Aptamer structures from A to ζ

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Solution structures of RNA aptamers for FMN, ATP, arginine, and citrulline reveal how oligonucleotides can fold to form selective binding pockets for biological cofactors and amino acids. These structures confirm old ideas and provide new insights about three-dimensional structures of nucleic acids and their possible role in chemical reactions.

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An archaic meaning of apt is 'suited' or 'adapted'. In modern biology, an 'aptamer' [1] is a DNA or RNA oligonucleotide that has been selected *in vitro* for specific binding to a target molecule. The process through which these molecules are isolated is called *in vitro* selection [2] or selective evolution of ligands by exponential enrichment (SELEX) [3]. It starts with synthesis of a pool of DNA oligonucleotides containing a region of randomized nucleotides (usually ~30–100 nucleotides) flanked by conserved sequences that contain primer-binding sites for use in the polymerase chain reaction (PCR). For selection of RNA aptamers, the conserved region also contains a T7 RNA polymerase promoter. An iterative process involving binding of the DNA or RNA (obtained by transcription from the T7 promoter) oligonucleotides to a target molecule, isolation of a few percent of the molecules representing the tightest binders, reverse transcription in the case of RNA selection, and amplification by PCR, is used to isolate those molecules in the pool of ~10^{14} different initial sequences that conform best to the selection criteria. This method is widely applicable to investigations of RNA catalysis, nucleic acid drug development, protein–nucleic acid recognition, and investigations of the 'RNA world' origin of life hypothesis [2,4–6].

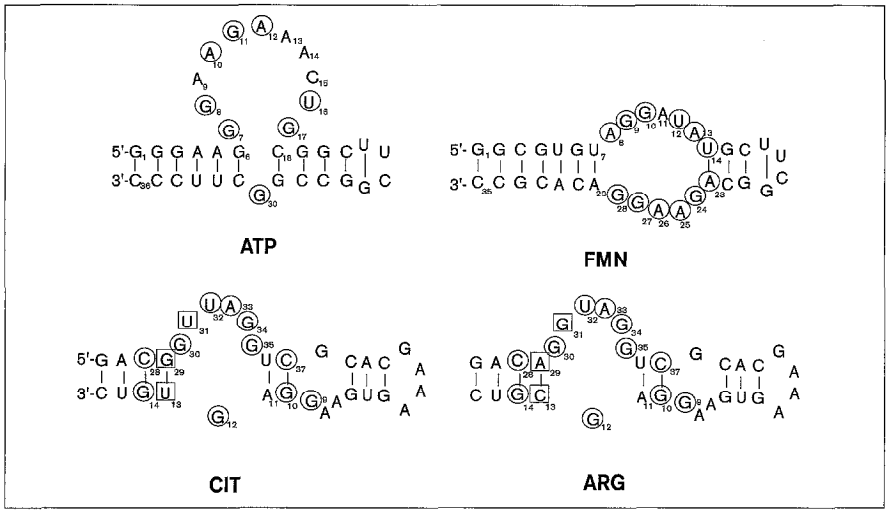
The nucleotide sequences of numerous aptamers have been reported, and secondary structures for these molecules have been proposed, based on consensus sequences from the pool of aptamers that bind to a particular ligand [2,4–6]. Yet until quite recently, the structure of only a single aptamer, a 15-nucleotide DNA molecule that specifically binds to and inhibits the blood-clotting protein thrombin [7], had been solved [8–10]. Now, in rapid succession, long-awaited RNA aptamer structures for three different types of small molecule targets have been reported, all solved using NMR spectroscopy [11–14]. The new structures reveal that the aptamers bind to their ligands via a combination of well-known structural motifs and unexpected interactions that provide a tantalizing glimpse into a world orchestrated by nucleic-acid chemistry.

