versions of the glycine riboswitches have been reported in the literature. The biological relevant full-length glycine riboswitches were reported recently by our group and the Das group (12, 13), in which the interaptamer linker forms a P0 duplex with the previously unrecognized 5' leader sequence. In over 50% of known tandem glycine riboswitches, P0 duplex complies with a kink-turn motif consensus, which increases glycine affinity, allows further RNA compaction upon binding with kink-turn motif binding proteins, and abolishes glycine-binding cooperativities (12–14). Interestingly, the previously reported glycine riboswitches lacking P0 duplex were reproducibly shown to bind two glycine molecules cooperatively (9, 10, 12, 16–18). Experiments showed that deleterious single-nucleotide mutation to individual aptamers not only disabled the mutated aptamer, but also greatly reduced the glycine-binding affinity of the other, indicating structure elements in one aptamer may control glycine binding in the other through interaptamer interaction (9, 12, 19, 20). Further analysis of the P0-lacking glycine riboswitches may shed light on the molecular basis of the ligand-binding cooperativity and help design novel sophisticated biosensing genetic circuits for application in synthetic biology.

In the present work, we choose to study the P0-lacking *Vibrio cholerae* glycine riboswitch (nts 1–225, referred to as VCIII hereafter) as this construct exhibits the highest ligand-binding cooperativity, with a

Hill coefficient of 1.64 (9). Our goal is to analyse the ligand-binding cooperativity in greater detail and identify structure elements that are not only responsible for allosteric control of glycine sensing but also amenable for future design of novel genetic circuits.

The Strobel group has proposed a sequential glycine-binding model for the ligand-binding cooperativity in VCIII glycine riboswitch (19). According to this model, one glycine molecule binds to the downstream aptamer II first and allows tertiary interactions to be made between the two aptamers, facilitating binding of a second glycine molecule to the upstream aptamer I, leading to glycine-binding cooperativity. This model provides explanation for the inhibition of aptamer I's glycine affinity by a deleterious mutation in aptamer II. However, this model does not provide explanation for the inhibition of aptamer II's glycine affinity by a deleterious mutation in aptamer I. Combining direct deletion studies, mutational analysis and sequence examination, we have identified and verified an aptamer I element which effectively controls the aptamer II glycine affinity, especially in the shortened VC glycine riboswitch. Interestingly, the inhibitive effect of this structural element can be rescued with a short nucleic acid strand, restoring aptamer II glycine binding. This provides a possibility of engineering shortened glycine riboswitch into a genetic control element that senses both glycine metabolite and a DNA input signal.

Materials and Methods

DNA constructs of the RNAs

The VC glycine riboswitch genes were generated by recursive PCR (21) from DNA oligonucleotides (Integrated DNA Technologies) with EcoRI restriction site and T7 promoter sequence engineered at the 5' terminus, and EarI and HindIII sites engineered at the 3' terminus similar to that described previously (22). Double digestion of the riboswitch DNA genes with EcoRI and HindIII (New England Biolabs) was then performed in the supplied NEB buffer 2 at 37°C for 1 h. Double-digested riboswitch DNA genes and pUC19 vector were ligated together using T4 DNA ligase (New England Biolabs) in the supplied T4 DNA ligation buffer at 16°C with overnight incubation to generate glycine riboswitch plasmids as reported previously (23). The glycine riboswitch mutants were constructed either by a similar method or by QuikChange site-directed mutagenesis (Stratagene) from the corresponding wild-type plasmids and proper DNA oligonucleotide primers. These plasmids were transformed into JM109 cells, amplified, purified with Qiagen plasmid preparation kits, and sequenced by Eton Bioscience Inc.

