Conformational adaptation of UNCG loops upon crowding

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ABSTRACT

If the A-form helix is the major structural motif found in RNA, the loops that cap them constitute the second most important family of motifs. Among those, two are overrepresented, GNRA and UNCG tetraloops. Recent surveys of RNA structures deposited in the PDB show that GNRA and UNCG tetraloops can adopt tertiary folds that are very different from their canonical conformations, characterized by the presence of a U-turn of a Z-turn, respectively. Crystallographic data from both a lariat-capping (LC) ribozyme and a group II intron ribozyme reveal that a given UUCG tetraloop can adopt a distinct fold depending on its structural environment. Specifically, when the crystal packing applies relaxed constraints on the loop, the canonical Z-turn conformation is observed. In contrast, a highly packed environment induces “squashing” of the tetraloop by distorting its sugar-phosphate backbone in a specific way that expels the first and fourth nucleobases out of the loop, and falls in van der Waals distance of the last base pair of the helix, taking the place of the pair formed between the first and fourth residues in Z-turn loops. The biological relevance of our observations is supported by the presence of similarly deformed loops in the highly packed environment of the ribosome and in a complex between a dsRNA and a RNase III. The finding that Z-turn loops change conformation under higher molecular packing suggests that, in addition to their demonstrated role in stabilizing RNA folding, they may contribute to the three-dimensional structure of RNA by mediating tertiary interactions with distal residues.

Keywords: UNCG tetraloop; Z-turn loop; lariat-capping ribozyme; group II intron

INTRODUCTION

To fulfill their biological roles, RNA molecules adopt specific structures able to interact with their molecular partners, change conformations and carry out catalytic reactions. Their structures result from folding, a process of condensation of the RNA chain in which concatenation of the Watson–Crick base pairs builds up A-form anti-parallel helices interspersed by single-stranded regions. Nevertheless, the experimentally determined RNA structures stored in the PDB indicate that single-stranded regions also adopt specific structures, which allow the RNA to acquire diverse and complex three-dimensional architectures (Masquida et al. 2010; Westhof et al. 2011; Koculi et al. 2012). One of the most abundant types of motifs found in the RNA structural repertoire is represented by apical loops, which cap helices and allow the outgoing RNA strand to be anti-parallel to the ingoing one.

Among the loop motifs, tetraloops of the GNRA and UNCG families are overrepresented. GNRA tetraloops have long been known to weave long-range tertiary contacts (Michel and Westhof 1990; Pley et al. 1994; Cate et al. 1996). The orientation of the adenosine nucleobases in the loop favor interactions involving their sugar or Watson–Crick edges, called A-minor interactions (Doherty et al. 2001; Nissen et al. 2001). The receptors of these loops have been the subject of many studies (for review, see Fiore and Nesbitt 2013), including by in vitro selection methods (Costa and Michel 1997; Robertson et al. 1999; Barrick et al. 2001; Geary et al. 2008). However, in sharp contrast with GNRA loops, UNCG tetraloops have been mostly considered as nucleating RNA folding due to their exceptional thermodynamic stability (Varani et al. 2019). This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
RESULTS

The circularly permuted (CP) form of the LC ribozyme presents a distorted UUCG loop

In vivo, the LC ribozyme (Fig. 1) catalyzes two reactions. The branching reaction results in the formation of a 3 nt lariat upon the nucleophilic attack of the phosphate group of C230 by the 2′ hydroxyl of U232. Due to the chemical reversibility of the transesterification reaction, the ligation is also observed, although the equilibrium is in favor of the branching reaction. In addition, in vitro only, hydrolysis at C230 is also noticeable (Nielsen et al. 2005). In vitro, the coexistence of three different reactions generates a variety of RNA products, which do not favor crystallization. In order to select a unique conformation and improve crystallization of the LC ribozyme, we engineered a circular
permutation so that the natural 5’ and 3’ ends are tethered by the UUCG loop, and the nucleotides at the catalytic cleavage site, C230 and G229, become the new 5’ and 3’ ends, respectively. An optimized hammerhead construct was added upstream of the unfavorable transcriptional sequence 5′-CAU 3′ corresponding to the lariat sequence (Meyer and Masquida 2014). No interference of the engineered UUCG loop with the overall architecture was expected.