Thrombin-binding DNA aptamer

The 15-base oligodeoxynucleotide that binds thrombin, dGGTTGGTGTGGTTGG, forms a folded unimolecular structure containing two G quartets (underlined bases) connected by a TGT and two TT loops [8–10]. The consensus sequences of a variety of DNA and RNA aptamers contain potential G-quartet motifs [15–17]. The structure of the thrombin-binding aptamer was solved both by itself in solution [8–10] and in complex with thrombin by X-ray crystallography [18]. This folded structure is of significant interest as an example of the range of conformations that nucleic acids can adopt, but the crystal structure of the complex [18], which has recently been reevaluated [19,20], does not reveal much about the specificity of binding and inhibition. In contrast, the RNA aptamers discussed below, which were selected for binding to small molecule targets, do not form ordered structures in the absence of ligand, and interactions with bound ligand that contribute to binding specificity are clearly found in the structures.

The solution of the structure of the relatively small thrombin-binding DNA aptamer foreshadowed some of the problems that would be encountered during structure determination of the larger RNA aptamers. The presence of noncanonical base pairs and short loops precluded the use of standard sequential assignments. To unambiguously identify specific nucleotides, single base substitutions of I for G or U for T were made in the thrombin-binding aptamer [8]. Comparison of the spectra of aptamers with different base substitutions assisted the assignments in all of the new RNA-aptamer structures. In addition, the most important development in obtaining the assignments for the flavin-mononucleotide- (FMN-) and ATP-binding aptamers was the use of uniformly ^{13}C,^{15}N-labeled RNA samples [21–23]. Exploitation of these labels required the development and modification of heteronuclear NMR pulse techniques for specific use with RNA [24,25]. In the FMN- and ATP-binding aptamers a particular problem was the identification of the exchangeable resonances and their crosspeaks, which could not be assigned by standard NOE techniques. Rather, their assignment depended on the application of through-bond NMR techniques developed very recently in several laboratories [26–28]. These assignments were essential in defining specific base-pairing interactions in these non-regular structures.

Figure 1



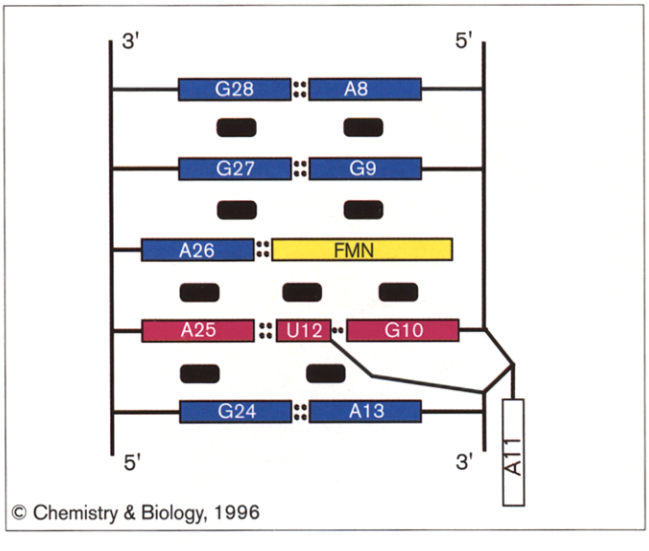
Sequence and secondary structure of minimal RNA aptamers used in the structural studies, targeted to ATP [11], FMN [12], citrulline [14] or arginine [14]. Conserved nucleotides are circled except for the three nucleotides that differ between the citrulline and arginine aptamers, which are boxed. The numbering systems shown are from the appropriate references. The ATP binder sequence is that used by Dieckmann *et al.* [11], which differs from that of Jiang *et al.* [13] only in the nonconserved stem regions.