In vitro transcription for the preparation of RNAs

Riboswitch plasmids were linearized by incubated with EarI (New England Biolabs) at 0.2 U/μg of plasmid in the supplied NEB buffer 4 at 37°C overnight. RNAs were transcribed in 1 ml reaction mixture containing 40 mM Tris-HCl (pH 7.9), 2 mM spermidine-3HCl, 10 mM DTT, 25 mM MgCl₂, 5 mM each of the nucleotide triphosphates, 50 μg/ml linearized plasmid, 0.01 U/μl 5 PRIME™ stop “RNase inhibitor RX (5 PRIME life science), 8 mU/μl thermostable inorganic pyrophosphatase (New England Biolabs), and 45 μg/ml T7 RNA polymerase (expressed and purified in-house) for 4 h to overnight at 37°C. The transcription mixture was incubated with 1 mM CaCl₂ and RNase-free DNase I (Promega) at a concentration of 0.5 U/μg of DNA plasmid at 37°C for 30 min. The RNA transcripts were purified by 6–8% denaturing polyacrylamide gel electrophoresis, excised under UV shadowing, electroeluted into TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA), PCA extracted, precipitated with ethanol and resuspended in TE buffer. RNA concentrations were determined by UV absorbance at 260 nm wavelength with extinction coefficients calculated by OligoCalc (24).

5' end ³²P labeling of RNAs

In a typical procedure, 40 pmol RNA transcript was dephosphorylated by incubation with 1 U of shrimp alkaline phosphatase (Fermentas Life Sciences) in the supplied phosphatase buffer in a total volume of 10 μl at 37°C for 30 min. After incubation at 65°C for 15 min to inactivate the phosphatase, 1 μl sterile H₂O, 1.5 μl 10× T4 polynucleotide kinase buffer (New England Biolabs), 2 μl γ-³²P ATP (Perkin Elmer), and 0.5 μl 10 U/μl T4-polynucleotide kinase (New England Biolabs) were added and the reaction mixture was incubated at 37°C for 30 min for RNA phosphorylation. For RNA transcripts with the 5' end buried by or too close to a duplex region, to improve the labeling yield, the transcripts were first annealed to a DNA oligonucleotide to free the 5' end before dephosphorylation and phosphorylation reactions. For example, to radiolabel the 5' end of VCII81 (nts 81–225), 40 pmol VCII81 was first incubated with 1.05 equiv of an annealing oligonucleotide (AACTACAGTCCTCG CTTATTC) at 95°C for 1 min, then at room temperature for 10 min before dephosphorylation and phosphorylation reactions. After phosphorylation, ³²P-labelled RNAs were purified by 6–8% denaturing polyacrylamide gel electrophoresis, visualized on an X-ray film, excised and passively eluted in 300 μl TE buffer on a rotator at 4°C overnight. After centrifugation, the radiolabelled RNAs were PCA extracted twice, precipitated with ethanol, and redissolved in TE buffer.

In-line probing assays and quantification

In-line probing assays were carried out similar to the reported procedure (25). Briefly, ~50 kcpm ³²P labelled RNA transcript was incubated at 25°C for ~48 h in 50 mM Tris-HCl (pH 8.3), 20 mM MgCl₂, 100 mM KCl under various glycine concentrations. For the in-line probing assays of VCII73 in the presence of complementary oligodeoxynucleotide (ACCTGAAAGATCAGAT), 12 μM complementary oligodeoxynucleotide was first annealed with 50 kcpm 5' ³²P radiolabelled VCII73 by incubation at 95°C for 1 min and then room temperature 5 min before addition of MgCl₂ and KCl. After 48 h incubation at 25°C, spontaneously cleaved RNA fragments were resolved by 8% denaturing PAGE, dried, exposed to PhosphorImager screens, scanned by PhosphorImager SI (Molecular Dynamics) and quantified by ImageQuant v5.2 (Molecular Dynamics). For VC glycine riboswitches and mutants, the following regions are quantified if contained on the constructs and well resolved: U74 (r2), A121-G123 (r3), nts 124–133 (for VCII81 and VCII73 when rescued by a complementary oligodeoxynucleotide), G136-G137 (r4), G146 (r5), G170-A172 (r6), C177-A178 (r7) and U207-C208. To control for loading differences, the band intensities for tight binding constructs ($K_d < 100 \mu\text{M}$, including VCII66P3a and VCII73M1) were normalized to reference bands (U156–U161). The reference bands do occasionally show small perturbation which has little effect on the glycine affinity of the tight binding constructs but can notably affect weak binders ($K_d > 500 \mu\text{M}$); to avoid this, weak binders were not normalized. K_d values of the individual glycine-perturbed regions were determined by non-linear regression fitting of each plot using the following equation: fraction bound = $m_1 \times [\text{gly}]/(K_{d1} + [\text{gly}]) + m_2$. The reported binding affinities were calculated as average K_d values obtained from the individual glycine-perturbed regions with curve fit coefficients better than 0.97 in at least two independent trials. For constructs that exhibit two glycine-induced transition due to two independent folding species, the following equation was used: fraction bound = $m_1 \times [\text{gly}]/(K_{d1} + [\text{gly}]) + m_2 \times [\text{gly}]/(K_{d2} + [\text{gly}]) + m_3$.

