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Crystallographic Pursuit of a Protein–RNA Aptamer Complex

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Summary

Only a few of the aptamers designed to selectively target proteins have been structurally characterized, such as those that target thrombin, von Willebrand factor, *Plasmodium falciparum* lactate dehydrogenase, interleukin 6, and platelet-derived growth factor B. Most of these aptamers are composed of DNA and were designed as therapeutics/diagnostics for targets found in human plasma. Recently, the crystal structure of a complex between an RNA aptamer and an intracellular target, G protein-coupled receptor kinase 2, was determined. Herein is described the overall approach used to isolate crystals that would allow the identification of the key interactions between aptamer and kinase. These strategies may be useful in structural characterization of other SELEX generated RNA aptamer complexes.

Keywords

Crystallography; aptamer; RNA; complex; inhibitor; kinase; GRK2

1. Introduction

There are only a handful of aptamer complexes listed among the ~4400 crystal structures of protein–nucleic acid complexes currently deposited in the Protein Data Bank (PDB), despite the fact aptamers have been developed for well over 1100 human proteins (www.somalogic.com). The paucity of protein–aptamer complexes in the PDB likely reflects inherent difficulties in crystallizing protein–nucleic acid complexes (1). One of the chief hurdles is the conformational heterogeneity of nucleic acid, in particular that of RNA, which stems from its relatively weak tertiary interactions and ability to form a multitude of different base pairing and stacking interactions of comparable free energy. For these reasons, the nucleic acid components of these complexes often dominate crystal lattice contacts. Although such conformational plasticity may facilitate crystal formation, it often leads to imperfectly formed crystals. Another technical hurdle is the fact that aptamers tend to be non-globular, in contrast to proteins, and the resulting crystals exhibit high solvent content due to lattice packing constraints. Both of these hurdles contribute to low diffraction resolution, and, in the worst case, no crystals at all.

These hurdles were all evident in the case of the C13 RNA aptamer, developed via a SELEX approach to bind selectively to G protein-coupled receptor kinase 2 (GRK2) (2). Crystals of GRK2 that diffracted to atomic resolution were only obtained after six years of effort, and ultimately required the inclusion of heterotrimeric G $\beta\gamma$ subunits, which stabilize the kinase

(3). In comparison, the C13.40 variant of the C13 aptamer (Fig. 1A) crystallized in complex with GRK2-G $\beta\gamma$ nearly instantaneously, but the resulting crystals, albeit large, diffracted only to low resolution (8.3 Å). By the time the dust settled (4), over twelve unique crystal forms of GRK2 in complex with C13 aptamer variants were obtained. In each the RNA formed significant crystal lattice contacts wherein structural heterogeneity was apparent, and only the highest resolution structure allowed definition of the key interactions between the aptamer and the GRK2 active site.

Many previously reviewed techniques for optimization of protein–RNA crystals were used in pursuit of the GRK2–C13 structure, such as those covered in (1). Thus, the focus of these procedures is on aptamer-specific crystallographic methods that proved helpful for the final successful structure determination. They may by extension be useful for the structural analysis of other protein–aptamer complexes.

2. Materials

2.1. Macromolecules

1. Aptamers. Ideally commercially synthesized and HPLC purified (e.g. by Integrated DNA Technologies) to help avoid RNase contamination, dissolved in 10 mM in DNase and RNase free ddH₂O (*see Note* ¹).
2. Protein target(s). Purified to homogeneity, passed through a 0.2 μ m spin filter, and concentrated typically to >1 mg/mL in size exclusion chromatography (SEC) buffer (see below) (*see Note* ²).

2.2. SEC

1. SEC buffer: 20 mM HEPES pH 7–8 (but *see Note* ¹), 50 mM NaCl, 5 mM MgCl₂ and 2 mM DTT (added fresh from a 1 M stock (*see Note* ³)).
2. Analytical SEC columns, such as S200 Superdex 10/300 (GE Healthcare) (*see Note* ⁴).

¹Although the C13 aptamer variants were very stable and did not require special consideration with regard to enzymatic degradation, it is advised that all solutions be treated so that they are RNase free (e.g. 0.1% diethylpyrocarbonate, but therefore avoid using buffers with primary amines like Tris and HEPES, which inactivate this chemical). Bake all glassware (e.g. 300 °C for 2 h) that comes into contact with the working solutions. Designate an RNase-free workspace and clean this space regularly. Also designate a set of pipettes for use only in this area and use barrier tips. Wear a lab coat and gloves to prevent user contamination.

