Published in final edited form as:

Methods Mol Biol. 2016 ; 1320: 219–232. doi:10.1007/978-1-4939-2763-0_14.

Soaking hexammine cations into RNA crystals to obtain derivatives for phasing diffraction data

Robert T. Batey^{1,*} and Jeffrey S. Kieft^{2,*}

¹Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, CO, 80309, USA

²Department of Biochemistry and Molecular Genetics and Howard Hughes Medical Institute, University of Colorado Denver School of Medicine, Aurora, Colorado, 80045, USA

Abstract

Solving the structure of a novel RNA structure by x-ray crystallography requires a means to obtain initial phase estimates. This is a challenge because many of the tools available for solving protein structures are not available for RNA. We have developed a reliable means to use hexammine cations to address this challenge. The process involves engineering the RNA to introduce a reliable hexammine binding site into the structure, then soaking crystals of these RNAs with an iridium (III) or cobalt (III) compound in a “directed soaking” strategy. Diffraction data obtained from these crystals then can be used in SAD or MAD phasing. In many cases, suitable derivatives can be obtained by soaking the hexammine into RNA crystals that have not been engineered. Considerations for using this method and example protocols are presented.

1. Introduction

Obtaining phase information is a necessary step to determine a macromolecular structure by x-ray crystallography. For proteins, this step in the process has become increasingly less challenging as the number of solved protein structures has grown. This is because phasing can often be readily accomplished using solved structures of homologous proteins to obtain initial phase estimates by the molecular replacement (MR) method and powerful programs such as PHASER (1, 2). For applications that require experimental phases, expressing the protein as a selenomethionine derivative is a robust method that now is used for over half of protein structures solved (3, 4). Therefore, only the most challenging problems—or an unusual situation—require different phasing approaches.

For RNA crystallography, the ability to use MR or biosynthetic incorporation of a heavy atom is significantly limited. With respect to MR, RNA crystallographers are less likely to use this approach, in part because the number and diversity of RNA structures available in the database is more limited than for proteins. Although molecular replacement-based

*To whom correspondence should be addressed: Jeffrey S. Kieft, HHMI/Department of Biochemistry and Molecular Genetics, University of Colorado Denver School of Medicine, Mail Stop 8101, Aurora, CO 80045, Telephone: 303-724-3257, Fax: 303-724-3215, Jeffrey.Kieft@ucdenver.edu, Robert T. Batey, Department of Chemistry and Biochemistry, University of Colorado at Boulder, Campus Box 596, Boulder, CO 80309-0596, USA, Telephone: 303-735-2159, Fax: 303-492-5894, Robert.Batey@colorado.edu.

methods using small A-form RNA helices have been developed (5-7), solving most RNA structures requires obtaining experimental phases using diffraction data from derivatized crystals. Synthetic approaches to the direct incorporation of selenium, bromine, or other anomalous scattering atoms are typically used on small oligonucleotides (8-18); for larger RNAs with complex tertiary architecture these approaches are less useful.

An alternative to the above approaches is a throwback to the earliest days of macromolecular crystallography: soaking. In this method, a crystal is derivatized by soaking the crystal in a heavy atom-containing solution (generally a cation to take advantage of the polyanionic nature of RNA), hoping that heavy atoms will bind to one or more specific locations within the RNA structure. Although often successful, a disadvantage to this method is that in most cases one cannot predict with any certainty if suitable specific binding sites exist, and hence is a trial-and-error method that can be time-consuming. The fickle and unpredictable nature of this process gave rise to the well-earned nickname of “soak-and-pray” (9, 19, 20).

To address the inherently uncertain and unpredictable nature of soaking heavy atoms into RNA crystals, we developed a highly general “directed soaking” method. This approach involves engineering of one or more reliable non-structure-perturbing cation binding sites into the RNA sequence of interest and then soaking hexammine cations suitable for phasing into crystals containing this RNA (21). The method described here is based on the observation that G-U wobble base-pairs in A-form RNA helices create a binding site for many cations (22-28), including hexammine complexes known to be very useful for phasing RNA diffraction data (Fig. 1) (examples: (27, 29-39)). Indeed, hexammine complexes have proven their worth in phasing diffraction data from crystals of complexes as large as the ribosome (examples: (40-42)). However, not all G-U wobble pairs work equally well to localize a hexammine cation because the identity and orientation of the base-pairs that flank the G-U pair matter. The reason for this effect has been analyzed elsewhere and versions appropriate for this method have been experimentally identified (21). Here, we recommend specific versions (see below). Overall, the method comprises mutating the RNA sequence of interest to include a G-U wobble motif that robustly binds a hexammine cation, crystallizing this RNA and then soaking hexammine complexes into the crystals to obtain the derivative (Note 1). We have had success with both cobalt (III) and iridium (III) hexammine, but even cesium has been used to phase when localized at this motif (43). Diffraction data collected from these crystals at appropriate x-ray energies is then used for multiple- or single-wavelength anomalous diffraction (MAD or SAD) phasing.