Native gel electrophoresis

5' Radiolabelled RNA was folded by the following procedure: 1 μl 20 μM RNA was added to 7 μl 10 mM Tris-HCl (pH 7.5) and incubated at room temperature for 2 min. The VCII73 rescue DNA oligo, where used, was also added to the mixture and incubated at either room temperature or 95°C for 2 min. 1 μl 100 mM MgCl₂ and 1 μl 0.1 M glycine were added to the mixture, followed by incubation at room temperature for 20 min. This mixture was then combined with 2 μl native gel loading dye (50% v/v glycerol, 0.1% w/v xylene cyanol) and loaded onto a 6% native polyacrylamide (29:1 acrylamide:bisacrylamide) gel in TB buffer (89 mM Tris base and boric acid) containing 5 mM MgCl₂ and 5 mM glycine. The electrophoresis was prerun with TB buffer containing 5 mM MgCl₂ for 2 h constant 25 W and conducted for additional 3.5 h after sample

loading at 4°C with constant 250 V. The gel was dried, exposed to PhosphorImager screens, scanned with PhosphorImager SI (Molecular Dynamics), and analysed by ImageQuant v5.2 (Molecular Dynamics).

Results

5-Extended aptamer II construct VCII66 lost most of its glycine-binding affinity

The sequential glycine-binding model proposed by the Strobel group (19) provided a great explanation for the inhibition of aptamer I's glycine affinity by a deleterious mutation in aptamer II of the P0-lacking VC glycine riboswitch. However, this model does not readily explain the inhibition of aptamer II's glycine affinity by a deleterious mutation in aptamer I. Breaker and coworkers showed that a deleterious mutation in aptamer I (VCIIIG17C, nts 1–225, G17C; Fig. 1, see figure legend for construct naming scheme) not only disabled the glycine-binding affinity of aptamer I, but also greatly reduced the glycine-binding affinity of aptamer II in the P0-lacking VC glycine riboswitch (9). This interaptamer inhibition, also reported by the Strobel group (19) and our group (12), is consistent with the ligand-binding cooperativity in the P0-lacking VC glycine riboswitch (VCIII). Ligand-binding cooperativity in a multi-subunit system typically suggests that binding to one site greatly enhances the binding at other sites (26). However, one version of the isolated aptamer II, VCII122, has been demonstrated to bind glycine even stronger than the double-aptamer riboswitch VCIII (59 ± 12 µM, Table I) with a binding affinity of 4.4 ± 2.0 µM (Table I) (12). The discrepancy between the glycine affinity of aptamer II in VCIIIG17C ($[2.3 \pm 0.5] \times 10^3$ µM, Table I, Supplementary Fig. S1) and that in VCII122 suggests that certain structural elements may exist in aptamer I and interfere with aptamer II function when aptamer I is disabled or not fully formed. To test this hypothesis, we extended the sequence of aptamer II at the 5' end to prepare the VCII66 construct (nucleotides, nts 66–225, Fig. 2), which contains the 3' half of aptamer I, the linker between two aptamers, and the fully functional aptamer II construct VCII122. As Kwon and Strobel (10) have indicated that P3a of aptamer I might be important to the glycine-binding cooperativity in VCIII, we included the 5' half of the P3a helix (nts 66–69) to accommodate the whole P3a helix in the construct. The glycine-binding affinity of VCII66 was then evaluated via in-line probing assays which map local structure changes by measuring the flexibility of the phosphodiester bond to achieve in-line geometry for non-enzymatic cleavage through internal transphosphorylation (25, 27). Results showed that the glycine affinity of VCII66 as calculated from aptamer II regions (Fig. 1) is much weaker than that of VCII122, with a K_d value of $(1.1 \pm 0.3) \times 10^3$ µM (Table I, Fig. 2). The weaker binding affinity could also result from a mixture of multiple folding species in which the misfolded species may exhibit high cleavage background and mask the cleavage pattern of the correctly folded species. The overall protection pattern of VCII66 in 100 mM glycine is similar to that of

