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Preliminary characterization of crystals of an *in vitro* evolved cyanocobalamin (vitamin B_{12}) binding RNA

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A 35-nucleotide pseudoknot that binds vitamin B_{12} been isolated using systematic evolution of ligands by exponential enrichment (SELEX). Affinity chromatography was used to purify functional, properly folded molecules and the hanging-drop vapor-diffusion method was used to crystallize this aptamer. Two crystal forms have been obtained and the preliminary crystallographic characterization is reported here. Both crystal forms (space groups $I222$ or $I2_12_12_1$ and $C222_1$) diffract to 2.9 Å and should prove sufficient for structure determination.

1. Introduction

Atomic resolution structures have been crystallographically determined for only a handful of different types of RNA with tertiary structure (Kim *et al.*, 1973; Robertus *et al.*, 1974; Pley *et al.*, 1994; Scott *et al.*, 1995; Cate *et al.*, 1996, for example). The small size of this database limits our ability to understand, in a general sense, the essential relationship between RNA structure and biological function. *In vitro* selection from pools of random-sequence molecules has yielded an assortment of structured RNA with receptor-like binding activities (aptamers) or catalytic activities (ribozymes) (*e.g.* Dai *et al.*, 1995; Lorsch & Szostak, 1994*a*,*b*; Morgan Conn *et al.*, 1996; Wilson & Szostak, 1995). In contrast to biologically derived RNA, the RNA motifs isolated by *in vitro* selection offer several experimental advantages as model systems for probing the relationships between RNA structure and function as outlined below.

The large size and complexity of many biological RNAs may contribute to the lack of diffraction-quality crystals. All of the *in vitro* selected aptamers and ribozymes isolated thus far can be reduced to minimal domains containing 35–100 nucleotides. By progressively trimming down non-essential nucleotides, it has been possible to design RNA in which every nucleotide is likely to be involved in folding to the stable structure. Additionally, the nature of the *in vitro* selection experiment implies that the selection 'winners' are well behaved: they typically fold spontaneously under the selection conditions with high efficiency. The same affinity methods initially used to isolate aptamers from the background of random, non-functional RNA can be applied to purify properly folded aptamers from misfolded ones, yielding a structurally homogeneous population of molecules. *In vitro* selection experiments allow one to rapidly generate artificial phylogenies, collections of related molecules whose sequences can be analyzed to determine which nucleotides must be absolutely specified to maintain function and which pairs of nucleotides interact with one another (*e.g.*, Bartel *et al.*, 1991). In combination with high-resolution structural studies, these genetic experiments have the potential to answer fundamental questions on the relationship between RNA structure and function.

We have chosen to focus on the cyanocobalamin (vitamin B_{12}) specific aptamer isolated by Lorsch & Szostak (1994*b*) as a model system for understanding how RNA folds to a defined tertiary structure and how it specifically interacts with small-molecule ligands. Adenosylcobalamin (the biologically active form of vitamin B_{12}) is believed to be one of the most evolutionarily ancient enzyme cofactors (Georgopapadakou & Scott, 1977) and has been postulated to have played a key role in the transition from RNA-based biology to modern DNA- and protein-dominated biology (Benner *et al.*, 1989). Using *in vitro* selection from a pool of 10^{15} random-sequence molecules, Lorsch and Szostak were able to isolate five RNA molecules that specifically recognize vitamin B_{12} (Lorsch & Szostak, 1994*b*). Within the 114-nucleotide-long RNA molecule that dominates the pool of RNAs selected in their experiment, an unusual 35-nucleotide pseudoknot is responsible for high-affinity binding (Fig. 1). As described below, this motif has several unique features that may be best understood through high-resolution structural studies.