Choosing the right version of the G-U wobble motif to engineer into the RNA sequence is important, as not all versions of the motif bind hexammine cations with the same characteristics. We undertook a systematic study of motifs containing single G-U wobble pairs and tandem G-U wobbles to find those that bound hexammines with the lowest B-factor (best localized) and with the highest anomalous signal (21). We found that the

¹Even if the G-U motif is not included in the RNA, soaking with hexammine cations can often lead to a derivative suitable for phasing. There are endogenous hexammine sites in many RNA structures that are robust enough for this purpose. However, inclusion of the engineered cation binding G-U motif ensures that a strong high-occupancy site is present. This, in turn, makes weaker endogenous sites easier to locate and use in phasing.

arrangement of both partially negatively and positively charged functional groups in the major groove affected cation binding. Based on these findings, we empirically identified both single and double G-U wobble motifs that could robustly and reliably bind a hexammine cation and could therefore be used to introduce a binding site for directed soaking (Fig. 2A&B).

This method has advantages and disadvantages. A strength is that it eliminates the need to try many different cations or to generate chemically modified RNAs (Note 2). The method has been used to successfully derivatize crystals grown at diverse ionic strengths, including very high monovalent concentrations that would be thought to be disruptive to electrostatic interaction between the hexammine and RNA (35). The method requires mutation of the RNA but because the mutations involve only a few nucleotides, can be introduced into parts of RNA that are nonessential for function and generally do not perturb the structure, this is not a significant concern. In some cases, like the SAM-I riboswitch, the site is already present in the natural sequence and thus no modification is required (27). Finally, no crystal soaking protocol can be truly general. Each crystal is different and thus the amount of soaking time, number of steps, concentration of heavy atom, etc. must be optimized. Here, we present two protocols that were successful for obtaining derivatives of RNA crystals grown in either high-salt or low-salt conditions; these protocols form the basis for protocols tailored to each crystal form. Finally, it should be noted that because of the polyanionic nature of RNA, that there are typically at least one or two hexammine binding sites in a typical RNA. A number of recent RNA structures were solved using hexammines without engineering specific binding sites into the sequence (examples: (33, 36-38, 44-47)). Together, these considerations now make iridium (III) hexammine, with or without prior engineering of the RNA, overwhelmingly the current method of choice for obtaining phase information.

2. Materials

2.1. Synthesis of iridium (III) hexammine chloride

1. Iridium (III) chloride (IrCl_3) (Aldrich 336807-2G)
2. Ammonium hydroxide
3. Teflon tape
4. Sintered-glass filters
5. Rotary evaporator
6. Concentrated hydrochloric acid (HCl)
7. Absolute ethanol
8. Ultrapure water

²The directed soaking method we describe here was originally designed to work with hexammine cations, but other cations are also likely to be useful. This is because the G-U motif is a fairly general cation binding site. Indeed, there is one example of cesium being used in conjunction with the motif for successful phasing (43). We have not, however, made an exhaustive survey of various cations.

2.2. Generation of hexammine acetate from hexammine chloride

1. Cobalt (III) hexammine chloride (Sigma) or iridium (III) hexammine chloride
2. Lead acetate (Sigma)
3. Ultrapure water