The crystal structure of the CP LC ribozyme (Meyer et al. 2014) shows that the DP2 UUCG loop adopts a squashed conformation where the backbone of residues 2 and 3 takes the place of the U–G pair formed between residues 1 and 4 characteristics of Z-turn loops, expelling nucleotides 1 and 4 into the solvent (Fig. 2A). Close inspection of the crystal packing indicates that the DP2 loop contacts two symmetry-related LC ribozyme molecules. One symmetry-related molecule (sym1) makes van der Waals contacts with residues 2 and 3 and the second symmetry-related molecule (sym2) provides stacking and H-bonding interactions to the expelled G4 (Fig. 3A,C).

Removal of one base pair from the DP2 stem restores the canonical UNCG loop structure

Following this observation, we engineered a CP LC ribozyme construct (CP LC DP2ΔUA) in which one base pair was removed from the DP2 stem (Fig. 1). This RNA crystallized under the same conditions and in identical space group P212121 with each parameter expanded by <4% with respect to the parent CP LC ribozyme construct (Table 1; PDB: 6gyv). The crystal structure of the CP LC DP2ΔUA construct reveals that the canonical structure of the UUCG loop capping the DP2 stem has been restored (Fig. 2B) and displays the typical Z-turn loop structural characteristics reported in D’Ascenzo et al. (2017). It appears that the removal of one base pair from the DP2 stem restores enough space so that residues U1 and G4 from the loop can stack within the helix and form the trans base pair between their Watson–Crick and sugar edges (Leontis and Westhof 2001), respectively. Although the crystal packing remains unchanged, the conformational change of the loop results in the loss of packing interactions due to the different spatial orientation of the fourth residue of the loop. While sym1 still provides van der Waals distance contacts, the increased distance between G4 and sym2 does not allow anymore any type of interactions (Fig. 3C).

Crystal packing induces a conformational change on a UUCG loop engineered at a peripheral section of a group II intron RNA

Group II introns are large self-splicing RNAs and mobile genetic elements of bacterial origin. They are usually regarded as the ancestors of spliceosomal introns and the spliceosome in eukaryotes. Similarly to nuclear pre-mRNA splicing, group II self-splicing proceeds through two consecutive transesterification reactions and the intron is released in a typical branched conformation called the “lariat.” The reversibility of the transesterification reactions allows the excised intron lariat to insert back into RNA or DNA targets through the reverse-splicing pathway. Reverse splicing into DNA is at the basis of the genomic mobility of group II introns. Recently, an engineered group II ribozyme derived from the O. iheyensis intron was used to obtain two crystal structures of a group II intron lariat primed for reverse splicing (Costa et al. 2016). The lariat form of this chimeric O. iheyensis intron was crystallized either alone or in the presence of a nonreactive 5’-exon analog RNA. Interestingly, each condition gave rise to a different crystal form having a distinct set of packing contacts. In the lariat-alone crystal structure, one of the packing interactions involves a UUCG tetraloop engineered at the tip of intron domain III. Remarkably, this crystal contact forces the UUCG loop to adopt a squashed conformation similar to the one observed in the DP2 UUCG loop from the CP LC ribozyme (Fig. 4A). Conversely, however, in
the context of the 5′-exon-bound lariat structure, the UUCG loop of domain III points toward the solvent and is seen to adopt the canonical conformation expected for the Z-turn tetraloop family (Fig. 4B). Noteworthy, the crystal packing variations do not influence the canonical conformation of the other UUCG loop engineered in intron domain IV.

