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Post-crystallization improvement of RNA crystal diffraction quality

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Summary

The crystallization and structural determination of large RNAs and their complexes remain major bottlenecks in the mechanistic analysis of cellular and viral RNAs. Here, we describe a protocol that combines post-crystallization dehydration and ion replacement that dramatically improved the diffraction quality of crystals of a large gene-regulatory tRNA-mRNA complex. Through this method, the resolution limit of X-ray data extended from 8.5 to 3.2 Å, enabling structure determination. Although this protocol was developed for a particular RNA complex, the general importance of solvent and counterions in nucleic acid structure may render it generally useful for crystallographic analysis of other RNAs.

Keywords

X-ray Crystallography; Crystal dehydration; Ion replacement; Riboswitch; T-box RNA; tRNA

1. Introduction

The rapid exploration of the non-coding genome using high-throughput technologies is revealing critical roles for RNA structure in a wide range of cellular processes (1). In addition, many DNA and RNA viruses utilize defined three-dimensional RNA folds to enable their lifecycle and achieve infectivity (2–4). Despite critical roles of structured RNAs in biology and their impact on human health, their functional elucidation is hampered by a paucity of available structural information (4, 5). One hundred years after its invention, X-ray crystallography still provides unparalleled structural information for macromolecules. The rarity of diffraction-quality crystals of larger RNAs (longer than 100 nucleotides) remains a major roadblock that hinders their structure determination (4). Compared to proteins, the polyanionic nature of the RNA backbone, reduced chemical diversity of the four nucleobases, reduced presence of long-range contacts, as well as inherent conformational flexibility, render it difficult for RNAs to form specific, stable crystal packing contacts (5).

Many RNA crystals only diffract X-rays to resolutions in the range of 5 to 8 Å, insufficient to provide biochemical insight (~3.5 Å or better is desirable). For protein crystals, many

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post-crystallization treatment strategies such as annealing, dehydration, supplementing ligands, etc., have been developed (6–9). For RNA crystals, the favorable response of crystals of *glmS* riboswitch-ribozyme to dehydration hints that the diffraction quality of RNA crystals could also be significantly improved by post-crystallization treatments (10, 11). In order to facilitate the development of general strategies and methods for crystallographic studies of larger RNAs, we detail and rationalize a protocol that enabled the crystallization and structure determination of a large tRNA-mRNA complex (12, 13). Exploiting the general importance of RNA solvation and counterions in stabilizing compactly folded RNAs (14), this method concurrently dehydrates the RNA crystals and substitutes the divalent cations in them. This two-pronged approach drives rigid-body movements of the RNA complexes in the crystal, causing them to achieve geometrically and energetically superior packing.

2. Materials

1. Oligonucleotides for PCR amplification.
2. *Taq* DNA polymerase, 5,000 U/mL (New England Biolabs).
3. T7 RNA polymerase, 50,000 U/mL (New England Biolabs).
4. Diethylpyrocarbonate (DEPC)-treated water. (See Note 1)
5. RNA Binding Buffer: 50 mM HEPES-KOH, pH 7.0, 100 mM KCl, 20 mM MgCl₂, 5 mM tris (2-carboxyethyl) phosphine (TCEP).
6. 20 mM spermine solution, in DEPC-treated water, filtered through 0.2 µm filter.
7. Crystallization Solution: 50 mM Bis-Tris (HCl) pH 6.5, 0.3 M Li₂SO₄, 20 mM MgCl₂, 20% (w/v) polyethylene glycol (PEG) 3350.
8. EasyXtal 15-Well Tool (Qiagen).
9. MicroSieves and MicroSaws (MiTeGen)
10. 90° angled MicroLoops or MicroMounts (MiTeGen)
11. Crystal Treatment Solutions: 50 mM Bis-Tris (HCl), pH 6.5, 100 mM KCl, 20–50 mM SrCl₂ or 20–100 mM MgCl₂, 40–45% PEG3350, 5 mM TCEP.