FMN-binding RNA aptamer

The FMN-binding aptamer [29] is one of several aptamers selected for specific binding to protein cofactors. One goal of such selections is to incorporate these binding motifs into selections for RNA aptamers with enzymatic activities that correspond to those of proteins that use these cofactors. FMN binds as a cofactor to flavoproteins and mediates oxidation–reduction reactions. The secondary structure of the FMN-binding aptamers is an internal loop composed of seven nucleotides on one strand, of which six are conserved amongst all the FMN aptamers isolated in this study, and six conserved nucleotides on the other strand, flanked by Watson–Crick paired stems of any sequence [29] (Fig. 1). As with the ATP- [30] and arginine- and citrulline- [31] binding aptamers, the consensus sequence is purine-rich. In the tertiary structure of the FMN-binding aptamer all but one of the internal loop nucleotides are involved in noncanonical base pairs, which effectively zipper the internal loop shut to form an RNA duplex along the entire length of the aptamer (Fig. 2) [12]. This is the simplest of the RNA aptamer structures in terms of the interactions with the ligand.

The FMN molecule binds to the aptamer by inserting its planar aromatic rings into the duplex in the familiar intercalation mode, but the uracil-like edge of the isoalloxazine ring is exploited by base pairing with A26 to form a Hoogsteen-like pair. Since the FMN molecule intercalates on the seven-base side of the internal loop, this leaves two more bases than can be accommodated by simple base pairs. G10 hydrogen bonds to U12 to form an A25•U12•G10 base triplet; this is possible because A11 is looped out and acts as a linker. The A25•U12 base pair has the same reverse-Hoogsteen orientation as the A26•FMN pair (see Fig. 5 below). Hoogsteen A•T base pairs are quite stable outside of the constraints of a standard double helix, and indeed were the first base pairs observed in crystal structures of nucleotides [32]. The base triplet provides additional stacking interactions for the three-membered FMN ring. Thus, the specificity of FMN for binding to the aptamer is determined by specific hydrogen bonds to one base and stacking interactions with bases above and below. The linker A11 nucleotide is the only nonconserved base in the consensus sequence. Nonconserved nucleotides that act as linkers are a recurring motif in these aptamer structures, as well as in the structures of biologically relevant RNAs [33].

Figure 2



Schematic drawing, indicating the stacking and tertiary interactions in the FMN binder. Conserved nucleotides are colored as follows: G28•A8, G27•G9, A26 and G24•A13 are blue, A25•U12•G10 is magenta, and the FMN is yellow. Stacking interactions are indicated by black rectangles. Schematic and color scheme are based on Fan *et al.* [12]