Fig. 1 Secondary structure of the P0-lacking VC glycine riboswitch (VCIII). r2–r8 indicate the representative glycine-induced perturbation regions in the in-line probing assay of VCIII; the corresponding nucleotides are either circled or boxed with a square, which represents either decreasing cleavage or increased cleavage with increasing glycine concentration, respectively. 'I' and 'II' refer to upstream aptamer I and downstream aptamer II, connected by a short linker. Definitions of construct lengths/mutations are given in the figure.

VCII122 in the overlapping regions (Fig. 2), indicating that aptamer II folds into similar overall structures in these two constructs at saturating glycine concentrations. In addition, we have analysed the riboswitch folding under in-line probing conditions with native gel electrophoresis. Results confirmed that VCII66 folds into a single species (Fig. 3, Lane 1). Combined together, these experiments suggest that there are critical structural elements between nt 66 and 122 interfering with glycine affinity of aptamer II in VCII66.

Identification of potential interaptamer interaction inhibiting the glycine affinity of VCII66

We denote the aptamer I structural element that interferes with aptamer II glycine binding as Region A, which is likely to interact with a specific region (or several regions) in aptamer II denoted Region B in the absence of glycine to reduce Region B's ability to participate in native structure formation and consequently decrease aptamer II's glycine affinity. Accordingly, in the in-line probing assays of VCIII or VCII66, Region B may interact with Region A in the absence of glycine while participating in the native structure formation in the presence of glycine. Thus, the in-line probing cleavage pattern of a potential Region B may appear to be unperturbed or only marginally perturbed at different glycine concentrations as either in the presence or in the absence of glycine it is structured. For VCII122, however, as Region A is not present in the construct, Region B may show structure perturbation from less structured in the absence of glycine to more structured in the presence of glycine. We compared the in-line probing patterns of the aptamer II regions in VCIII and VCII66 with that of VCII122 (Figure 1 and Figure S2 in the report by Breaker and

Table I. Binding affinities of glycine riboswitches and mutants^a.

RNA	K_d (μ M)	Description of the constructs
VCII122	4.4 ± 2.0^b	nts 122–225
VCIII	59 ± 12^b	nts 1–225
VCIIIG17C	$(2.3 \pm 0.5) \times 10^3$	nts 1–225, G17C
VCII66	$(1.1 \pm 0.3) \times 10^3$	nts 66–225
VCII81	6.1 ± 1.9	nts 81–225
VCII73	$(1.5 \pm 0.2) \times 10^3$	nts 73–225
VCIIIG17CM1	$(1.3 \pm 0.5) \times 10^3$	nts 1–225, G17C, CUUU75-78AAAA
VCII66P3a	Misfold: $(1.5 \pm 0.3) \times 10^3$ correct fold: 15.5 ± 4.2	nts 66–225, GAAG66-69UUUU, CUUU75-78AAAA
VCII73M1	Misfold: $(2.5 \pm 1.1) \times 10^3$ correct fold: 34.0 ± 31.6	nts 73–225, CUUU75-78AAAA
VCIIIG17CP3a	$(1.2 \pm 0.2) \times 10^3$	nts 1–225, G17C, GAAG66-69UUUU, CUUU75-78AAAA
VCII73 (+DNA)	12.5 ± 5.8	nts 73–225, with 12 μ M complementary DNA ^c

^aDetermined by in-line probing assays. Binding affinities were calculated as average K_d values (and $\pm$ their standard deviation) obtained from the individual glycine-perturbed regions with curve fit coefficients better than 0.97 in at least two independent trials.