Occurrences of “squashed” loop conformations within other RNAs

To find biologically relevant examples of our observations, we then investigated whether other squashed tetraloops of any sequence could be found in the PDB. We took advantage of the complete and recent data set gathered by Bottaro and Lindorff-Larsen (2017). In this study, a clustering approach identifies all the structurally distinct RNA tetraloops. Among the 44 clusters reported, four gather loops with structures based on Z-turns typical of UNCG loops (clusters 2, 5, 37, and 44). Three clusters gather conformations close to the squashed loop observed in the LC ribozyme crystal structure (clusters 16, 19, and 41). The contents of the clusters encompassing the Z-turn and the squashed loops are biased at two levels. First, redundancy of crystal or cryo-EM structures of ribosomes contributes most of the yet independent observations. Second, NMR structures usually bring >10 models, which cannot be considered as independent since they result from calculations obtained from a given set of restraints. Consequently, after pruning NMR structures, the final data set contains 1504 Z-turn and 380 squashed loops, respectively. Squashed loops represent ∼25% of the Z-turn loops, pointing to the significance of this RNA motif.

Considering squashed loops, the first example extracted from cluster 41 with a UACG loop sequence obeys the UNCG consensus (Fig. 5 in green). This loop belongs to the 1450 region of the Thermus thermophilus 16S rRNA, located at the periphery of the 70S ribosome (Maehigashi et al. 2014; Rozov et al. 2015, 2016a,b). The main structural features of this loop are due to its first (U1450) and last (G1453) residues, which bulge out. G1453 interacts with ribosomal protein RPS20. Despite its peripheral location, this loop is not involved in packing. Strikingly, it adopts a canonical UNCG structure in the original crystal structure of the 30S particle alone (1fgw, Wimberly et al. 2000), providing an additional potential biological role for the switch between canonical and squashed conformations. Interestingly, a 3 nt bulge is located exactly 3 bp away from the 1450 loop. This bulge bridges the 23S rRNA (2850 region) and also interacts with RPL19. Inspection of PDB files point to a squashed loop structure during elongation, while a Z-turn loop is preferred during other phases of translation, including initiation, termination, and stress-dependent ribosome stalling. These observations are nonetheless restricted to the T. thermophilus ribosome, and require additional data to be confirmed.

The NGNN tetraloop from the double-stranded RNA substrate of the yeast Rnt1p (RNase III family homologous to human droscha and dicer) also adopts the squashed conformation from cluster 41 in the crystal structure of the complex (Liang et al. 2014; Song et al. 2017). This tetraloop (AGUC in pdb file 1k6g) adopts a regular

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**FIGURE 2.** Secondary structures and conformations of the DP2 stem–loops. (A) The UUCG tetraloop of the CP LC ribozyme presents a squashed conformation (PDB: 6gyv). Squashing results from the proximity between the backbone of residues U2 and C3 and the last base pair of the stem (nucleotides with filled rings), which expels the residues from the loop toward the solvent. The helical conformation is interrupted at the level of U1 and restored only after G4. (B) The loop from the CP DP2ΔUA ribozyme adopts the canonical UUCG conformation with a maintained helical continuity at U1 and G4 (PDB: 6g7z). The Z-turn allows reorientation of the RNA chain toward the second strand of the stem. On each panel, the tetraloop is surrounded by a maximum likelihood map 2mFobs − DFcalc contoured at 1.5 σ.
conformation in the free form of the RNA (Lebars et al. 2001), in the sense that the first and fourth residues from the loop form a base pair, which stacks upon the last base pair of the stem. Nevertheless, the kink in the backbone is mediated at the phosphate group between residues 2 and 3, a situation neither typical of U-turn nor Z-turn loops. Mapping the NMR distance constraints from Lebars et al. (2001) onto a Z-turn loop points to only two which cannot be accommodated between the 2′ and 3′ hydroxyl atoms from residue L4 and the H6 atom from L3. Since the L4 residue is a purine in the Z-turn loop and not a pyrimidine as in the 1k6g NMR structure, these two constraints may be specific from this sequence. The torsional angles around the phosphate group (α and ζ) remained unconstrained, indicating that most of the torsion angles result from the distance constraints. Moreover, this loop conformation is not represented by any cluster from Bottaro and Lindorff-Larsen (2017), indicating that it does not represent a characteristic fold.

