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New molecular engineering approaches for crystallographic studies of large RNAs

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Abstract

Crystallization of RNAs with complex three-dimensional architectures remains a formidable experimental challenge. We review a number of successful heuristics involving engineering of the target RNAs to facilitate crystal contact formation, such as those that enabled the crystallization and structure determination of the cognate tRNA complexes of RNase P holoenzyme and the Stem I domain of the T-box riboswitch. Recently, RNA-targeted antibody Fab fragments and Kink-turn binding proteins have joined the ranks of successful chaperones for RNA crystallization. Lastly, we review the use of structured RNAs to facilitate crystallization of RNA-binding proteins proteins and other RNAs.

Introduction

Crystallization of large RNAs for structure determination by X-ray crystallography remains a very substantial technical challenge, similar to the crystallization of membrane proteins. Crystals of RNAs with complex three-dimensional structures rarely diffract X-rays to resolutions useful for biochemical insight (better than $\sim 3\text{\AA}$), because the surface of these molecules is dominated by a poorly differentiated, regular array of negatively charged phosphates, because their long-range structure is typically stabilized by a limited number of tertiary interactions, and because, intrinsically, many RNAs are conformationally heterogeneous [1] [2]. Moreover, near-universal strategies for *de novo* phase determination such as selenomethionine substitution [3] used for protein crystallography [2] are not yet available for RNA. These RNA-specific challenges are reflected in a paucity of structures of large RNAs and of co-crystal structures of proteins bound to their RNA substrates and partners in the structural databases.

A variety of molecular engineering approaches have been developed to assist with the crystallization and crystallographic phase determination of RNAs, such as the use of protein chaperones [1,2,4–6], grafting of stable and intermolecular contact-prone RNA motifs such as tetraloops (with their receptors) and kissing loops [7–10] and introduction of covalently

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or non-covalently bound heavy- or anomalously-scattering atoms useful for *de novo* structure determination [11–16]. Common modes of intermolecular RNA-RNA contacts observed in well-diffracting crystals include base stacking, backbone hydrogen bonds, and van der Waals packing of nucleobases with riboses. This latter type of interaction has been repeatedly observed, *e.g.* in crystals of tRNAs [17,18], ribosome-tRNA complexes [19,20], and the T-box-tRNA complex [6].

The past three years have witnessed continued progress in the determination of structures of complex RNAs, including those of several riboswitches, *in vitro* selected aptamers, and ribozymes. Innovations in RNA engineering to facilitate crystallization continue to play an important role [21]. Of particular interest for this review are the structure determinations of two large RNAs in complex with tRNAs: the RNase P holoenzyme [8,22] and the T-box riboswitch [6], because both required innovative engineering of each RNA partner. These structure determinations illustrate recent advances in RNA-engineering strategies for crystallization.

Native structural features of tRNA support tRNA-tRNA crystal contacts

Crystallographic studies of tRNA pioneered the field of nucleic acid structural biology [18,23–25]. Analysis of crystal structures of tRNAs and tRNA complexes reveals their intrinsic propensity to form certain crystal contacts. Crystals of tRNA^{Asp} and initiator tRNA^{Met}, for instance, feature kissing-loop-type anticodon-anticodon base-pairing arrangements that are reminiscent of the anticodon-codon decoding site in the ribosomal P-site [19,20], as well as the anticodon-“specifier” recognition in the T-box-tRNA complex [26,27] (Figure 1a, b). It follows that, by controlling the sequence of the anticodon, the tendency for this type of crystal contact to form can be encouraged or suppressed. A different, “interlocked” tRNA dimer configuration previously observed in tRNA^{Asp} crystals has recurred in the T-box-tRNA complex crystals [6,24,26] (Figure 1b, c). These examples support the existence of certain preferred packing arrangements for a given RNA, the knowledge and appreciation of which could inform rational design of crystallization constructs, elucidation of non-crystallographic symmetry, and interpretation of experimental electron density maps.

New engineering strategies for tRNA co-crystallization

The conservation of the canonical L-shaped architecture of mature elongator tRNAs suggests that the “elbow” region, formed by joining the apices of the D- and T-loops, is structurally constrained in the face of genetic drift [24,28] (Figure 1d). In contrast, alterations including long appendages inserted at the distal regions of the anticodon-stem loop (ASL) and the acceptor stem (AS) are expected not to drastically compromise the tRNA core structure (Figure 1d). Indeed, tRNA has been used as a robust scaffold to present and help fold many RNA sequences inserted into the ASL [29]. Engineering of the ASL and the AS led to formation of crystal contacts that enabled the recent structure determinations of RNase P holoenzyme and the T-box-tRNA complex, respectively.

The universal ribozyme RNase P cleaves the 5' leader sequence of pre-tRNA regardless of its anticodon sequence. Thus, alterations to the ASL do not impact the interaction of pre-

tRNA with RNase P. In order to promote crystal contact formation, Reiter et al., capped a functionally dispensable stem-loop of RNase P (the P12 helix) with a GAAA tetraloop and inserted a tetraloop receptor (TR) motif [7,30,31] in the pre-tRNA ASL [22] (Figure 1d, e). Then, a series of constructs differing in the number of “spacer” base pairs between these elements and the bodies of the respective RNAs [7] were generated. Systematic screening of forty-two combinations of variant RNase P and pre-tRNA molecules yielded one crystal form that allowed this long-awaited structure to be solved at 3.8 Å resolution [8,22].