3. Methods

3.1. Design of the RNA/introduction of the G-U wobble motif

1. The directed soaking method described here can be employed in two ways (Fig. 3). In the first, the cation-binding G-U wobble motif is included in the design of the library of RNAs used in initial crystal screens. The advantage is that if diffracting crystals are obtained from this library, no additional RNAs need to be made to generate crystals suitable for directed soaking. In the second method, libraries of RNAs that do not contain the motif are screened to identify diffracting crystals. Once crystals are found, new versions of the crystallizing RNA are made that contain the motif in one or more places and this RNA is used to grow crystals that then can be used in directed soaking. An advantage to this second method is that if the wild type and motif-containing RNAs crystallize under similar conditions, it provides evidence that introduction of the motif does not alter the structure of the RNA (Note 3).
2. In either of the two manifestations described above, an important decision is where to place the cation-binding G-U wobble motif. The goal is to place the motif where it will not affect crystallization, structure, or function. In every RNA there are likely many suitable locations and in most cases several should be tried. Guiding principles are:
 - a. Place the motif into existing Watson-Crick paired helical elements.
 - b. Iridium hexammine bound with high occupancy to one motif is likely enough to provide sufficient phasing power when combined with weaker endogenous sites for RNAs below 150 nucleotides. However, when possible, include multiple sites in different helices, especially for larger RNAs.
 - c. Place the motif at least 1-2 base-pairs from the end of helices. The G-U wobble can introduce slight local changes in helical conformation that could affect end-to-end helical stacking often important for crystallization.
 - d. Place the motif in parts of the RNA not essential for function or for formation of the global fold (Fig. 4A). This information must be gleaned from other studies (phylogeny, mutagenesis coupled with biophysical, biochemical, and functional studies, etc.).

³If crystals of the RNA of interest have been obtained using RNA that does not contain the G-U motif for cation binding, the same or very similar crystallization conditions are likely to be successful in crystallizing the RNAs that contain the motif. Inclusion of the motif, in our experience, does not significantly alter the conditions under which an RNA crystallizes or the quality of the crystals.

- e. Attempt to find locations where the motif can be introduced with a minimum of mutation. A location in the sequence where a point substitution converts the wild type sequence to a cation binding motif is ideal (Fig. 4B).
 - f. RNA is often systematically engineered to enhance crystallizability by altering the lengths of helical elements that are not critical for function (48-50); if this is done, the motif can be included within the engineered helix or helices.
3. RNA containing the motif is transcribed using T7 RNA polymerase or chemically synthesized, purified, and crystallized using methods beyond the scope of this protocol (Note 4).

3.2. Synthesis of iridium (III) hexammine

Unfortunately, at the time of this writing, iridium (III) hexammine is not commercially available. This protocol is an adaptation of that presented by Galsbol *et al.* (51). In particular, the glassware and methods used in this protocol are revised to use readily available materials and ease of implementation for any biochemistry laboratory.

1. In a heavy-walled Ace pressure tube (Aldrich, Z181080-1EA), add 2 g of IrCl_3 to 35 mL (fill tube almost to top) of ammonium hydroxide. Seal the tube well with Teflon tape, particularly around the O-ring seal (Note 5). Screw the top on tight and set halfway into a silicone oil bath set at 150 °C. Incubate for 4 days. If volume decreases due to a leak in the seal, remove, cool down and refill with fresh ammonium hydroxide.
2. Over the course of the first few days, most of the solid should dissolve and the solution transition from a dark brown to an orange/yellow solution and finally to a nearly colorless solution. These changes reflect the formation of colored tri-, tetra-, and penta-ammine compounds prior to the hexammine compound, which is colorless.
3. When synthesis is complete, remove the tube from the silicon oil bath and allow to completely cool. Then incubate on slushy ice.
3. Pass solution through a sintered glass filter to remove solid material.
4. Remove solvent by rotary evaporation, with the water bath set at 50 °C to heat the solution while evaporating.
5. Resuspend solid in 5 mL of water and place in a polypropylene 50 mL conical tube. Note that the solid material will not completely go into solution; this is normal.

⁴Protocols for crystallizing various RNAs are beyond the scope of this chapter. Discussions of the many strategies and protocols used to crystallize RNAs can be found in this book and other useful reviews (50, 52, 53).

⁵A difficulty commonly encountered with the Ace pressure tube is that the FETFE O-ring seal is not compatible with ammonium hydroxide at high temperature and rapidly degrades. This is resolved by wrapping the O-ring in Teflon tape prior to sealing the tube. An alternative and more expensive option is a Teflon-lined hydrothermal synthesis reactor (Deschem Equipment, Ltd.). The advantage to the use of this vessel is that it is simpler to use and the seal is completely lined with inert materials.