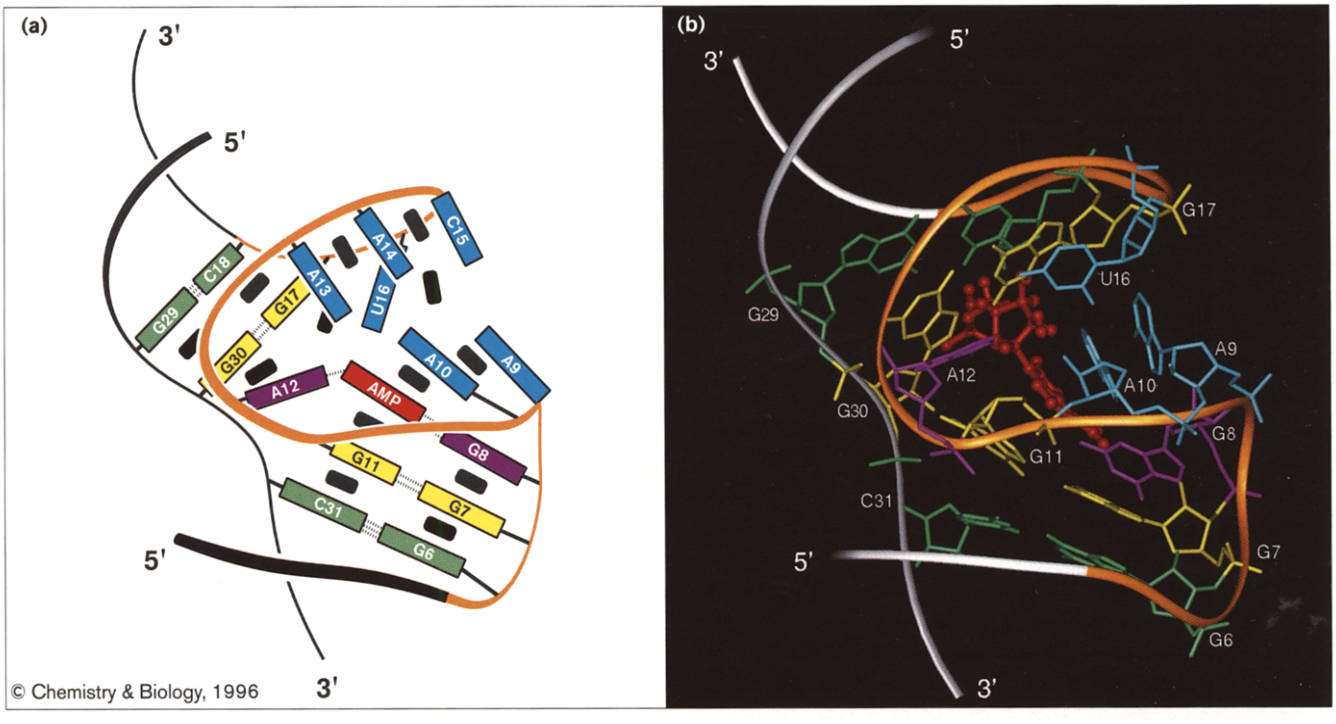
ATP-binding RNA aptamer

ATP was the first biological co-factor for which an aptamer was selected [30]. The consensus sequence for the ATP-binding aptamers was subsequently incorporated into oligonucleotides used in *in vitro* selections for RNAs with kinase activity. Some of the several classes of isolated kinases retained this secondary structure motif for ATP binding.

The selection for ATP-binding aptamers was carried out with ATP covalently bound to a column via a linker attached at the C8 position [30]. Isolated aptamers bind ATP, ADP, AMP and the adenosine moiety of FAD tightly and specifically [29,30]. The consensus sequence and secondary structure for the ATP-binding RNA aptamers is a purine-rich, 11-base loop opposite a bulged G nucleotide, flanked by nonconserved Watson–Crick stems that anchor the internal loop (Fig. 1). Structures of the ATP aptamer complexed to AMP have recently been solved by two groups [11,13]. The oligonucleotides used in the two studies had different stem sequences and lengths but identical sequences for the internal loop, which conform to the consensus sequence. The structures described by the two groups for the internal loop, which forms the binding pocket for the AMP molecule, are the same within experimental error.

A schematic drawing of the ATP-binding aptamer structure, with the nucleotide numbering from Dieckmann *et al.* [11], is shown in Figure 3. (The numbering differs from Jiang *et al.* [13] only for the stem nucleotides and for the bulged G34 [13] versus G30 [11].) The internal loop forms a compact, folded structure around the AMP molecule; the backbone tracing of this structure can be described by a ζ (Greek letter zeta). The invariant nucleotides in the internal loop form a binding pocket, which completely buries the AMP molecule, leaving only the C8 and N7 atoms and the phosphate group accessible to solution. Two asymmetric G•G base pairs, formed between G7 and G11 and between G17 and the single bulged G30 (G34 in Jiang *et al.* [13]), close the two stems flanking the internal loop (see Fig. 5 below). The bottom loop of the ζ is formed by G7 through G11. The hairpin turn in this region forms a so-called U-turn (G/UNR, where N is any nucleotide and R is a purine nucleotide), which was first recognized in a tRNA crystal structure 20 years ago [34]. The U-turn is characterized by a turn in the backbone between a G or U nucleotide and N, stacking of the R nucleotide under N, and some specific hydrogen bonds [35]. The features of the U-turn motif have more recently been identified in the stable GNRA tetraloops, which also contain a G•A base pair [35].