A useful feature of many RNAs with complex folds, such as tRNA and RNase P, is that circular permutation often does not alter their overall structure or biochemical function [32,33]. This allows circular permutation to be employed as part of molecular engineering heuristics aimed at obtaining high quality crystals (*e.g.* [34]). Genetic analysis demonstrated that the T-box riboswitch Stem I domain decodes the tRNA anticodon, but does not interact with the acceptor stem [28,35]. To promote crystal contact formation, the tRNA was circularly permuted to eliminate the potentially disordered acceptor stem -UCCA-3' terminus and to replace it with a GAAA tetraloop (Figure 1d). This did not adversely affect tRNA folding, or its ability to bind to the T-box Stem I domain [6]. Crystallization screening of a battery of such circularly permuted tRNA constructs differing in the spacing between the tetraloop and the tRNA body combined with a set of Stem I constructs differing in the length of their proximal region, as well as the presence and specific nature of a K-turn binding protein (see below) eventually yielded crystals that allowed the structure of this mRNA:tRNA complex to be solved at 3.2 Å resolution, twenty years after its discovery (Figure 1d, f) [6].

An expanded repertoire of protein crystallization chaperones for RNA

Use of the RRM-I domain of human spliceosomal U1A protein and its cognate 10-nt stem-loop binding site as a crystallization chaperone has enabled successful structure determination of many RNAs such as the HDV ribozyme [1,4,36], the group I intron [37] the flexizyme [38], the cyclic-di-GMP riboswitch [39,40], the class I RNA ligase [41], the *glmS* ribozyme [42–44], etc. (Figure 2a, d). The stable and versatile U1A crystallization module has recently been complemented by several new protein-based tools that facilitate crystallization and structure determination of RNA.

Developments in immunological technologies allowed for the phage-display based selection of Fabs (immunoglobulin fragments, antigen-binding) that specifically bind structured RNAs [5]. Some of these recombinant Fabs, in conjunction with their RNA epitope, can function as portable RNA chaperones, *i.e.* crystallization modules [2] (Figure 2b, e). An arginine-enriched Fab that recognizes a stem loop with a terminal heptanucleotide GAAACAC has been successfully used to facilitate the crystallization of the P4-P6 domain of the *Tetrahymena* group I intron and of a class I RNA ligase ribozyme; the latter was also independently crystallized using the U1A crystallization module [5,41] (Figure 2b, d, e). The use of the Fab as compared to U1A module is potentially advantageous because of the larger size (50 versus 11 kDa) and β -rich structure of the former, which would facilitate structure determination by molecular replacement, enhance anomalous signal (due to more

numerous selenomethionine sites), and provide opportunities to form intermolecular β sheets as crystal contacts [2].

The continuing discovery of RNA structural motifs and their cognate protein partners have produced additional protein-RNA modules that hold great promise as crystallization chaperones [21,45,46]. The Kink turn (K-turn) motif is widespread RNA structural motif that plays key roles in globally organizing many large RNAs [47–51]. K-turns are binding sites for the L7Ae family of proteins. These share a compact fold that specifically recognizes K-turns, often with extraordinary affinities (picomolar to low nanomolar K_d) [52–54]. Although K-turns are intrinsically flexible, dimorphic structures, protein binding typically stabilizes their canonical, kinked conformation [54–57]. The fact that a large number of RNAs naturally contain K-turns, the ease with they can be introduced into peripheral helical elements, and the ready availability of a number of L7Ae homologs with different molecular properties, make L7Ae family proteins promising general crystallization chaperones. The inclusion of the YbxF protein, a bacterial L7Ae homolog, in the T-box riboswitch Stem I-tRNA complex, not only stabilized the functionally important K-turn in Stem I, but also directly contributed to favorable crystal packing and supplied selenomethionines critical for obtaining experimental phases and solving the structure of the 177-nt mRNA-tRNA complex [6] (Figure 2f). Interestingly, when several other L7Ae homologs (including archaeal hyperthermostable proteins) were also tested for their chaperone efficacy, they produced distinctly different crystal morphologies, consistent with their involvement in crystal packing (Zhang and Ferré-D'Amaré, unpublished).