Loops from clusters 16 and 19 also adopt squashed conformations, even though their sequences depart from the UNCG consensus (Table 2; Fig. 5 in cyan and purple, respectively). Cluster 16 characterizes GGAU loops in the 23S rRNA from T. thermophilus and cluster 19, CGAA, UGAG and UUAG loops in the same region of the 23S rRNA from three different organisms, Haloarcula Marismortui (Ban et al. 2000), Deinococcus radiodurans (Schlüenzen et al. 2001), and T. thermophilus (Maehigashi et al. 2014; Rozov et al. 2015). In the case of cluster 16, the loop is situated at the solvent interface of the rRNA, close to ribosomal protein L28. The expelled first residue (G2210) interacts by stacking with U1493 (T. thermophilus numbering, pdb 1vvj) and hydrogen-bonds with a non-bridging atom of the phosphate group from G1492. The fourth residue of the loop, also ejected into the solvent, interacts with an arginine residue (R52) from L28. No crystal packing interaction is observed, but the RNA is not fully modeled in this region opening the possibility that the conformation of the loop may be restrained by unobserved contacts.

In contrast, the loops from cluster 19 are well buried in the 50S subunit and interact in the same way in the three considered organisms. The first and fourth residues of the UUAG loop stack on A2430 (T. thermophilus numbering) and G2448, respectively. In addition, the WC edge from the fourth residue interacts with the sugar edge of G2445. The second and third residues make shallow groove contacts with nucleotides 2246 to 2248. The very same arrangement is observed for the other loops CGAA and UGAG. Only RNA contacts are observed in this case, reminding of the different situations observed in the crystal packing that drove our study.

The structural characteristics of these loops can thus be summarized as follows. L1 and L4 are bulged out with a strong clustering for the latter. L2 and L3 are mostly located on the shallow groove and deep groove sides, respectively. The conformations for L3 residues are more

| TABLE 1. Crystallographic data for CP LC ribozyme DP2ΔUA |
|----------------|----------------|
| PDB id | 6g7z |
| Resolution range | 41.17 – 3.337 (3.456 – 3.337) |
| Space group | P 2_1 2_1 |
| Unit cell | 59.945 88.791 110.038 90 90 90 |
| Completeness (%) | 92.64 (33.98) |
| Wilson B-factor | 88.10 |
| Reflections used in refinement | 8336 (299) |
| Reflections used for R-free | 417 (15) |
| R-work | 0.2351 (0.3748) |
| R-free | 0.2933 (0.5942) |
| Number of nonhydrogen atoms | 3995 |
| Macromolecules | 3970 |
| Ligands | 25 |
| RMS (bonds) | 0.002 |
| RMS (angles) | 0.55 |
| Clashscore | 8.91 |
constrained than for L2 residues. L4 residues are often engaged in tertiary interactions with neighboring residues, which defines a set of clustered conformations.

**DISCUSSION**

It is usually accepted that UNCG tetraloops adopt a unique fold with characteristics accurately described elsewhere (D’Ascenzo et al. 2017), in brief, a Z-turn favoring the formation of a trans sugar edge-Watson–Crick pair between residues U1 and G4 of UUCG loops, provided that the G residue adopts a syn conformation. This conformation of the loop also presents a head-to-tail orientation of the third and fourth ribosomes reminiscent of Z-RNA (Hall et al. 1984; Davis et al. 1986). In another study, Bottaro and Lindorff-Larsen (2017) have shown that tetraloops with sequences distinct from UNCG could also adopt the canonical UNCG loop geometry and that, on rare occasions, UNCG sequences could acquire other conformations. A UUAG loop sequence compatible with the presence of a Z-turn between residues 3 and 4 has also been reported (D’Ascenzo et al. 2018), indicating that under relaxed constraints, loops UGAG and UUAG from cluster 19 may adopt a Z-turn-based conformation.