The aptamer binds cyanocobalamin with relatively high affinity ($K_d \simeq 90$ nM), suggesting a large number of specific interactions stabilize the RNA–ligand complex. Cobinamide dicyanide (lacking the dimethylbenzimidazole ribonucleotide substituent) binds ~200 times more weakly than cyano-

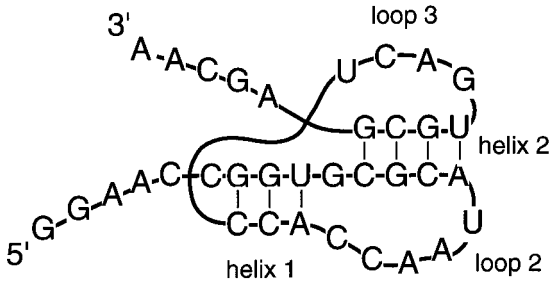

Figure 1
The minimized form of the vitamin B_{12} aptamer utilized in these crystallization studies.

cobalamin while adenosylcobalamin (the biologically active form) fails to bind with any measurable affinity. These results indicate that substituents on both sides of the corrin ring are specifically recognized by the RNA, suggesting that the RNA may wrap around the large cofactor to achieve both high-affinity and high-specificity binding. The presumed topology of the B_{12} pseudoknot is based on a combination of phylogenetic data (confirming helix one) and chemical modification experiments (suggesting helix two) (Lorsch & Szostak, 1994*b*). In most pseudoknots, loop two (connecting the 5′-strand of the second pseudoknot helix with the 3′-strand of the first helix) is virtually non-existent, and the two helices are thought to be coaxially stacked on each other (Shen & Tinoco, 1995). In contrast, the B_{12}-binder pseudoknot has a single nucleotide in loop one and a large loop two. Model building suggests it is impossible to coaxially stack the two helices given the short length of the linking loops. Crystallographic studies will demonstrate whether such pseudoknots can actually form and how they assemble into a defined tertiary structure. The aptamer requires 1 *M* lithium chloride for proper folding and binding. Lithium ions form tight complexes with phosphates (exchange measurements suggest that the interaction resembles covalent bonding more than ionic pairing) (Cotton & Wilkinson, 1988). As such, the presence of lithium may neutralize the charged RNA backbone, enabling the RNA to fold into a more compact and stabilized structure than it might otherwise be allowed to adopt. Determination of the structure of this molecule would provide the first insights into how a small ion such as lithium, which cannot coordinate functional groups, acts to promote the folding of an active RNA structure.

In the present study we report the growth of two different crystal forms of the vitamin B_{12} pseudoknot. Characterization of the X-ray diffraction from each is also presented. Both crystal forms diffract to approximately 2.9 Å and should prove sufficient for determining the aptamer structure to atomic resolution. Methods applied to obtain these crystals are likely to be generally applicable to other RNA isolated by *in vitro* selection.

2. Methods

2.1. Materials

DNA oligonucleotides were synthesized by standard β-cyanoethyl phosphoramidite methods using a Millipore 8909 Expedite DNA synthesizer. Vitamin B_{12} and vitamin B_{12} agarose were purchased from Sigma Chemical Company (St Louis, MO, USA).

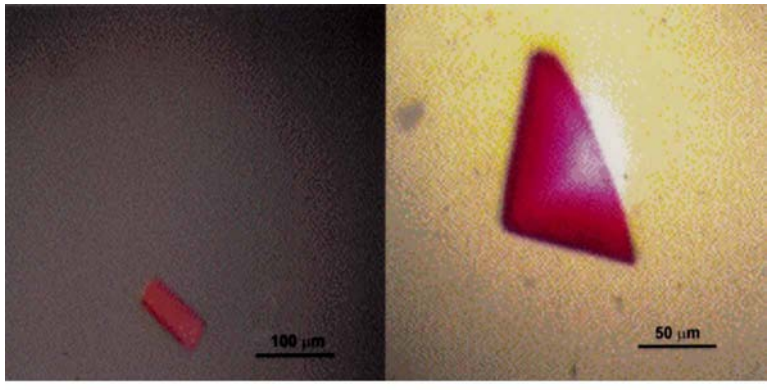

Figure 2
The two crystal forms obtained by factorial screening. (*a*) Crystal form *A*, rod-shaped crystals with dimensions 120 × 30 × 30 mm. (*b*) Crystal form *B*, plate-like crystals with dimensions 150 × 150 × 10 mm.