^bData were taken from (12).

^cComplementary oligodeoxynucleotide sequence is ACCTGAAAGATCAGAT.

Fig. 2 The glycine affinity of VCII66 is drastically reduced compared with that of VCII122. (A) Secondary structure of VCII66. r5–r8 and circled nucleotides are the same as defined in Fig. 1. (B) Representative semi-log plots of the normalized fraction of RNA bound versus glycine concentration for r5 and r6 of VCII66. (C) In-line probing gel image of VCII66. NR indicates no reaction.

coworkers (9) and our in-line probing results) and found that nts 147–150 (AGAG) is the best region fit to this description of a candidate Region B. Examination of the corresponding regions in two other well-characterized glycine riboswitches from *B. subtilis* (9) and *Fusobacterium nucleatum* (10) reveals the identical AGAG sequence; thusly nts 147–150 (AGAG) in VCIII is chosen as Region B. As long-range interactions in RNA structure involving a stretch of nucleotides often are through duplex

formation with Watson-Crick or Wobble base pairs, such as kissing-loop interactions (28–31) pseudoknots (31–34), etc., we postulated that Region A and Region B interact through a simple duplex formation. Considering both Watson-Crick and Wobble pairing, Region B (AGAG) could pair with CUCU, CUUU, UUCU or UUUU. Searching the primary sequence of VCIII with these four short oligonucleotide sequences, we found three candidates in aptamer 1: nts 35–38 (UUUU), nts 75–78 (CUUU), nts 91–94 (UUCU).

Fig. 3 Native gel with 5' ^{32}P radiolabelled RNA constructs in this article showing the existence of a single species, with the exception of Lane 2 and 4 showing an additional slower migrating band. Lane 1, VCII66; Lane 2, VCII66P3a; Lane 3, VCII73; Lane 4, VCII73M1; Lane 5, VCII81; Lane 6, VCIIIG17CP3a; Lane 7, VCIIIG17C; Lane 8, VCIIIG17CM1.

Comparing the corresponding sequences in the glycine riboswitches from *B.subtilis* and *F.nucleatum*, we found that only nts 75–78 is conserved to pair with Region B. Importantly, nts 75–78 locates between 66 and nt 122 as predicted in the previous section. Therefore, nts 75–78 (CUUU) is assigned as Region A in VC glycine riboswitch. Furthermore, nts 75–78 (CUUU) and nts 147–150 (AGAG) locate in the highly conserved regions of the 3' half of the P3a helix on aptamer I and J1/2 of aptamer II, respectively, strengthening their potential functional roles as Region A and Region B.

Verification and characterization of the inhibitory effect of Region A on aptamer II function in the shortened glycine riboswitch

First, we prepared a construct VCII81 (nts 81–225, Fig. 4A), removing the entire predicted Region A and taking advantage of the consecutive guanines at position 81 and 82 for efficient *in vitro* transcription. If Region A is fully removed in this construct, we expect VCII81 to show significant rescue in the glycine-binding affinity; in-line probing assays were used to determine the glycine binding K_d of VCII81. Results (Fig. 4B and C) showed that VCII81 retains the full ligand-binding capacity of VCII22 with a K_d of $6.1 \pm 1.9 \mu\text{M}$ (Table I), consistent with the complete removal of Region A. Native gel electrophoresis results confirmed that VCII81 folds into a single species (Fig. 3 Lane 5). Interestingly, in the cleavage pattern of the in-line probing assay of VCII81 (Fig. 4C), in addition to the expected glycine-perturbed regions r5–r8, we observed that nts 123–133 and nt 135 exhibit glycine-induced structural perturbation with similar glycine affinities. These observations rationalize the loss of glycine affinities in aptamer II glycine riboswitches shorter than VCII22 reported previously (12). Particularly, nts 123–124 are located within region r3, a glycine-perturbed single-stranded aptamer I region in the double-aptamer glycine riboswitches. Observation of glycine-induced structural perturbation in this region for VCII81 is reminiscent of the similar observation in the previously reported VCLDG17C construct (nts –7–225, G17C), in which region r3 exhibits similar glycine-induced structural perturbation as other aptamer II regions (r5–r7) although aptamer I is disabled (12). These results, combined together, confirm that aptamer II requires structural elements

from aptamer I to bind glycine properly. In turn, this also rationalizes the potential existence of the aptamer I region (Region A) to modulate the glycine affinity of aptamer II.