- 6 . Add 2 mL concentrated HCl to resuspension. A large amount of white precipitate should appear. Centrifuge at 5000xg for five minutes to pellet the precipitate.
- 7 . Remove supernatant (usually light yellow in color) and wash pellet with 5 mL of 2:1 (v/v) water:concentrated HCl by vigorously vortexing. Centrifuge to pellet the precipitate and remove supernatant.
- 8 . Repeat step 7 two times.
- 9 . After last spin, remove supernatant and wash the pellet three times with 5 mL of absolute ethanol.
- 10 . Air dry and resuspend in ultrapure H₂O. Add sufficient water (total of ~15 mL) to bring most of the white solid into solution. Spin to pellet the insoluble material and transfer the supernatant to fresh microfuge tubes.
- 11 . Collect an absorbance spectrum of the material. There should be an obvious peak at 251 nm. Calculate the concentration of iridium (III) hexamine chloride using the literature value for the extinction coefficient of 92 M⁻¹cm⁻¹ at 251 nm (51).
- 12 . Store solution at -20 °C.

3.3. Introducing the hexamine into crystals by soaking: low ionic strength example

The following protocol is based on a successful example for soaking hexamine cations into crystals grown under relatively low ionic strength conditions (34). Several parameters must be optimized for each crystal form (Note 6 **and** ⁷).

1. Grow crystals of the RNA of interest of suitable size and quality for diffraction data collection. For this example, the crystals were grown in 50 mM Na-MES pH 5.6, 100 mM Mg-acetate, 15% 2-methyl-2,4-pentanediol (MPD) in a sitting-drop vapor diffusion experiment at 30 °C.
2. Unseal the well containing the crystals and add solution matching the conditions of the drop plus 1 mM hexamine chloride and any cryoprotecting agents (soaking solution) to the crystallization drop. Add enough to double the size of the drop (e.g. add 1 µL soaking solution to a 1 µL drop).
3. Reseal the well and allow the drop and crystals to equilibrate for 5 minutes.

⁶Parameters that may vary for different crystal forms include:

- a. Concentration of the hexamine cation.
- b. Time of soaking.
- c. Number of “steps” in the procedure; i.e., is the cation concentration increased in steps and if so, how many?
- d. Concentration and identity of any included cryoprotectants.

⁷Here, we present procedures for soaking cations into the crystal after they have grown. It is also possible to co-crystallize the hexamine with the RNA (27). If the initial crystals were grown without hexamine, then including them in the drop during crystallization may mandate some re-optimization of the crystallization conditions to account for the increased ionic strength association with the trivalent hexamine cation.

4. Unseal the well containing the crystals and add 20 μ L of the soaking solution.
5. Reseal the well and allow the drop and crystals to equilibrate for 5 minutes.
6. Unseal the well containing the crystals and remove 15 μ L of the well volume, then add 15 μ L of the soaking solution.
7. Reseal the well and allow the drop and crystals to equilibrate for 10 minutes.
8. Harvest the crystals with a cryo-loop (Hampton Research) and flash-cool in liquid nitrogen.

3.4. Generating cobalt (III) hexammine acetate from the chloride salt (Note 8)

1. Dissolve 0.668 g of cobalt (III) hexammine chloride in 25 mL ultrapure water to generate a solution of 100 mM hexammine.
2. Dissolve 1.42 g lead acetate in 5 mL ultrapure water.
3. Combine the lead acetate and cobalt (III) hexammine solutions in a 50 mL conical tube. Lead chloride will immediately precipitate. Allow 30 min at room temperature for complete precipitation.
4. Centrifuge the conical tube in a clinical centrifuge to pellet the precipitate. Collect the supernatant which contains the hexammine acetate salt and transfer to a clean large plastic weigh boat.
5. Allow the weight boat and solution to sit on the bench until the water has completely evaporated. Dissolve the resultant crystals in 5 mL of ultrapure water, resulting in a solution of cobalt (III) hexammine acetate at ~500 mM.
6. Store at -20°C .

3.5. Generating iridium (III) hexammine acetate from the chloride salt (Note 8)

This is based on the same procedure used to produce cobalt (III) hexammine acetate. However, because iridium (III) hexammine is not commercially sold and thus not available in large amounts, we adapted the protocol slightly to use smaller amounts of starting material.