In the present study, we report observations from crystal structures of two different ribozymes, where a given UUCG loop adopts two distinct conformations according to the crystal packing variations resulting from slight structural changes of the RNA in the asymmetric unit. In each case, the UUCG loops were engineered in order to stabilize the underlying stem and thus, facilitate crystallization of the RNA molecule. Although the aim of these strategies was not to promote inter-molecular interactions, the engineered UUCG loops become involved in crystal packing contacts, which resulted in squashing these loops. The conformations induced by these interactions are very similar, and reveal that the squashed conformation is specifically obtained in response to the backbone–backbone interaction through contacts between phosphate and 2’ hydroxyl groups. In the two occurrences of this interaction observed in our crystal structures, a helix of a symmetry-related molecule contacts the tip of the loop formed by the second and third residues (Fig. 3). However, in the crystal structure of the group II ribozyme, the guanine (L4) of the squashed loop does not interact with a symmetry-related RNA like it does in the CP LC ribozyme crystal structure. This indicates that G-mediated tertiary interaction is not a prerequisite to the folding of the squashed conformation.

The relevance of the distorted UUCG loop conformation we observe is supported by the behavior of specific loops in ribosomal RNAs. Interestingly, the UACG loop from cluster 41 in the study of Bottaro and Lindorff-Larsen (2017) adopts the squashed conformation only in the context of the 70S ribosome since the same loop adopts a canonical conformation in the original structure of the 30S ribosomal subunit (Wimberly et al. 2000). Subunit association is well known to induce RNA conformational changes, suggesting that the one we describe in the context of the ribosome may have a biological significance. Inspection of the other clusters reveals that loops with other sequences can indeed adopt conformations very much related to the squashed loop from the LC ribozyme. The L2 nucleotide presents the most variable position. Residues at positions 3 and 4 occupy very identical positions in spite of belonging to different clusters (Table 3). It appears that the centroid approach developed by Bottaro and Lindorff-Larsen may be too sensitive, and that some clusters could actually be merged under more general structural features.

The resolution of the structures can also be questioned. Usually, structural data mining is performed on structures with resolution better than 2.5–3 Å. In this case, we also took into account structures with resolution worse than 3 Å. Although the positions of loop residues may result from the geometric restraints applied on the models during refinement, the generally crowded surroundings of
FIGURE 5. Comparison between the conformations of the squashed loops from clusters 41 (green, PDB: 4v6f), 16 (cyan, PDB: 1vvj), and 19 (purple, PDB: 1j5a) from the Bottaro and Lindorff-Larsen study (Bottaro and Lindorff-Larsen 2017) and the squashed loop from the LC ribozyme (orange, PDB: 6gyv). Superimposition of the different types of loops seen from the shallow groove side shows that nucleotides cluster individually in spite of the large differences observed for the torsional angles. Emphasis is given for nucleotides from the loops at first (A, L1), second (B, L2), third (C, L3), and fourth position (D, L4).

TABLE 2. Contacts between squashed loops in ribosome structures

| PDB     | Location                   | RNA contacts                                      | Protein contacts |
|---------|----------------------------|--------------------------------------------------|------------------|
| C41: UACG*  |                            |                                                  |                  |
| 4v8b UACG | Loop 1448 (16S)            | L2 Hbond with 16S-G147GA149                       | RPS20. No packing interaction |
| C16: GGAU (23S)b |                        |                                                  |                  |
| 1vvj GGAU | G2210GAU2214                | G1 stack with U1493                               | Shallow groove contact W70 of RPL2 loop close to RPL28 |
| C19: CGAA, UGAG, UUAG (23S) |             |                                                  |                  |
| 1fk CGAA | C920GAA923 (well buried into the structure) | L2–L3 interact with RNA backbone (2279–2280) L1 stack with A2467 Adenine L4 WC edge with S edge of G2480 | None |
| 1j5a UGAG | U840GAG843 (23S) (well buried into the structure) Same position regardless of nucleotide numbering | L2–L3 interact with RNA backbone (2225–2227) L1 stacks with A2409 Guanine L4 wc edge with s edge of G2424 (opposite strand from Adenine L4 from CGAA loop) | None |
| 1vvj UUAG | U827UAG830 (23S) Same location as CGAA and UGAG (Same file as for C16) | L2–L3 interact with RNA backbone (2246–2248) L1 stacks on A2430 L4 stacks on A2448 Guanine L4 wc edge with s edge of G2445 (same strand as for CGAA) | None |