2.2. RNA synthesis

A template oligo corresponding to the bottom strand of the T7 promoter and the reverse complement of the aptamer sequence (5′-TTG CTC GCA CTG AGG TGG TTA TGC GCA CCG GTT CCT ATA GTG AGT CGT ATT ACA TAT GCG TGT TAC C-3′) was synthesized and purified by PAGE. Large scale transcription reactions (typically 10–20 ml per preparation) contained 5 m*M* NTP, 35 m*M* $MgCl_2$, 1 m*M* spermidine, 0.01% Triton, 40 m*M* Tris–HCl, 5 m*M* DTT, pH 8.1, 0.5 µ*M* template oligo and 0.5 µ*M* T7-promoter top strand. Following overnight incubation with purified recombinant T7 RNA polymerase at 310 K, the transcription reaction was stopped by the addition of 0.6 g ml^{-1} urea and the magnesium pyrophosphate precipitate was removed by centrifugation.

2.3. RNA purification

RNA was purified initially by denaturing 15% polyacrylamide gel electrophoresis. Because of the large volume of the transcription reaction and the large yield of RNA, preparative-scale gels were used. Following elution of gel slices encompassing products of length of both *N* and *N* + 1 (from T7 run-off transcription) into 0.3 *M* NaCl, RNA was ethanol precipitated and resuspended in B_{12}-binding buffer (1 *M* LiCl, 25 m*M* Na-HEPES. pH 7.4). B_{12} RNA was affinity purified by passing the resuspended RNA over a vitamin B_{12} agarose column, washing with ten column volumes of B_{12}-binding buffer, and eluting with five column volumes of elution buffer (B_{12}-binding buffer plus 5 m*M* vitamin B_{12}). Peak fractions from the affinity elution were pooled and concentrated with Centriprep-3 and a Centricon-3 (Amicon Inc., Beverly, MA, USA) to a usable final concentration of 1.1 mg ml^{-1} RNA (1:50 RNA to ligand). RNA was stored at 277 K prior to use in crystallization trials.

2.4. Factorial screening for crystallization conditions

Crystallization conditions were obtained by screening using the hanging-drop method. An adaptation of the factorial screen used by Doudna *et al.* (1993) was used to identify suitable crystallization conditions. Our protocol was identical to that described previously with the following modifications.

(i) In the original protocol, RNA is resuspended in a low-salt buffer and annealed by heating and slow cooling. We presumed that purification by affinity chro-

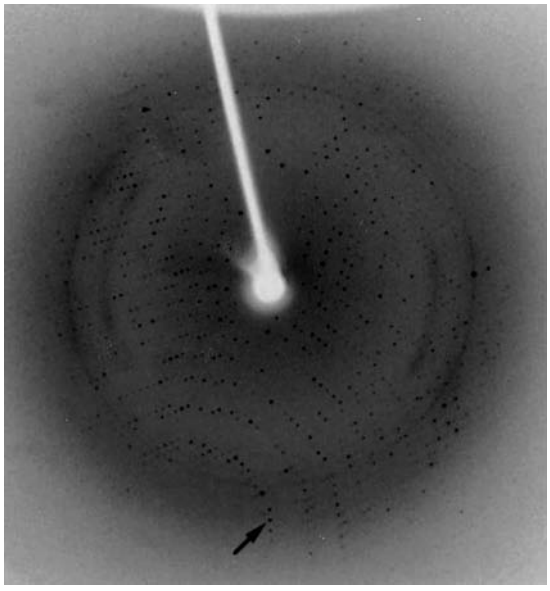

Figure 3
Diffraction from crystal form *A*. A 20 min 1.5° oscillation image was collected from 150 µm crystals as described in §2. A sample spot at 2.9 Å resolution is indicated by the arrow.

matography would preselect those RNA molecules that were correctly folded (since they had adopted a functional conformation) and made no additional efforts to optimize folding by annealing. As such, 1.1 mg ml^{-1} affinity-purified RNA was diluted directly with each of the sparse-matrix stock solutions to prepare the hanging drops.