A nucleic acid renaturation step may also be useful before attempting to isolate complexes with proteins. We did not perform this step for crystallographic work because we assumed mis-folded aptamers would not bind and be separated from properly folded RNA by SEC. However, renaturation could be required for more complex aptamers, or when one is trying to just “mix and go” (see 3.1 step 7). What follows is an example protocol:

- a. Incubate 100 μ M aptamer at 95 °C for 5 min in 20 mM Tris pH 7.0, 50 mM NaCl
- b. Incubate on ice for 2 min.
- c. Supplemented to 5 mM MgCl₂ final concentration and incubate at 37 °C for 15 min.

²Often proteins are buffer exchanged in a final polishing step using SEC. This can be omitted if the protein–aptamer complex is to going be purified via SEC because this will reduce loss of material.

³The MgCl₂ is included as a counter ion for the nucleic acid phosphodiester backbone. When G $\beta_1\gamma_2$ was to be co-crystallized, the buffer also contained 10 mM CHAPS to stabilize the geranylgeranyl group at the C-terminus of the G γ_2 subunit.

⁴Two columns connected in tandem will improve resolution of bound from unbound species, but may not be necessary if their molecular weight differences are large enough.

3. UV spectrophotometer to measure A_{260} and A_{280} readings from SEC fractions.
4. Protein concentrators (e.g. Amicon Ultra), ideally with molecular weight cutoffs below the molecular weight of the smallest species.

2.3 Crystallization Reagents

1. Commercial sparse matrix screens. The Natrix and Natrix 2 screens (Hampton Research) are a good starting point.
2. High purity precipitants such as 30% (w/v) stocks of PEG 3350 and 8000 (available from many vendors) and 1 M stocks of crystallization buffers in ddH₂O, all sterilized through 0.2 μ M filters.
3. 96 well sitting drop plates with three depressions for robotic screens (e.g. 96-3 well, INTELLI-PLATE[®] sold by Hampton Research). Sealing (packing) tape.
4. 24 well hanging drop plates (e.g. VDX sold by Hampton Research) and siliconized cover slips.

3. Methods

3.1. Isolation and Evaluation of Protein–Aptamer Complexes

1. In a typical reaction, combine ~1 mg protein (e.g. GRK2, 80 kDa) in a 1:1.5 molar ratio with aptamer in 0.5 mL. Incubate on ice for 30 min (*see Note* ⁵). When G β ₁ γ ₂ was included for co-crystallization, approximately 0.7 mg was also added and the mixture incubated on ice for an additional 30 min.
2. Inject no more than 0.5 mL of the sample onto the SEC column using an appropriate HPLC system. We use a BioLogic DuoFlow, but AKTA systems are also popular. Use a slow flow-rate (~0.3 mL/min) and collect 300 μ L fractions at 4 °C (*see Note* ⁶).
3. Determine peak protein fractions from A_{280} values. Using a standard curve (e.g. linear regression of log(Da) vs. (elution volume-void volume), determine estimated molecular weight of the peaks (*see Note* ⁷).
4. Determine the A_{260}/A_{280} ratio in each peak estimated to have close to the correct complex molecular weight (*see Note* ⁸).

⁵The strategy is to saturate the higher molecular species with the lower molecular weight one, driving all the higher molecular weight species into a complex. This allows for better separation from the unbound lower apparent molecular weight species, which could ultimately inhibit crystallization by “poisoning” the growing lattice. For small proteins or large aptamers, the protein may need to be the component in excess.

⁶In principle, the smaller the injection volume, the better the peak resolution. Smaller amounts of material may also reduce peak broadening, but 250 μ g of material is suggested at minimum because in all runs, up to 100 μ g sample is nonspecifically absorbed by the column, filters, and tubing.

⁷Asymmetric protein complexes will often elute before the volume predicted for their molecular weight, and macromolecules that nonspecifically interact with the resin will elute after. Check for such aberrant behavior in a control run with protein (or aptamer) alone.

⁸For example, the C13.40 RNA aptamer, a 40 nucleotide truncation of C13 (Fig. 1A), eluted as a complex with GRK2–G β ₁ γ ₂ at ~140 kDa, consistent with its predicted molecular weight of ~140 kDa (126 kDa protein and 13 kDa RNA). The A_{260}/A_{280} ratio in peak fractions was 1.5, corresponding to ~85% protein and ~15% nucleic acid, close to the 90% protein and 10% RNA predicted if the molecules are in a stoichiometric complex (7).