3. Methods

3. 1. Design and Synthesis of T-box RNA and tRNA for Crystallization

1. Initial biochemical and biophysical characterization of T-box RNA-tRNA complexes (15) was essential for design and engineering of crystallization constructs (Fig. 1). Glycine-specific *glyQ/glyQS* T-box constructs from 20

¹DEPC is a toxic alkylating agent. It should be handled with appropriate personal protective equipment in a chemical fume hood. DEPC-treated water is non-toxic, because after mixing, the water-DEPC mixture is autoclaved. Heating in the presence of water converts DEPC into non-toxic carbon dioxide and ethanol.

species were selected from a multiple sequence alignment, with preference given to thermophilic, extremophilic, and pathogenic organisms. The T-box and tRNA constructs were transcribed *in vitro* using T7 RNA Polymerase, purified by denaturing Urea-PAGE, electroeluted, washed once with 1M KCl and extensively with DEPC-treated water, concentrated and stored at 4° C or –20° C before use (11, 16).

2. T-box RNAs from a range of bacterial species were evaluated for their propensity to form monodisperse, stoichiometric complexes with tRNA using non-denaturing PAGE. T-boxes from a handful of bacterial species, such as the extremely halotolerant and alkaliphilic *Oceanobacillus ihayensis* eventually used in structural determination, exhibited robust tRNA binding, forming tRNA-mRNA complexes that migrated as relatively sharp bands on non-denaturing gels.
3. Full-length T-box RNAs that contain both the Stem I and the antiterminator domains (Fig. 1a) exhibited a tendency to form dimers, presumably due to the thermodynamic instability of the antiterminator (17). Therefore, T-box RNAs were truncated at a series of lengths and their affinities towards tRNA and tendency to form monodisperse complexes evaluated using isothermal titration calorimetry (ITC) and non-denaturing gels. This analysis demonstrated that Stem I is the minimal T-box domain that is both necessary and sufficient for high-affinity, specific binding to tRNA (Fig. 1b–c) (12).
4. To aid crystallization of RNA, several RNA-binding proteins have been successfully used as crystallization chaperones, such as the human spliceosomal U1A protein (18), recombinant antibody fragments (Fabs) (19, 20), etc. The Kink-turn (K-turn) is a widespread bistable RNA structural motif initially discovered on the ribosome that sharply kinks the RNA duplex backbone by 120° and is the landing platform to recruit several conserved proteins to accomplish a range of cellular functions (21–24). The T-box Stem I domain harbors a conserved, functionally important K-turn (22). To stabilize the bistable K-turn structure, provide added opportunities for crystal packing, and allow for phasing using selenomethionines, a panel of K-turn binding proteins were tested for their ability to support crystal growth and improve crystalline order. Interestingly, the choice of K-turn binding protein appreciably influenced the crystal morphology. While the presence of thermophilic *Methanococcus jannaschii* L7Ae protein (25) (and other species of L7Ae) yielded star-shaped non-single crystals (Fig. 1d), the addition of mesophilic *B. subtilis* YbxF (26) produced single, square-plate-shaped crystals (Fig. 1e). The latter is much more amenable to diffraction data collection. In the absence of any K-turn-binding protein, only non-diffracting crystals of T-box-tRNA binary complexes were occasionally observed.

3.2. Crystallization of the T-box Stem I-tRNA-YbxF Ternary Complex

1. Dilute concentrated tRNA^{GAAA} (~ 1 mM; 24 g/L) to ~20 μ M using DEPC-treated water to reduce intermolecular interaction and dimerization.
2. “Snap-cool” tRNA^{GAAA} by incubating at 90 °C for 3 min followed by rapid cooling to 4 °C using a thermocycler (See Note 2).
3. Concentrate refolded tRNA to ~12 g/L (500 μ M) using Amicon spin concentrators (10 kD MWCO; 0.5 mL).
4. Mix 200 μ M each T-box Stem I RNA snap-cooled tRNA in RNA Binding Buffer (Materials), incubate first at 50 °C for 10 min and then at 37 °C for 30 min.
5. Add one equivalent selenomethionyl *B. subtilis* YbxF to the RNA complex.
6. Add spermine to 2 mM. The mixture may become transiently cloudy. Mix gently with a pipette tip.
7. Heat to melt a stock of 2% low-melting-point agarose solution and allow it to cool to 37 °C using a heat block to prevent it from solidifying.
8. Mix 1:1 the sample solution and Crystallization Solution, keep at 37 °C.
9. Add 1/10 volumes of 2% low-melting-point agarose solution and gently mix by pipetting up and down. The presence of agarose fibers in crystal solvent channels has been shown to lend mechanical support to the crystals (27, 28). The presence of 0.2% low-melting-point effectively prevents the T-box co-crystals from cracking induced by the sudden change in osmolarity (Fig. 2a).
10. Transfer the crystallization mixture onto cover slides and initiate crystallization experiments by hanging drop vapor diffusion.