Figure 3



(a) Schematic drawing and **(b)** lowest energy structure of the ATP-binding aptamer. The ζ fold of the backbone is highlighted in orange. Stem nucleotides are colored green, the G7•G11 and G17•G30 base pairs are yellow, G8 and A12 are purple, A9, A10, A13–U16 are cyan, and the AMP is red. Stacking interactions are indicated by black rectangles in (a). In (b), the nonconserved linker nucleotides A13-A14-C15 have been deleted for clarity. After Dieckmann *et al.* [11].

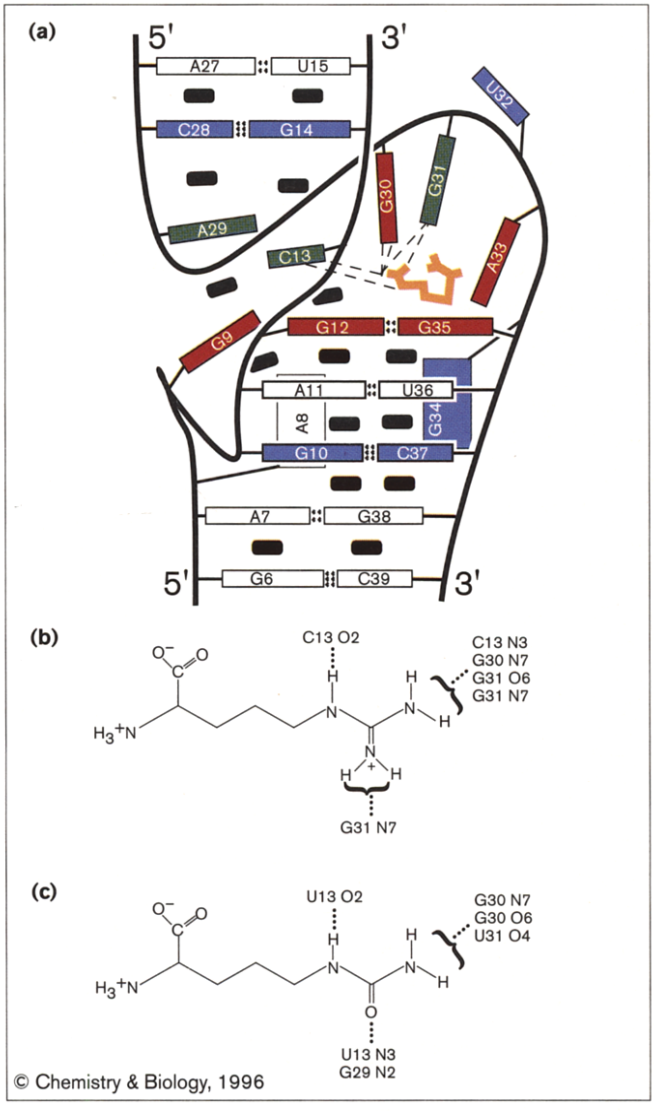
In the ATP-binding aptamer, G8-A9-A10 form the U-turn and the AMP molecule hydrogen bonds to the G8 nucleotide such that the turn can be described as a GNR(A) tetraloop, although the G8•AMP base pair geometry (see Fig. 5 below) is different from the sheared G•A base pair found in other GNRA tetraloops. The large central part of the ζ shape is composed of nucleotides A12–C15. The invariant A12 nucleotide hydrogen bonds via its NH_2 group to the AMP N3 atom, while the non-conserved A13, A14 and C15 nucleotides form a single-stranded stack, which makes a left-handed turn from A12. These nucleotides form a linker between the conserved nucleotides A12 and U16, which together with G17 and G30 form the back side of the AMP-binding pocket. U16 and G17 make the little right hand turn at the top of the ζ. This turn is a simple rotation of these nucleotides from the stacked A13–C15, placing G17 in position to form the G17*anti*•G30*syn* base pair, which closes the second stem. The tertiary interactions derived from the structure calculations are supported by additional NMR studies of mutant aptamers containing base substitutions [11]. All of the invariant nucleotides in the aptamer are involved in formation of the binding pocket, whereas the four non-conserved bases are in the three-base linker and the N nucleotide in the GNR(A) 'tetraloop'.