1. Dissolve 0.0535 g lead acetate in 0.5 mL of 188 mM iridium (III) hexammine chloride in a micro-centrifuge tube (188 mM was the concentration of our stock of iridium (III) hexammine chloride solution; adjust the amount of lead acetate for other concentrations).
2. Lead chloride will immediately precipitate. Allow 30 minutes at room temperature for complete precipitation.

⁸The acetate salts of hexammines are more soluble than the chloride salts, and the sulfate salts are insoluble. For most applications, the chloride salts are acceptable as the hexammine concentration used is well within the range of solubility. However, for some applications higher concentrations of hexammines may be needed. The example presented here is one where the crystal was grown under very high salt conditions and the hexammine concentration must be high to compete for the cation binding sites (35).

3. Centrifuge tube in a micro-centrifuge at maximum velocity to pellet the precipitate. Collect the supernatant which contains the hexammine acetate salt and transfer to a small clean plastic weigh boat.
4. Allow the weigh boat and solution to sit on the bench until the water has completely evaporated. The resultant crystals can then be stored at -20°C until needed. Before use, dissolve in ultrapure water to the desired concentration.

3.6. Introducing the hexammine by soaking: high salt example

Crystals grown under higher ionic strength (high salt) present a challenge because the high concentration of cation can compete for the hexammine binding sites, precluding specific binding. This mandates using much higher concentrations of hexammine in the soaking solution; however, because the hexammine chloride salts are only soluble to $\sim 100\text{ mM}$ under these conditions, a suitable concentration may not be achievable. In addition, hexammine sulfates are relatively insoluble and thus crystals grown under high sulfate conditions have the additional complication that the hexammine cations precipitate when added to the crystallization drop. The following is a protocol that was used to overcome these challenges by replacing the sulfate in the crystallization drop with acetate and using hexammine acetate salts (35). Again, several parameters must be optimized (Note 6).

1. Grow crystals of the RNA of interest of suitable size and quality for diffraction data collection. For this example, the crystals were grown against a well solution of 1.4 M lithium sulfate, 40 mM Mg-acetate, 50 mM HEPES-NaOH pH 7.5 in a hanging-drop vapor diffusion experiment at 30°C .
2. To stabilize the crystals, unseal the well, remove the well solution, and replace with 2.0 M lithium sulfate, 40 mM Mg-acetate, 50 mM HEPES-NaOH pH 7.5, 0.5 mM spermidine-HCl (“well solution”).
3. Reseal the well and allow the drop to equilibrate for 24 hours at 30°C .
4. Unseal the well and transfer the crystals to a second soaking tray containing $50\text{ }\mu\text{L}$ of the well solution. This is most readily done using a cryo-loop.
5. Cover/seal the soaking tray and allow the drop and crystals to equilibrate for 10 minutes.
6. Uncover the soaking tray and add $50\text{ }\mu\text{L}$ of soaking solution “10%”. This “10%” solution is made of 10% 3.0 M lithium acetate, 40 mM Mg-acetate, 0.5 mM spermidine-HCl, 50 mM HEPES-NaOH pH 7.5, 200 mM hexammine acetate (“derivative solution”) and 90% well solution.
7. Reseal the well and allow the drop and crystals to equilibrate for 10 minutes.
8. Unseal the well and remove $50\text{ }\mu\text{L}$ of the solution, then immediately add $50\text{ }\mu\text{L}$ of solution “20%” (20% derivative solution + 80% well solution).
9. Reseal the well and allow the drop and crystals to equilibrate for 10 minutes.

10. Repeat steps 8 and 9, each time increasing the percentage of derivative solution by 10% until the crystals are in essentially a 100% derivative solution (10 incremental soaks).
11. Unseal the well and remove most of the solution surrounding the crystals, then add 100 μ L of 100% derivative solution. Repeat.
12. Reseal the well and allow the drop and crystals to equilibrate for 1 hour.
13. Harvest the crystals with a cryo-loop and flash-cool in liquid nitrogen.

Acknowledgements

The authors thank current and former members of our labs for thoughtful discussions and technical assistance and David Costantino for critical reading of this manuscript. RTB is supported by NIH grants GM073850 and GM083953. JSK is supported by NIH grants GM097333 and GM081346. JSK is an Early Career Scientist of the Howard Hughes Medical Institute.