5. Pool fractions that have closest to the expected A_{260}/A_{280} ratio and/or most consistent ratios. Concentrate to 5–10 mg/mL (*see Note* ⁹).
6. Pass sample through a 0.2 μ m spin filter prior to crystallization (*see Note* ¹⁰).
7. In the event that the aptamer affinity is too low or complex formation is ambiguous by SEC, it is reasonable to attempt to crystallize a complex of sample prepared by mixing the aptamer and protein together, and then proceeding directly to concentration and filtering. We had success via this approach using a 1:1.5 molar ratio of GRK2 to the C13.20 aptamer. However, there is always the danger that one might crystallize excess aptamer, necessitating proper controls (*see section 3.2* step 1).

3.2. Crystallization

There are many approaches to crystallizing macromolecules (5), but the traditional hanging drop method is one of the most robust. For example, the GRK2–aptamer complexes were typically mixed 1:1 (v/v) with well solution consisting of precipitant (such as polyethylene glycol (PEG) 3350 or 8000) in a 2 μ L drop that was suspended over a 1 mL well. Incubation temperature is an important variable in screening for crystals, but for RNA aptamers 4 °C is the best initial choice because this may slow the rate of degradation and/or reduce conformational heterogeneity.

1. Set up a sparse matrix screen using various commercial kits and a crystallization robot (Art Robbins Gryphon is a popular choice). This allows one to assess a wide variety of conditions using smaller amounts of sample in shorter amounts of time than can be set up by hand. Robots such as this use a sitting drop format wherein there can be up to three depressions for samples. Thus, for each well solution to be tested, one could: screen three different protein concentrations; or two different protein concentrations and a buffer blank; or complex in one, aptamer in the second, and protein in the third. Thus one can easily control for (potentially) undesirable crystals that result from nonstoichiometric amounts of one component, from components that dissociated under the crystallization conditions, or from buffer components.
2. Set up more focused homemade screens around promising conditions from the commercial screens or around those that are known to yield crystals of related samples. Below are standard initial grids we use in our studies. The recipes are for the well solutions (1 mL final volume) in a 24 well hanging drop plate (4 rows, 6 columns). Typically two trays of each grid are prepared so that one can be incubated at 4 °C and the other at 20 °C (*see Note* ¹¹):

⁹There is no absolute rule of thumb for what concentration of complex is optimal for crystallization, because the ideal value is closely linked to the solubility of the complex being isolated. This parameter is often optimized during the crystallization process. For GRK2–aptamer complexes, we have had good luck in the range of 6–10 mg/mL.

¹⁰It is advised to set up crystallization screens immediately, as opposed to freezing the complex in liquid N₂ for another day, because freeze thaw cycles likely damage samples. That said, it is probably a good idea to save enough protein from each preparation so one can try to repeat a few crystallizations in the event that future preparations of the complex fail to produce crystals.

¹¹Keep the complex on ice whenever possible. When mixing complex with well solution on cover slips, add the complex to drop last because this will help draw the sample out of the pipette tip. If the drops are small, and if you are operating at room temperature (not advised for sensitive samples), one has to work quickly because the volume change that occurs via evaporation will be greater and

Grid 1: PEG 8000 versus pH, low salt (see Note ¹²):

30% PEG 8000: 5%, 10%, 15%, 20% (w/v) (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns) (see Note ¹³)

Grid 2: PEG 8000 versus pH, high salt (NaCl):

30% PEG 8000: 5%, 10%, 15%, 20% (w/v) (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

NaCl: 1 M NaCl (e.g. 250 µl of 4M in a 1.0 mL well) (all wells)

Grid 3: PEG 3350 versus pH, low salt:

30% PEG 3350: 5%, 10%, 15%, 20% (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

Grid 4: PEG 3350 versus pH, high salt (NaCl):

30% PEG 3350: 5%, 10%, 15%, 20% (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

NaCl: 1 M NaCl (e.g. 250 µl of 4M in a 1.0 mL well) (all wells)

Grid 5: NaCl versus pH:

4M NaCl: 2M, 2.5M, 3M, 3.5M (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

Grid 6: Ammonium sulfate versus pH:

3.5M ammonium sulfate: 1.5M, 2.0M, 2.5M, 3M (rows) (see Note ¹⁴)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

Grid 7: PEG 400 versus pH, low salt:

100% PEG 400: 20%, 30%, 40%, 50% (v/v) (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

potentially irreproducible results may be obtained. The coverslips bearing the sample are suspended over the well solution and sealed, typically with vacuum grease. When setting the coverslips, try not to let the coverslips overlap and be sure each well is sealed. Too much grease will lead to wandering coverslips and air gaps. Inspect each tray via microscope immediately after setup. Mark anything that could later be mistaken for a crystal in your notes. Check daily for 3 days, then every other day, then weekly, etc. If certain pH ranges or precipitant concentrations invariably precipitate the complex (heavy white snow-like material), adjust the grids accordingly to avoid these conditions and screen more useful chemical space. If the sample does not precipitate under any condition, try increasing either the protein or the precipitant concentration.