Arginine- and citrulline-binding RNA aptamers

Aptamers selected for specific binding to amino acids are of interest as a minimalist approach to understanding RNA–protein recognition. In the context of small molecule recognition, arginine and citrulline differ from the nucleotide cofactors because they lack an aromatic ring for stacking interactions. Thus, the binding pocket for the ligand might be expected to be quite different. The citrulline- and arginine-binding aptamers were isolated in two successive selections, carried out with the amino acids immobilized on an agarose column via a linker to their amino group [31]. First a citrulline-binding aptamer consensus was identified; this was then mutagenized to a level of about 30 % and used as the starting point for a selection for arginine-binding aptamers. The resulting aptamers share a conserved secondary structure containing two internal loops separated by two base pairs and flanked by Watson–Crick helices. The secondary structure of the larger and more conserved of the two internal loops resembles that of the ATP-binding aptamer; a single bulged G nucleotide lies opposite a purine-rich loop. Two base pairs on either side of the large internal loop form part of the consensus secondary structure. The consensus sequences of the two aptamers differ by only three conserved nucleotides. The oligonucleotides studied by NMR represent the minimal aptamers as determined by a variety of enzymatic and chemical modification studies [36] (Fig. 1).

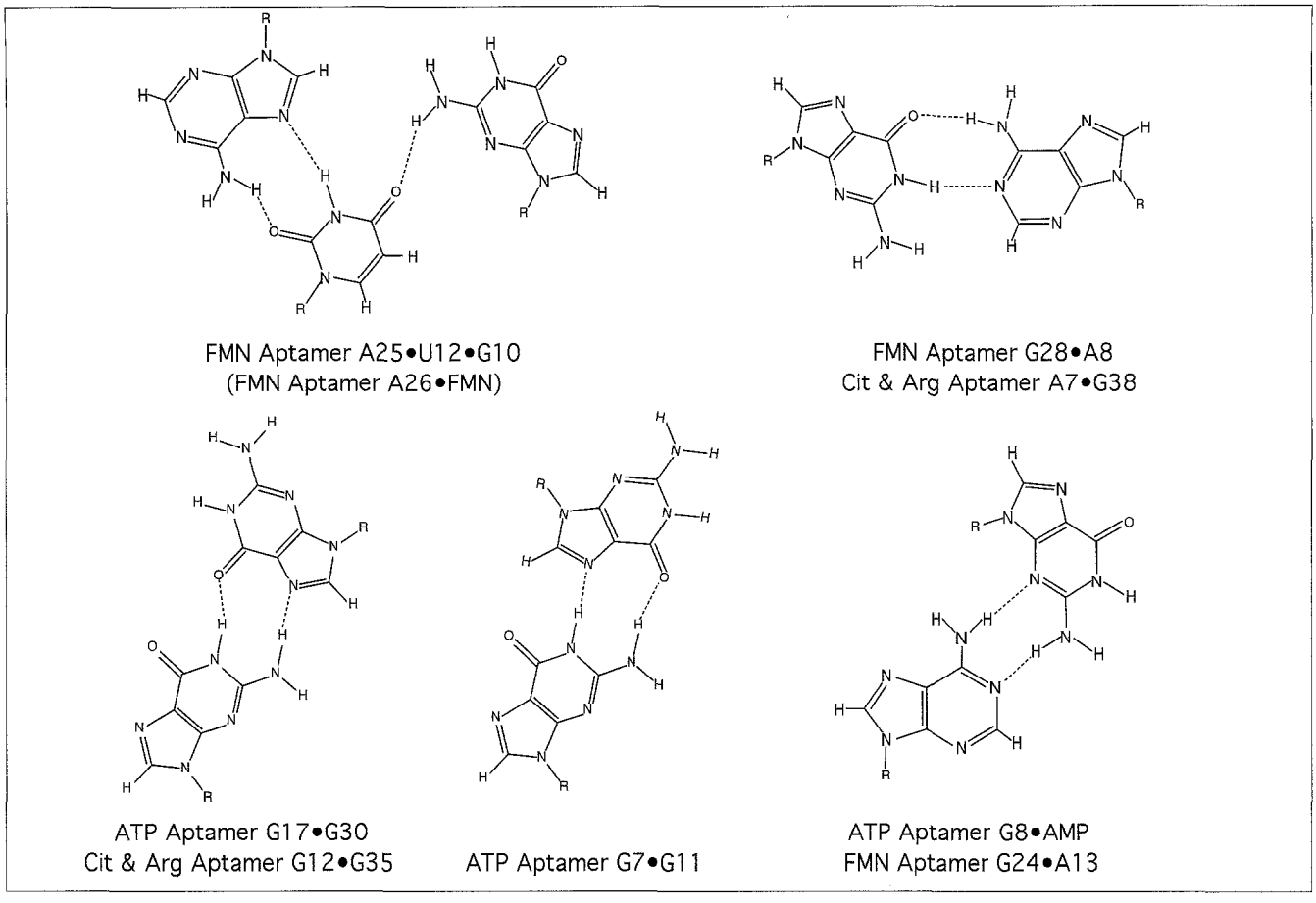
The binding pockets for arginine and citrulline are essentially the same, with specificity for the two different ligands being conferred largely by hydrogen bonds between the amino acids and different functional groups in the three bases that differ in the two aptamers (Fig. 4) [14]. The second stem is closed by a G12•G35 base pair that is hydrogen bonded in exactly the same way as the G30•G17 base pair found in the same position of the ATP aptamer, but in