References

1. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr.* 2007; 40:658–74. [PubMed: 19461840]
2. McCoy AJ. Solving structures of protein complexes by molecular replacement with Phaser. *Acta Crystallogr D Biol Crystallogr.* 2007; 63:32–41. [PubMed: 17164524]
3. Doubie S. Preparation of selenomethionyl proteins for phase determination. *Methods Enzymol.* 1997; 276:523–30. [PubMed: 9048379]
4. Hendrickson WA, Horton JR, LeMaster DM. Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. *Embo J.* 1990; 9:1665–72. [PubMed: 2184035]
5. Robertson MP, Scott WG. The structural basis of ribozyme-catalyzed RNA assembly. *Science.* 2007; 315:1549–53. [PubMed: 17363667]
6. Robertson MP, Scott WG. A general method for phasing novel complex RNA crystal structures without heavy-atom derivatives. *Acta Crystallogr D Biol Crystallogr.* 2008; D64:738–44. [PubMed: 18566509]
7. Robertson MP, Chi YI, Scott WG. Solving novel RNA structures using only secondary structural fragments. *Methods.* 2010; 52:168–72. [PubMed: 20541014]
8. Brandt G, Carrasco N, Huang Z. Efficient substrate cleavage catalyzed by hammerhead ribozymes derivatized with selenium for X-ray crystallography. *Biochemistry.* 2006; 45:8972–7. [PubMed: 16846240]
9. Golden BL. Heavy atom derivatives of RNA. *Methods Enzymol.* 2000; 317:124–32. [PubMed: 10829276]
10. Jiang J, Sheng J, Carrasco N, Huang Z. Selenium derivatization of nucleic acids for crystallography. *Nucleic Acids Res.* 2007; 35:477–85. [PubMed: 17169989]
11. Carrasco N, Buzin Y, Tyson E, Halpert E, Huang Z. Selenium derivatization and crystallization of DNA and RNA oligonucleotides for X-ray crystallography using multiple anomalous dispersion. *Nucleic Acids Res.* 2004; 32:1638–46. [PubMed: 15007109]
12. Hobartner C, Micura R. Chemical synthesis of selenium-modified oligoribonucleotides and their enzymatic ligation leading to an U6 SnRNA stem-loop segment. *J Am Chem Soc.* 2004; 126:1141–9. [PubMed: 14746483]
13. Hobartner C, Rieder R, Kreutz C, Puffer B, Lang K, Polonskaia A, et al. Syntheses of RNAs with up to 100 nucleotides containing site-specific 2'-methylseleno labels for use in X-ray crystallography. *J Am Chem Soc.* 2005; 127:12035–45. [PubMed: 16117544]