The difference in the glycine-binding affinities between VCII66 and VCII81 suggests that the structural elements modulating aptamer II function locate between nt 66 and nt 80, which contains the predicted Region A (nts 75–78, the 3' half of P3a), L3a, and the 5' half of P3a. To test whether L3a and the 5' half of P3a are responsible in modulating aptamer II function, we have prepared a construct VCII73 (nts 73–225), removing the 5' half of P3a and L3a. In-line probing experiments showed that VCII73 binds glycine much more weakly than VCII22 or VCII81 with a K_d value around $(1.5 \pm 0.2) \times 10^3 \mu\text{M}$ (Table I, Supplementary Fig. S2), even weaker than VCII66. Similar to VCII66, native gel electrophoresis showed that VCII73 folds into a single species (Fig. 3, Lane 3), ruling out of the possibility of a masking effect of the misfolded species. The weaker affinity of VCII73 compared with VCII66 may be due to the fact that the 5' portion of the P3a stem is no longer present in VCII73 (as it is in VCII66) to competitively duplex with Region A, allowing Region A to inhibit aptamer II function even further through better interaction with Region B.

Another way to verify the inhibitory effect of Region A on aptamer II glycine binding is to directly mutate Region A in VCII66 or VCII73 to prevent the interaction between Region A and Region B and analyse whether there is any rescue in aptamer II glycine affinity. We therefore prepared mutants VCII73M1 (nts 73–225, CUUU75-78AAAA) and VCII66P3a (nts 66–225, GAAG66-69UUUU, CUUU75-78AAAA). Here VCII66P3a contains extra mutations GAAG66-69UUUU to maintain possible P3a duplex formation. We then analysed the glycine-binding properties of these constructs with in-line probing assays. Unfortunately, both constructs exhibit two glycine-induced transitions, making it difficult to evaluate the glycine affinity of these constructs. Native gel electrophoresis showed that in addition to the band at the expected location, a new slower migrating band was observed for each of these two constructs (Fig. 3, Lane 2 and 4). We attribute this second species to either a simply misfolded or oligomeric form that may exhibit glycine-induced transition at a higher glycine concentration. In-line probing data fit quite well with the equation describing two independent glycine-folded species (Materials and Methods section). The correctly folded species showed a glycine affinity of $15.5 \pm 4.2 \mu\text{M}$ for VCII66P3a (Supplementary Fig. S3) and $34 \pm 32 \mu\text{M}$ for VCII73M1 (Supplementary Fig. S4), both exhibiting a significant rescue from their parent constructs (Table I).

Collectively, these results confirmed the predicted Region A (nts 75–78) to be the structural element responsible for inhibiting aptamer II glycine affinity. In addition, Region A is solely responsible for the inhibition without the need for loop L3a or P3a duplex formation.

Fig. 4 VCIH81 retains glycine-binding affinity of VCIH122. (A) Secondary structure of VCIH81. r5–r8 and the nucleotides circled or boxed with a square are the same as defined in Fig. 1. Additional glycine-induced perturbation regions nts 123–133 and nt 135 are also denoted. (B) Representative semi-log plots of the normalized fraction of RNA bound versus glycine concentration for r7, r6 and U128 of VCIH81. (C) In-line probing gel image of VCIH81. NR, T1 and OH represent no reaction, partial digestion with RNase T1, and partial digestion with alkali, respectively.