3.3. Post-Crystallization Treatments

1. Square-plate-shaped crystals of the T-box-tRNA-YbxF ternary complex start appearing as early as 1–2 days. Diffraction quality crystals tend to grow more slowly, reaching final dimensions of $300 \times 300 \times 50 \mu\text{m}^3$ over the course of 1–3 weeks (Fig. 2a). These crystals have the symmetry of space group $C222_1$, with unit cell dimensions of $a=108.7 \text{ \AA}$, $b=108.8 \text{ \AA}$, $c=291.8 \text{ \AA}$. As do many other macromolecular crystals with relatively long unit cell edges, the longest unit cell edge (291.8 $\text{\AA}$) of these crystals is parallel to the shortest physical dimension of the crystals, *i.e.* the edge that describes the thickness of the rectangular or rhombic plates. Thus, oscillation diffraction images that result from incident X-rays that traverse

²tRNA^{Gly} and other tRNAs are known to form dimers in solution depending on conditions used for folding the RNA. To reduce dimerization, tRNAs are diluted in DEPC-treated water and “snap-cooled”, that favors tRNA folding while suppressing intermolecular association.

through the broad faces of the plates suffer from significant overlap of neighboring reflections. Such overlap is circumvented by the use of 90° bent crystal loops, which restrict the incident X-rays to only enter and exit the crystals through their shortest physical “edges” but not their “faces” (Fig. 2a).

2. Depending on the final concentration of low-melting point agarose in the crystallization drop and temperature, the entire drop may exhibit consistencies ranging from fluid liquid to viscous liquid, to jelly-like solid to robustly solid. Select appropriate tools to transfer crystals into ~200 µL Crystal Treatment Solutions in glass depression plates, i.e., use conventional nylon loops to transfer individual crystals from non-viscous liquid drops, and use tools such as MicroSieves (MiTeGen) to transfer whole, solidified drops. The composition of Crystal Treatment Solutions will vary as it is based on both the RNA Binding Solution and the Crystallization Solution. A gradient of concentrations of the primary precipitant (20–50% PEG3350 in this example) is scouted to achieve a range of final solvent contents and the effect on diffraction quality is measured. Different concentrations of a panel of divalent cations in particular the alkaline earth metals (Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+}) should be screened, both for supporting crystal growth and for post-crystallization treatment. In the case of the T-box complex crystals, crystal growth in Mg^{2+} combined with post-crystallization treatment in Sr^{2+} stands out as the optimal procedure, producing the best Bragg spots profiles required for *de novo* phasing using single-wavelength anomalous dispersion (SAD).
3. Seal each well of the depression plate using a glass cover slide and Vaseline. Incubate the crystals in Crystal Treatment Solution (Materials) for 16 hours. For the crystals of the T-box ternary complex, shorter treatments (i.e. less than 4 hours) generally do not produce the full effect of the treatment.
4. Carefully dissect the crystals out from their surrounding agarose network using MicroSaws (MiTeGen) and remove as much as agarose as possible (Fig. 2a). As the orientation of the crystals in the crystal loop is critical for reducing overlap during data collection, it is essential to trim nearly all agarose away from the crystal faces so that the plate-like crystals would be mounted parallel to the plane of the 90° bent loop due to surface tension.
5. Using a 90° bent loop such as the angled MicroLoops or MicroMounts (MiTeGen), pick up single, trimmed crystals and immediately plunge into liquid nitrogen for vitrification. As the Crystal Treatment Solution already contains at least 40% (w/v) polyethylene glycol (PEG) 3350, no additional cryoprotective agent is necessary.