¹²Many complexes are less soluble in solutions with low ionic strength. The low salt concentration may have to be adjusted upwards (e.g. to 100 mM) or the maximum precipitant concentration downwards in order to keep the complex in solution long enough for crystals to nucleate.

¹³It is best to use buffers that have broad useful pH ranges because this reduces the number of parameters that vary across the tray. Good buffers are preferred (8), although Na-citrate is a very useful reagent for the lower pH ranges given its broad buffering range (3–6.2). However, because Mg^{2+} will typically be included as a counter ion for the nucleic acid, phosphate buffers should be avoided because $MgPO_4$ forms really beautiful, yet ultimately very disappointing crystals.

¹⁴Ammonium sulfate stocks should be remade with more frequency than other precipitants. Stock solution could either be used as is (will be acidic) or individual stocks adjusted with NaOH to the designated pH of each column of the grid.

Grid 8: PEG 400 versus pH, high salt:

100% PEG 400: 20%, 30%, 40%, 50% (v/v) (rows)

pH: 100 mM buffer (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5) (columns)

NaCl: 1 M NaCl (e.g. 250 μ l of 4M in a 1.0 mL well) (all wells)

3.3. Improvement of Low Quality Crystals

Although rapidly forming large trigonal plate crystals, the C13.40-GRK2-G β ₁ γ ₂ complex had 86% solvent content and diffracted to only 8.7 Å, which is insufficient for determining the molecular basis of aptamer selectivity, especially when the aptamer structure is unknown. There are however many ways to improve crystal quality. Below are aptamer-specific steps we undertook in pursuit of our highest resolution structure at 3.5 Å spacings. These approaches can likely be tailored to structure determinations of other protein-aptamer complexes.

1. Trim unnecessary portions/projections of the aptamer. This will likely reduce flexibility and make the aptamer more globular, but may come at the price of lower affinity. For example, the C13.40 aptamer, which forms a relatively simple hairpin-loop/stem structure (Fig. 1A), made lattice contacts via end-to-end contacts by its flexible stem. Such contacts are frequently observed in RNA crystals (6). The stem did not correspond to the selected region of the aptamer and thus was dispensable except for its potential role in stabilizing the fold of the aptamer. We therefore designed C13.28, wherein the stem was shortened by six base pairs compared to C13.40 (Fig. 1A, B). As hypothesized, C13.28 retained similar lattice packing to the C13.40 crystals and had reduced solvent content (83%) and RNA flexibility because the end-to-end dimer contact was reduced by 12 base pairs, or approximately one full helical turn of A-form RNA. The diffraction limit was improved to 4.5 Å (Fig. 1C).
2. Engineer tighter junctions. For example, a 5' G overhang was included in the C13.29 variant of the aptamer to try to stabilize the end-to end junction in the C13.28-GRK2-G β ₁ γ ₂ crystals with a G-G base pair (6). Sequences in the stem can also be optimized to contain more stable, GC-rich Watson-Crick base pairs, such as in the C13.28 variant (Fig. 1B), where 5' GGCA 3'/3' CCGU 5' was introduced at the termini.
3. Eliminate promiscuous lattice forming motifs. Removal of non-essential portions of the aptamer that favor heterogeneous or flexible lattice contacts, such as the stem in C13.28 (Fig. 1C), is probably one of the most effective strategies. For example, the best diffracting crystals for the GRK2-aptamer complex were obtained using C13.18, which contained only 18 bases of the 20 selected nucleotides of the original aptamer (56% solvent content). A 3.5 Å structure was achieved, but at the price of 35-fold less binding affinity and 10-fold lower IC₅₀ against kinase activity.
4. Pray to a different deity, but meanwhile keep engineering and screening!

Acknowledgments

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Figure 1. GRK2-binding aptamers. A) Secondary structure of the C13.40 aptamer, as revealed by structural studies. The nucleotides in red correspond to those that were altered by SELEX. The inset shows a representative crystal of the C13.40–GRK2–Gβγ complex, which diffracted to only 8.3 Å. B) Secondary structure of the C13.28 aptamer. Note the altered stem nucleotides. The inset shows representative crystals of the C13.28–GRK2–Gβγ complex, which diffracted to 4.5 Å resolution. C) Two-fold crystal contact of the C13.28–GRK2–Gβγ complex mediated by base stacking at the 5′ end of the stem of the aptamer (orange phosphodiester backbone with bases represented as cyan rods). Mg²⁺ is shown as a black sphere.