*No deformation of the loop in the original T. thermophilus crystal structure of 30S subunit (4kvb).

bNo crystal packing interactions.
the squashed loops indicate that these conformations more likely result from molecular adaptation. Consequently, squashed loops gather similar structural features, which are (i) residues L1 and L4 bulging out, (ii) the backbone from residues L2 and L3 oriented right above the last helical base pairs, and (iii) the base from residue L3 pointing toward the deep groove side. These features allow residues 1 and 4 to interact with neighboring molecules, either RNAs or proteins, and the backbone of residues 2 and 3 to interact with the shallow groove of helices in the vicinity. Most importantly, the squashed conformation is reversible at least in four occurrences, including one in the ribosome, and one in the dsRNA substrate from Rnt1p.

In summary, our study presents for the first time evidence that loops belonging to the UNCG family can adopt more than one conformation according to the variations of their structural context. Considering that RNA molecules are highly dynamic and that they fulfill their biological functions through complex folding pathways and structural rearrangements, conformational UNCG loop flexibility may play an important role in RNA biology by allowing them to fulfill particular functional needs through their engagement in specific tertiary interactions with neighboring residues. The fact that the loops from cluster 19 are embedded in the same kind of structural environment than the crystal packing from the LC and group II ribozymes corroborates our conclusions.

Interestingly, a parallel can also be drawn between UNCG tetraloops and those belonging to the GANC consensus, which specifically caps the catalytic domain V hairpin in subgroup IIC self-splicing introns. Comparison of the crystal structures of the chimeric O. iheyensis intron revealed that the GANC tetraloops are flexible and adopt two different conformations according to the presence or absence of the 5′-exon substrate (Costa et al. 2016). In the presence of the 5′-exon, the GAAC loop of domain V acquires an alternate conformation that allows it to contact a specific intron receptor sequence through a single base stack interaction. Thus, in this case, the flexibility of the GAAC loop plays a crucial biological role by participating in the proper folding of the catalytic center of the intron. It is also interesting to note that this GAAC-receptor interaction is for the moment restricted to group IIC introns since it is specifically adapted to function in the particular structural context of domain V found in these introns.

Finally, the recent description of Z-turn specific receptors (D’Ascenso et al. 2018) also supports the idea that the different conformations of tetraloops of a given sequence may have distinct receptors. The squashed tetraloops can still interact with macromolecules in the vicinity, especially with the shallow groove of RNA helices, while expelled nucleobases 1 and 4 can stack or H bond with residues in the vicinity.

The present work can thus be seen as the description of a new kind of adaptive tetraloop interaction for which more occurrences may be found in RNA structures that will be solved in the future.

### MATERIALS AND METHODS

#### In vitro transcription, crystallization, and structure resolution of the CP LC DP2ΔUA construct

The CP LC DP2ΔUA was cloned, transcribed and purified as described in Beckert and Masquida (2011) and Meyer and Masquida (2014, 2016). The purified RNA was crystallized under conditions obtained from the crystallization of the CP LC ribozyme (Meyer et al. 2014). One volume of 100 µM of RNA was mixed to one volume of crystallization solution containing 200 mM NaCl, 100 mM HEPES pH 7.5 and 5%–25% PEG 3350. The structure was solved by the molecular replacement method and refined using Refmac (Murshudov et al. 2011) and Phenix (Table 1; Adams et al. 2010).

#### Data analysis

Representative loops from the various clusters presented in the Bottaro and Lindorf-Larsen study (Bottaro and Lindorf-Larsen 2017) were superimposed to the squashed loop of the LC ribozyme to pick up clusters with similar folds. Torsion angles were determined with xDNA-DSSR (Lu and Olson 2003). Figures, superimpositions and RMSD calculations were made in PyMol (Schrodinger 2010).

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