3.4. Understanding the Basis of Treatment-induced Improvement of Crystal Quality

1. Structure determination of as-grown, untreated crystals, and a number of crystals subjected to various combinations of post-crystallization treatments (Fig. 2b & Table 1) allow the tracking of macromolecular movements in these crystals in response to the treatments received (12, 13).
2. Structural alignment of untreated and optimally treated crystals reveal that the ternary complexes of T-box-tRNA-YbxF shift closer to each other in the crystal as rigid bodies (Fig. 3a; see Note 3), producing superior packing contacts such as three intimate base-stacking interactions between symmetry-related complexes (Fig. 3b) as well as a stable A-minor interaction between the engineered GAAA tetraloop on tRNA acceptor stem and the minor groove of the proximal region of T-box Stem I (Fig. 3c).
3. The unique preference for Sr^{2+} in post-crystallization treatments of T-box co-crystals may be rationalized by its specific association with the 3' *cis*-diols of neighboring symmetry-related T-box RNAs (Fig. 3d), its frequent bidentate innersphere interactions with the Hoogsteen faces of purines (Fig. 3e), or its presence at bulges and junctions where phosphates cluster, or bridging across the narrow major groove. The ability of Sr^{2+} to bind RNA 3' termini and its flexible coordination geometry are properties that may allow it to improve crystalline packing of RNA (29).

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³Note that the space group as well as the unit cell dimensions of the crystals have changed significantly in response to the post-crystallization treatments (Table 1).

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Fig. 1. Sequences and secondary structures of full-length and truncated T-box riboswitch RNA used for crystallization. **(a)** Secondary structure and sequence conservation of a full-length *B. subtilis* glycine-responsive *glyQST*-box riboswitch and its cognate tRNA^{Gly}. Circles with dark and light orange shades indicate highly conserved (>80%) and moderately conserved (50–80%) sequences, respectively. Salient structural features on both RNAs are boxed and annotated. Intermolecular T-box-tRNA base-pairing interactions are indicated by solid lines connecting the boxed sequences. **(b)** Secondary structure of *Oceanobacillus iheyensis* *glyQ* T-box Stem I domain used for co-crystallization. The italic, red sequences and red arrows denote engineered regions. **(c)** Secondary structure of engineered *B. subtilis*/*O. iheyensis* tRNA^{Gly} (identical sequences) used for co-crystallization. The original tRNA acceptor stem sequence is circularly permuted and capped with a stable GAAA tetraloop (red, italic sequences). The bidirectional arrow denotes the length variations to screen for optimal crystal contacts. **(d)** Representative crystals of T-box-Stem I-tRNA complexed with *Methanococcus jannaschii* L7Ae. All scale bars represent 200 μm. **(e)** Representative crystals of T-box-Stem I-tRNA complexed with *B. subtilis* L7Ae. Note the differences in crystal morphology as dictated by the protein component in the complex.

Fig. 2.

Post-crystallization treatments dramatically improve diffraction quality of large RNA complexes. **(a)** Post-crystallization treatment procedures and effects on the crystal appearance. Due to the drastic changes in osmolarity (e.g., induced by a 20 to 40% change in PEG3350 concentration), pervasive crystal cracking and even disintegration occurs. Cracking is effectively prevented by the mechanical support from the agarose fibers in the solvent channels of the crystals. Note the agarose network that transferred together with the embedded crystals. All scale bars denote 200 μ m. **(b)** Comparison of magnified portions of

diffraction oscillation photographs of untreated (as-grown) crystals (left, PDB: 4TZP), partially treated crystals (middle panels and top right panel, PDB: 4TZV, 4TZW and 4TZZ), and crystals that were subjected to full cation replacement and dehydration (lower right panel; PDB: 4LCK) to demonstrate the improvement in spot profile and order-to-order separation. Arrows indicate progressive additions of treatments. Diffraction limits are indicated below each panel. Post-crystallization treatment and resulting crystal properties are summarized in Table 1.

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Fig. 3.