Figure 4



The structure and interactions in the citrulline- and arginine-binding aptamers. **(a)** Schematic drawing, indicating the stacking and tertiary interactions in the arginine-binding aptamer. Conserved nucleotides, which form the binding pocket, are colored red (G9, G12, G30, A33, G35) and green (C13, A29, G31) and other conserved nucleotides are colored blue (G10, G14, C28, U32, G34, C37). Stacking interactions are indicated by black rectangles. Dashed lines indicate hydrogen bonds between the nucleotides and the arginine. For the citrulline-binding aptamer, the same overall fold is observed but specific interactions with the three noncommon residues U13, G29, and U31 are different. **(b)** Proposed H-bond interactions between arginine and bases. **(c)** Proposed H-bond interactions between citrulline and bases. Schematic and hydrogen-bond interactions are based on data reported in Yang *et al.* [14].

Figure 5



Noncanonical base pairs in the FMN-, ATP-, citrulline-, and arginine-binding RNA aptamers. The uracil-like isoalloxazine ring of the FMN hydrogen bonds to A26 in the same orientation as the A25•U12 interaction shown.

this case the loop G35 nucleotide rather than the bulge G12 is *syn* (Fig. 5). No other base pairs were identified in the arginine- and citrulline-binding pockets. The two stems, separated by the small internal loop in the secondary structure, form a continuous helix including a G38•A9 base pair from the internal loop; the other bases from the small internal loop, G9 and A8, are looped out of the helix. The A8 nucleotide is situated in the major groove of the stem, perpendicular to the base-pair planes and opposite G34, which also lies along the groove. The G9 nucleotide is tilted up to the plane of the G12•G35 base pair, and these three nucleotides form part of the bottom of the binding pocket for the amino acid. The rest of the binding pocket is formed by the conserved nucleotides C/U13, A/G29, G30, G/U31, and A33. Nucleotides 30–33 form a right-handed bulge in the nearly A-form RNA helix, and all but U32, which is looped out, surround the amino acid at various angles above the G12•G35 base pair. Hydrogen-bond interactions from the residues C/U13, G30, G/U31, and from A29 in the case of the citrulline aptamer, confer specificity. All of the hydrogen-bond interactions are with the functional groups of the amino-acid sidechain (Fig. 4b,c). The amino, carboxyl, and Cα groups stick out of the binding pocket into solution, consistent with the attachment of the linker to the amino group for the selection. The two structures establish specific roles for most of the conserved nucleotides in the arginine- and citrulline-binding aptamers.