14. Salon J, Sheng J, Jiang J, Chen G, Caton-Williams J, Huang Z. Oxygen replacement with selenium at the thymidine 4-position for the se base pairing and crystal structure studies. *J Am Chem Soc.* 2007; 129:4862–3. [PubMed: 17388591]
15. Sheng J, Jiang J, Salon J, Huang Z. Synthesis of a 2'-Se-thymidine phosphoramidite and its incorporation into oligonucleotides for crystal structure study. *Org Lett.* 2007; 9:749–52. [PubMed: 17263541]
16. Baugh C, Grate D, Wilson C. 2.8 Å crystal structure of the malachite green aptamer. *J Mol Biol.* 2000; 301:117–28. [PubMed: 10926496]
17. Kieft JS, Zhou K, Grech A, Jubin R, Doudna JA. Crystal structure of an RNA tertiary domain essential to HCV IRES-mediated translation initiation. *Nat Struct Biol.* 2002; 9:370–4. [PubMed: 11927953]
18. Martick M, Scott WG. Tertiary contacts distant from the active site prime a ribozyme for catalysis. *Cell.* 2006; 126:309–20. [PubMed: 16859740]
19. Golden BL, Gooding AR, Podell ER, Cech TR. X-ray crystallography of large RNAs: heavy-atom derivatives by RNA engineering. *Rna.* 1996; 2:1295–305. [PubMed: 8972777]
20. Wedekind JE, McKay DB. Purification, crystallization, and X-ray diffraction analysis of small ribozymes. *Methods Enzymol.* 2000; 317:149–68. [PubMed: 10829279]
21. Keel AY, Rambo RP, Batey RT, Kieft JS. A general strategy to solve the phase problem in RNA crystallography. *Structure.* 2007; 15:761–72. [PubMed: 17637337]
22. Masquida B, Westhof E. On the wobble GoU and related pairs. *Rna.* 2000; 6:9–15. [PubMed: 10668794]
23. Varani G, McClain WH. The G x U wobble base pair. A fundamental building block of RNA structure crucial to RNA function in diverse biological systems. *EMBO Rep.* 2000; 1:18–23. [PubMed: 11256617]
24. Kieft, JS. Structure and Thermodynamics of a Metal ion Binding Site in the RNA Major Groove: Cobalt (III) Hexamine as a Probe.[Thesis]. Type, University of California; Berkeley, Berkeley: 1997.
25. Cate JH, Doudna JA. Metal-Binding Sites in the Major Groove of a Large Ribozyme Domain. *Structure.* 1996; 4:1221–9. [PubMed: 8939748]
26. Colmenarejo G, Tinoco I Jr. Structure and thermodynamics of metal binding in the P5 helix of a group I intron ribozyme. *J Mol Biol.* 1999; 290:119–35. [PubMed: 10388561]
27. Montange RK, Batey RT. Structure of the S-adenosylmethionine riboswitch regulatory mRNA element. *Nature.* 2006; 441:1172–5. [PubMed: 16810258]
28. Stefan LR, Zhang R, Levitan AG, Hendrix DK, Brenner SE, Holbrook SR. MeRNA: a database of metal ion binding sites in RNA structures. *Nucleic Acids Res.* 2006; 34:D131–4. [PubMed: 16381830]
29. Batey RT, Gilbert SD, Montange RK. Structure of a natural guanine-responsive riboswitch complexed with the metabolite hypoxanthine. *Nature.* 2004; 432:411–5. [PubMed: 15549109]
30. Cate JH, Gooding AR, Podell E, Zhou K, Golden BL, Kundrot CE, et al. Crystal structure of a group I ribozyme domain: principles of RNA packing. *Science.* 1996; 273:1678–85. [PubMed: 8781224]
31. Cochrane JC, Lipchock SV, Strobel SA. Structural investigation of the GlmS ribozyme bound to its catalytic cofactor. *Chem Biol.* 2007; 14:97–105. [PubMed: 17196404]
32. Kazantsev AV, Krivenko AA, Harrington DJ, Holbrook SR, Adams PD, Pace NR. Crystal structure of a bacterial ribonuclease P RNA. *Proc Natl Acad Sci U S A.* 2005; 102:13392–7. [PubMed: 16157868]
33. Toor N, Keating KS, Taylor SD, Pyle AM. Crystal structure of a self-spliced group II intron. *Science.* 2008; 320:77–82. [PubMed: 18388288]
34. Pfingsten JS, Costantino DA, Kieft JS. Structural basis for ribosome recruitment and manipulation by a viral IRES RNA. *Science.* 2006; 314:1450–4. [PubMed: 17124290]
35. Costantino DA, Pfingsten JS, Rambo RP, Kieft JS. tRNA-mRNA mimicry drives translation initiation from a viral IRES. *Nat Struct Mol Biol.* 2008; 15:57–64. [PubMed: 18157151]