Treatment-induced, in-crystal movements of T-box ternary complexes produce superior crystal contacts. **(a)** In-crystal redistribution of T-box ternary complexes as rigid bodies driven by dehydration and cation replacement. Overlay of T-box ternary complexes in untreated (as-grown) crystals (light blue, PDB: 4TZP) and fully dehydrated and cation-exchanged crystals (dark blue, PDB: 4LCK). The corresponding crystallographic unit cells are also shown, indicating close to ~10% compression along both *a* and *c* axes. The reference complexes in the center of the panel superimpose well (RMSD for 172 C1' < 1.4

Å), but the neighboring four complexes shift substantially closer as a result of the post-crystallization treatment (RMSDs range from 3 to 10 Å and 10 to 19 Å, for RNA C1' and protein Ca, respectively). Red arrows denote directions of displacement (translation and rotation) of the four neighboring complexes. **(b)** Treatment-induced formation of an intimate crystal contact involving three symmetry-related T-box ternary complexes, shown in blue, green, and teal, respectively. Molecules from the untreated (PDB: 4TZP) and fully cation-replaced and dehydrated crystals (PDB: 4LCK) are overlaid and colored in pastel and solid colors, respectively. Parallel lines denote intermolecular stacking between nucleobases of symmetry-related complexes. Arrows indicate displacements between the untreated and fully treated states. The rear face of the interdigitated T-loops of Stem I distal region (opposite the face interacting with the tRNA elbow)(30) form a prominent flat surface available for crystal packing (12). Two patches of this flat surface (A39 and A60 respectively), upon full treatment, engage in direct stacking contact with the apical adenine of the GAAA tetraloop capping the tRNA acceptor stem (tA73) of a second complex (green), and with the terminal base pair of T-box Stem I (G1•C102) of a third complex (teal), respectively. Dotted triangle surrounds an intermolecular A-minor interaction (detail in **c**) present only in the fully treated crystals. tRNA residue numbers are preceded by 't'. **(c)** Detail of the intermolecular class-I A-minor interaction formed between the tetraloop of a tRNA and the minor groove of the proximal region of Stem I, colored as in **(b)**. **(d)** Interfacial Sr^{2+} ions bridge symmetry-related T-box RNA molecules by binding to their 3' termini. A well-defined Sr^{2+} ion (green sphere) is seen bound to the *cis*-diol of the T-box RNA 3' terminal nucleotide (C102, marine), and two non-bridging oxygen atoms of a symmetry-related T-box molecule (cyan), through inner-sphere coordination. Similar inner-sphere coordination between Sr^{2+} and two 3' *cis*-diol groups bridge two symmetry-related heptanucleotide derived from tRNA^{Ala} acceptor stem (31). The Sr^{2+} bridging two T-box 3' termini thus may have contributed significantly to the improved crystal quality through Sr^{2+} soaking. **(e)** Pervasive Sr^{2+} binding to RNA nucleobases and backbone, such as a pair of well-defined Sr^{2+} ions next to T-box G43, one of which makes bidentate innersphere interactions with the Hoogsteen face of G43. The electron density shown in **(d)** and **(e)** is a portion of a composite simulated anneal-omit $2|F_o|-|F_c|$ synthesis contoured at 1.5 s.d. overlaid with the final refined model.

Table 1

Select properties of crystals treated with varying degrees of ion replacement and dehydration.

PDB code	Li ₂ SO ₄ (mM)	MgCl ₂ (mM)	SrCl ₂ (mM)	PEG 3350 (% w/v)	Resolution (Å)	Space Group	Unit cell dimensions (Å)	V _M (Å ³ /Da)	V _S (%)
4TZP	300	20	0	20	8.5	C222 ₁	108.7, 108.8, 291.8 [*]	3.26	74.6
4TZV	0	20	0	20	5.0	P4 ₃ 2 ₁ 2	75.7, 75.7, 270.2 [*]	2.93	71.7
4TZW	0	0	50	20	4.7	P4 ₃ 2 ₁ 2	75.3, 75.3, 268.9 [*]	2.89	71.3
4TZZ	0	100	0	48	3.6	P2 ₁	70.6, 260.7, 70.7 [†]	2.46	66.3
4LCK	0	0	40	40	3.2	C222 ₁	100.8, 109.7, 268.1 [*]	2.81	70.4

^{*} $\alpha = \beta = \gamma = 90^\circ$

[†] $\alpha = \gamma = 90^\circ$, $\beta = 92.8^\circ$

V_M Matthews coefficient (Matthews, 1968) V_S Calculated solvent content