Structure determination of the aptamers

Until now, relatively few RNA structures of any kind have been determined by either NMR spectroscopy or X-ray crystallography. NMR methods [24,25] for obtaining the maximum number of assignments, nuclear Overhauser effect (NOE) restraints, and dihedral-angle restraints had to be developed in parallel with the determination of the structures discussed here, and progress continues apace. The same is true for methods for calculation and refinement of nucleic-acid structures. Thus, at the present time there is no universally accepted protocol for NMR-derived structure calculation and refinement of nucleic acids. In general it is agreed that the most rigorous approach to providing an initial, reasonable sampling of conformational space is to start with structures generated by distance geometry or with methods for producing randomized starting structures.

Distance geometry starting structures were generated for all but the FMN-binding aptamer, for which a partial randomization approach was used. Specific details of the methods for structure calculation and refinement vary significantly among the aptamer structures reported. For the ATP-binding aptamers, the structures were solved by two groups using different methods for structure calculation and input restraints, but the final structures are the same in all important features. Reasonable pairwise root mean square deviations, of generally ~1.5 Å for all heavy atoms of the family of structures generated, were obtained for the ligand-binding pockets. More importantly, the three-dimensional structures of the aptamers, which could not be predicted on the basis of the secondary structures, to a large extent explain their consensus sequences and ligand specificities. Thus, although some of the 'higher resolution' features of these aptamer structures may not ultimately stand the test of time, in general they are a good representation of the quality of RNA structures that can be obtained with the current technology. For the arginine and citrulline aptamers, where no ^{13}C,^{15}N-labeled nucleotides were used, additional restraints derived from experiments exploiting these labels may lead to even better structures.

How should the biologist or chemist interpret these structures? The best bet is to look at the family (generally 5–20) of refined structures and keep in mind that this represents the limit of the 'resolution' of these structures, in other words, the minimum range of conformational space that satisfies the input restraints. Specific hydrogen-bond interactions are reported for all of the aptamer structures, but it is important to realize that there are currently no uniform criteria for defining hydrogen bonds based on NMR structures. Although both the distance and the angle between atoms determine whether a hydrogen bond is formed, often only the distance is used as a criterion, and even this may not be reliable since there may be insufficient NOE restraints to adequately define the location of certain groups. Additional criteria, such as the observation of slowly exchanging imino or amino groups or chemical shifts of the protons or nitrogen atoms, may be used to decide whether a hydrogen bond is present or even whether it is put explicitly into the restraints. Hydrogen bonds are difficult to determine by NMR, and should be interpreted with caution.

Recurring structural motifs

RNA biochemists have long recognized that RNA nucleotides are promiscuous in their base pairing. A common feature of all of the aptamers are noncanonical and, in particular, purine•purine base pairs (Fig. 5). G•G base pairs are found in all of the RNA aptamers as well as in the DNA thrombin-binding aptamer. Nonconserved nucleotides within the consensus most often are important in the aptamer structures as linkers or spacers, allowing placement of conserved bases into position for specific interactions with the ligand. As expected, stacking interactions and hydrogen bonds contribute to binding specificity and stability, but sometimes in unexpected ways. The appearance of the well-known U-turn in the familiar GNRA tetraloop motif in the ATP-binding aptamer supports the view that what we learn from aptamer structures may indeed have general relevance to biologically derived RNAs. Yet aptamer structures cannot be expected to completely mimic RNA biology, since they are selected for a single function and do not have to survive in a cell and interact with other cellular components. In this respect, they may provide insight into the prebiotic roles of RNA. As more aptamer structures are solved it may indeed be possible to construct a realistic picture of both prebiotic and modern RNA structure and chemistry.

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