36. Peselis A, Serganov A. Structural insights into ligand binding and gene expression control by an adenosylcobalamin riboswitch. *Nat Struct Mol Biol.* 2012; 19:1182–4. [PubMed: 23064646]
37. Smith KD, Lipchock SV, Ames TD, Wang J, Breaker RR, Strobel SA. Structural basis of ligand binding by a c-di-GMP riboswitch. *Nat Struct Mol Biol.* 2009; 16:1218–23. [PubMed: 19898477]
38. Garst AD, Heroux A, Rambo RP, Batey RT. Crystal structure of the lysine riboswitch regulatory mRNA element. *J Biol Chem.* 2008; 283:22347–51. [PubMed: 18593706]
39. Huang L, Serganov A, Patel DJ. Structural insights into ligand recognition by a sensing domain of the cooperative glycine riboswitch. *Mol Cell.* 2010; 40:774–86. [PubMed: 21145485]
40. Ban N, Nissen P, Hansen J, Moore PB, Steitz TA. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science.* 2000; 289:905–20. [PubMed: 10937989]
41. Cate JH, Yusupov MM, Yusupova GZ, Earnest TN, Noller HF. X-ray crystal structures of 70S ribosome functional complexes. *Science.* 1999; 285:2095–104. [PubMed: 10497122]
42. Clemons WM Jr, May JL, Wimberly BT, McCutcheon JP, Capel MS, Ramakrishnan V. Structure of a bacterial 30S ribosomal subunit at 5.5 Å resolution. *Nature.* 1999; 400:833–40. [PubMed: 10476960]
43. Gilbert SD, Rambo RP, Van Tyne D, Batey RT. Structure of the SAM-II riboswitch bound to S-adenosylmethionine. *Nat Struct Mol Biol.* 2008; 15:177–82. [PubMed: 18204466]
44. Edwards AL, Reyes FE, Heroux A, Batey RT. Structural basis for recognition of S-adenosylhomocysteine by riboswitches. *Rna.* 2010; 16:2144–55. [PubMed: 20864509]
45. Ren A, Rajashankar KR, Patel DJ. Fluoride ion encapsulation by Mg²⁺ ions and phosphates in a fluoride riboswitch. *Nature.* 2012; 486:85–9. [PubMed: 22678284]
46. Serganov A, Huang L, Patel DJ. Coenzyme recognition and gene regulation by a flavin mononucleotide riboswitch. *Nature.* 2009; 458:233–7. [PubMed: 19169240]
47. Johnson JE Jr, Reyes FE, Polaski JT, Batey RT. B12 cofactors directly stabilize an mRNA regulatory switch. *Nature.* 2012; 492:133–7. [PubMed: 23064232]
48. Kieft JS, Costantino DA, Filbin ME, Hammond J, Pfingsten JS. Structural methods for studying IRES function. *Methods Enzymol.* 2007; 430:333–71. [PubMed: 17913644]
49. Edwards AL, Garst AD, Batey RT. Determining structures of RNA aptamers and riboswitches by X-ray crystallography. *Methods Mol Biol.* 2009; 535:135–63. [PubMed: 19377976]
50. Reyes FE, Garst AD, Batey RT. Strategies in RNA crystallography. *Methods Enzymol.* 2009; 469:119–39. [PubMed: 20946787]
51. Galsbøl FH, Simonsen K. The Preparation, Separation and Characterization of some Ammine Complexes of Iridium(III). *Acta Chem Scand.* 1990; 44:796–801.
52. Golden BL. Preparation and crystallization of RNA. *Methods Mol Biol.* 2007; 363:239–57. [PubMed: 17272845]
53. Golden BL, Kundrot CE. RNA crystallization. *J Struct Biol.* 2003; 142:98–107. [PubMed: 12718923]

Fig. 1. Binding of hexamines to the cation binding G-U wobble motif. A. Idealized G-U wobble pair. The small balls on the nucleobases indicate functional groups in the major groove with a partial negative charge form a surface for cation binding. The large sphere denotes the location of the central metal atom of a bound hexamine complex. For simplicity, the amines are not shown. B. Example of cation binding to a single G-U wobble motif within an RNA helix. The G-U pair and flanking sequences are labeled. The orange sphere is the central atom of the iridium (III) hexamine complex, the amines have been removed for simplicity.

Fig 2.

Empirically determined versions of the G-U wobble motif that bind hexamines suitable for phasing (21). A. Single G-U motif versions that can robustly bind a hexamine cation. The rightmost was used to solve two different RNA structures: domain 3 from the Cricket paralysis virus internal ribosome entry site (35), and the *S*-adenosylmethionine riboswitch type I (27). B. Two tandem G-U motifs that robustly bind a hexamine cation.

Fig. 3. Different ways to integrate the use of the cation binding G-U motif and hexammines into an overall RNA crystallization and structure-determination strategy.

Fig. 4.

Examples of how to engineer RNA to include the cation binding motif. A. A stylized RNA secondary structure is shown. Black portions are conserved regions known to be essential for structure and function and gray regions are variable that can be altered without effect. Two good locations for a cation binding G-U wobble motif (a successfully used version is boxed) are shown with hatched boxes. B. Examples of ways an RNA helix could be mutated to introduce a good hexamine cation binding site. In one, a point mutation is used to convert a stretch of Watson-Crick base-pairs to the motif. In the other, three sites are mutated to give the same motif. Both methods work, but the first could be simpler to accomplish with standard mutagenesis methods.