Characterization of the interaptamer effect of Region A in double-aptamer glycine riboswitches

Having established the modulating role of Region A on the glycine-binding affinity of aptamer II in the single aptamer constructs, next we investigate its role on aptamer II in the double-aptamer glycine riboswitches. According to the crystal structure of the glycine riboswitches (11, 20), the corresponding Region A directly participates in the glycine-binding pocket of aptamer I. This means direct mutation of Region A on the wild-type double-aptamer VCIH glycine construct will likely diminish or disable aptamer I glycine binding, making it difficult to precisely dissect the effect of Region A on aptamer II glycine affinity as deleterious mutation in aptamer I was demonstrated to reduce the glycine affinity in aptamer II previously (9, 10, 12). To circumvent this complication, we have examined the effect of Region A on the double-aptamer VC glycine riboswitch (VCIH) in the presence of G17C mutation, which rendered aptamer I incapable of binding glycine regardless of the presence or absence of Region A. As reported previously (9, 10, 12), mutation in VCIHG17C disables aptamer I function and greatly reduced the glycine affinity of aptamer II. If Region A remains functional and modulates the glycine-binding activity of aptamer II in VCIHG17C, one would expect that a mutation in Region A would

remove this modulation and rescue the glycine affinity in aptamer II. We therefore prepared VCIHG17CM1 (nts 1–225, G17C, CUUU75–78AAAA) and analysed its glycine-binding properties. Native gel showed that VCIHG17CM1 folded into a single species similar to VCIHG17C (Fig. 3, Lane 7 and 8) and in-line probing experiments showed that mutation in Region A had a modest rescuing effect (~ 2 -fold) and increased the glycine affinity of aptamer II in VCIHG17CM1 to $(1.3 \pm 0.5) \times 10^3 \mu\text{M}$ (Fig. 5A; Supplementary Fig. S5) from $(2.3 \pm 0.5) \times 10^3 \mu\text{M}$ in VCIHG17C. In addition, we prepared VCIHG17CP3a (nts 1–225, G17C, GAAG66–69UUUU, CUUU75–78AAAA) which contains additional mutations to maintain a possible P3a duplex. Native gel showed that VCIHG17CP3a folded into a single species (Fig. 3 Lane 6) and in-line probing experiments showed that VCIHG17CP3a bound glycine with a modestly rescued affinity of $(1.2 \pm 0.2) \times 10^3 \mu\text{M}$ (Supplementary Fig. S6), similar to VCIHG17CM1.

The drastically different modulating effect of Region A in two versions of constructs, VCIHG17C versus VCIHG17CM1/VCIHG17CP3a (~ 2 -fold modulation, Fig. 5A, Table I) and VCIH73 versus VCIH81 (~ 200 -fold modulation, Fig. 5B, Table I), shows that Region A modulates aptamer II activity in a context dependent manner and truncation has a large effect on the

Fig. 5 Effect of Region A on the glycine-binding affinity of aptamer II. Representative semi-log plots of the normalized fraction of RNA bound versus glycine concentration for r6 of VCIIG17CM1 compared with that of VCIIG17C (A) and VCII73 compared with VCII81 (B).

interaptamer interaction and glycine binding. In the context of the double-aptamer glycine riboswitches, Region A plays a partial but not a sole role in the interaptamer inhibition of aptamer II. What else can cause the interaptamer inhibition of aptamer II in the double-aptamer glycine riboswitches? It is possible that when the aptamer I glycine-binding site is not occupied, interaptamer interaction is not formed properly for optimum glycine binding in the aptamer II site. This is not completely surprising as we have demonstrated that glycine binding acts synergistically with interaptamer interaction in the glycine riboswitch (12). The smaller rescuing effect of Region A mutation in the double-aptamer glycine riboswitch may be more biological relevant to the naturally occurring glycine riboswitch, where the full-length P0-containing VC glycine riboswitch does not exhibit glycine-binding cooperativity (12, 14). However, this result also shows that the shortened VC glycine riboswitch VCII73 is more suitable for future engineering into a novel genetic circuit.