Reciprocal use of structured RNAs as crystallization chaperones for proteins

As our knowledge of RNA structure expands, it has become practical to use RNAs of known structure to facilitate crystallization and structure determination of challenging proteins and RNAs. Attempts to crystallize *B. subtilis* YbxF alone bore no fruit whereas a ternary complex of S-adenosylmethionine (SAM), an engineered SAM-I riboswitch with an endogenous K-turn, and YbxF readily produced diffraction-quality crystals, enabling structure determination of YbxF by molecular replacement using the known riboswitch [54]. Because of its facile crystallization [58], the SAM-I riboswitch has also been used as a crystallization scaffold to characterize natural, non-canonical K-turns motifs [59]. Conceivably, a well-characterized structural RNA that presents a protein-binding site (such as the K-turn) can be used as a ubiquitous scaffold to crystallize a large number of RNA-binding proteins that bind similar RNA elements. For instance, a general, readily packable RNA scaffold that presents a tRNA-like structure may allow the co-crystallization and structural determination (by molecular replacement using the scaffold structure) of many tRNA-binding proteins from modification enzymes to aminoacyl-tRNA synthetases. This would be a simple extension of the DNA cage concept pioneered by Seeman [60,61].

In a remarkable example of convergent evolution, the ribosome L1 stalk, RNase P, and the T-box riboswitch recognize the characteristic flat surface formed by a tertiary base pair at the tRNA elbow using a common structural motif [6,62]. This compact, independently folding structure is formed by interdigitation of a pair of pentanucleotide T-loops [63],

producing flat surfaces formed by base pairs or base triples on both flanks. While one side is used to recognize the tRNA elbow, the other side participates directly in stacking with a terminal base pair and a GAAA tetraloop in a T-box-tRNA co-crystal [6], or to another copy of the interdigitated T-loop motif itself in a distal T-box Stem I fragment crystal [64], or in a truncated Stem I-tRNA co-crystal [65]. Thus the interdigitated T-loop module could potentially act as a crystallization chaperone that facilitates crystal packing of other RNAs and proteins.

Conclusion

Effective engineering strategies to aid RNA crystallization include taking advantage of intrinsic RNA packing preferences and opportunities and facilitating formation of additional crystal contacts exploiting well-characterized, packing-friendly RNA motifs such as kissing loops, pseudoknots, tetraloops and tetraloop receptors, interdigitated T-loops, etc. In addition, the expanded arsenal of available protein chaperones provides excellent alternatives to form crystal contacts for challenging RNAs. As more RNA-protein complex structures become available, additional protein chaperones will emerge, analogous to the MS2 [66] and PP7 [67] coat proteins that are widely used in *in vivo* tagging of RNAs [68]. The modular nature of RNA structural motifs and an ever-expanding range of capabilities of RNA to recognize metabolites, catalyze chemical transformations, and regulate gene expression, are expected to permit rational design and construction of RNA nano-structures and machines [69,70], as exemplified by the recent construction of a polyhedron made entirely from tRNAs [71].

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Highlights

- Construct engineering is a key strategy in RNA crystallization
- Natural and engineered intermolecular contacts form ordered crystals
- An expanded arsenal of protein chaperones to facilitate RNA crystallization
- Readily crystallized RNAs can aid structure determination of RNA-binding proteins

Figure 1. Intrinsic and engineered crystal contacts enable crystallization of tRNA and tRNA complexes

(a–c) Prominent crystal contacts of native tRNAs include kissing-loop-type anticodon-anticodon base-pairing in initiator tRNA^{Met} (a, magenta; PDB: 1YFG [27]) and tRNA^{Asp} (b, red; PDB: 1VTQ [26]), and a head-to-tail, "interlocked" dimer observed in crystals of tRNA^{Asp} (b, red) and the T-box Stem I-tRNA complex (c, green; PDB: 4LCK [6]). Symmetry-related molecules are denoted "sym". (d) Secondary structure schematics of a tRNA (middle) and engineered tRNAs in the anticodon stem loop (ASL, left), and in the acceptor stem (AS, right). The black arrowheads indicate the direction of RNA chain from 5'

to 3'. The bidirectional red arrows denote systematic helical length (straight arrows) and rotation (curved arrows) alterations. The apices of D-stem loop (DSL) and T-stem loop (TSL) join through tertiary base pairs to form the "elbow" of tRNA. **(e)** Prominent tRNA crystal contacts in the RNase P holoenzyme crystals. An engineered GAAA tetraloop capping the RNase P P12 helix contacts the tetraloop receptor inserted in the tRNA ASL. The distal nucleobase of the ASL stacks with a single nucleotide from the L15 loop. **(f)** Prominent tRNA crystal contacts in the T-box riboswitch Stem I-tRNA complex co-crystals. Interdigitation of the two T-loops of Stem I produces two flat surfaces (blue). One of these is used to stack against and recognize the tRNA elbow (green); the flat surface on the opposite side stacks with the terminal base pair of Stem I (light blue) and the apical adenosine of the GAAA tetraloop of the permuted tRNA (lime green), both from symmetry-related molecules.

Figure 2. More protein chaperones to aid RNA crystallization

(a–c) Secondary structure of the human U1A binding site (a), Fab binding site (b), and a typical K-turn (c). Non-conical base pairs in the K-turn are denoted using Leontis-Westhof symbols [72]. (d–f) Crystal contacts mediated by U1A (d, green, bound to L1 RNA ligase; PDB: 3HHN [41]), Fab (e, cyan, bound to L1 RNA ligase; PDB: 3IVK [41]), and YbxF (f, magenta, bound to T-box riboswitch Stem I-tRNA complex [6]).