Allosteric inhibition of aptamer II function by Region A may be rescued by a DNA oligonucleotide

Our finding of the inhibitory role of Region A on aptamer II glycine affinity in the shortened VC glycine riboswitch is intriguing. The ~ 200 -fold effect provides a good window for potential gene expression control. However, in order to harness this effect, we need to be able to reverse this inhibition by an exogenous effector, a signal can be sensed by an *in vitro* or *in vivo* system in the future. Therefore, we investigated whether the modulation effect of Region A on aptamer II can be rescued by a simple input signal. Our previous analysis indicates that Region A may regulate aptamer II function by duplexing with sequences on aptamer II. This suggests that a short nucleic acid strand complementary to Region A may competitively remove the inhibitory effect and rescue the glycine-binding affinity of aptamer II. To test this, the glycine-binding affinity of VCII73 in the presence of complementary oligodeoxynucleotide (ACCTGAAAGATCAGAT, Fig. 6A) was determined with in-line probing. Results showed that in the presence of $12 \mu\text{M}$ complementary oligodeoxynucleotide, VCII73 exhibited a

similar glycine perturbation pattern as VCII81 and bound glycine with an affinity of $12.5 \pm 5.8 \mu\text{M}$ (Fig. 6B and C). This corresponds to a 120-fold increase of glycine affinity from that of VCII73 and an almost complete restoration of the glycine affinity of VCII81.

Discussion

Glycine riboswitches are most often located upstream of the *gcvTHP* operon controlling the expression of glycine cleavage system (9, 35), which breaks down glycine and produces carbon dioxide, ammonia and 5–10-methylenetetrahydrofolate in the presence of Tetrahydrofolate (36). 5–10-methylenetetrahydrofolate can also serve as a source of methyl groups for methionine, serine and 2'-deoxythymidine (37). Glycine riboswitches have also been identified upstream of the *glcB* gene in the most abundant marine bacterium *Candidatus Pelagibacter ubique* (18), controlling the expression of malate synthetase which makes malate from exogenous glycine derivatives. These show that naturally occurring glycine riboswitches are important regulators of carbon metabolism in bacteria. Besides the natural gene control functions, glycine riboswitches have been used for controllable overproduction of recombinant proteins using the inexpensive glycine inducer (15). This has demonstrated application of the glycine riboswitch as a general genetic control element. However, the ligand-binding cooperativity in the P0-lacking glycine riboswitch suggests that this system may be able to offer more than just a basic metabolite-sensing genetic control gate to synthetic biology.

In this work, we have identified a structural element (Region A) located in aptamer I that regulates the ligand-binding affinity of aptamer II in the shortened glycine riboswitches, VCII66 and VCII73. Removal of Region A from VCII73 resulted in a construct VCII81 which showed fully restored (~ 200 -fold rescue) glycine-binding affinity. The interaction partner of Region A in aptamer II is proposed as Region B (nts 147–150) for this aptamer II modulation. However, this is not experimentally tested because the proposed Region B is highly conserved, and the mutations in

Fig. 6 The glycine-binding affinity of VCII73 can be rescued by the addition of a complementary oligodeoxynucleotide. (A) Secondary structure of VCII73 when annealed to complementary oligodeoxynucleotide ACCTGAAAGATCAGAT. r5–r8 and the nucleotides circled or boxed with a square are the same as defined in Fig. 1. Additional glycine-induced perturbation regions nts 123–133 are also denoted as defined in Fig. 1. (B) Representative semi-log plots of the normalized fraction of RNA bound versus glycine concentration for r7, r6 and U128 of VCII73 rescued by complementary oligodeoxynucleotide ACCTGAAAGATCAGAT. (C) In-line probing gel image of VCII73 rescued by complementary oligodeoxynucleotide ACCTGAAAGATCAGAT. NR represents no reaction.

Region B are likely to diminish the glycine affinity of aptamer II, making any rescue of aptamer II's glycine affinity due to the disruption of Region A and B interaction unobservable. Interestingly, a short oligodeoxynucleotide complementary to Region A and neighboring nucleotides can rescue the glycine-binding activity of VCII73 by ~120-fold. Given the demonstrated importance of the gene control functions of natural and synthetic glycine riboswitches, our findings could pave the path for developing a new genetic circuit sensing two mechanistically distinct signals, a short nucleic acid strand and small molecule metabolite (glycine).

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

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