(ii) Previous studies have shown that 1 *M* lithium chloride is required for binding. As such, all trials were carried out in the presence of high concentrations of lithium chloride. RNA in an elution buffer containing lithium chloride was used in the hanging drops. To compensate for this high salt concentration, one-tenth volume of 10 *M* lithium chloride was added to the reservoir.

(iii) 16 additional conditions representing random combinations of other conditions in the screen were added to sample more fully parameter space, bringing the total to 60 trials. No crystals grew in these additional conditions.

2.5. Data collection

All data were collected using an R-AXIS IIc imaging-plate detector. X-rays were generated by a Rigaku RU200 rotating-anode generator equipped with a rotating copper anode, a graphite monochromator, a 0.5 mm filament and a 0.3 mm collimator, operated at 50 kV and 180 mA. Crystals were equilibrated in a stabilizing solution consisting of the equilibration reservoir (5 *M* LiCl and 6% 2-propanol) containing 10% glycerol prior to flash freezing in a stream of boiling liquid nitrogen at 98 K generated by the MSC cryocooling system. All data were collected from crystals maintained at this temperature. Data were processed using the *HKL* package (MSC).

3. Results

Factorial screening was carried out at 277, 288 and 298 K. The presence of lithium chloride in the crystallization trials seems to generally exert a solubilizing effect on RNA, causing less aggregation to appear at high precipitant concentrations. Crystals of the RNA–ligand complex were obtained in three different screen conditions after prolonged incubation at 277 K. No crystals were obtained in the initial screening at higher temperatures or in trials containing the RNA alone (Fig. 2). The crystals are believed to be a stable RNA–ligand complex due to the red color that is retained after extensive washing (over several days) in vitamin-B_{12}-free solution.

Characterization of the different crystals by X-ray diffraction reveals that they correspond to two distinct but related crystal forms. Crystal form *A* was isolated from a screen condition containing 7% 2-propanol, 5 m*M* $MgCl_2$, 20 m*M* potassium cacodylate, pH 6.0. Preliminary characterization of diffraction from these crystals indicate unit-cell parameters $a = 91$, $b = 99$, $c = 221$ Å, $\alpha = \beta = \gamma = 90^\circ$. Laue symmetry and systematic absences in the diffraction are consistent with either space group $I222$ or $I2_12_12_1$. Assuming six molecules per asymmetric unit, V_m is calculated as 3.13 Å^3 Da^{-1}. As shown in Fig. 3, diffraction extends to beyond 2.9 Å, with 77% of the spots at this resolution having $I/\sigma > 2$. Crystal form *B* was isolated from two screen conditions containing either 7% 2-propanol, 15 m*M* $MgCl_2$, 20 m*M* K-PIPES, pH 6.5, 0.5 m*M* $BaCl_2$ or 20% dioxane, 5 m*M* $MgCl_2$, 1 m*M* spermine, 1 *M* LiCl, 20 m*M* K-MOPS, pH 7.0. These crystals similarly diffract to 2.9 Å, although they clearly correspond to a different crystal form with unit-cell parameters $a = 89$, $b = 159.8$, $c = 100.3$ Å, $\alpha = \beta = \gamma = 90^\circ$, space group $C222_1$, $V_m = 3.36$ Å^3 Da^{-1} assuming four molecules per asymmetric unit. Given the similarity of several of the unit-cell parameters and symmetry operators, we speculate that aspects of the molecular packing within the crystal lattice are conserved between the two crystal forms.

We have shown that factorial screening readily identifies conditions that yield strongly diffracting crystals of this affinity purified *in vitro* selected RNA. Given the resolution limit of diffraction for these crystal forms, both should prove sufficient for solving the aptamer structure. We are currently attempting to obtain phase information through conventional heavy-atom soaking methods. Additionally, we aim to utilize the cobalt ion coordinated by the bound vitamin B_{12} as a potential anomalous scatterer in MAD experiments.

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References

Bartel, D. P., Zapp, M. L., Green, M. R. & Szostak, J. W. (1991). *Cell*, **67**, 529–536.
Benner, S. A., Ellington, A. D. & Tauer, A. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 7054–7058.
Cate, J., Gooding, A., Podell, E., Zhou, K., Golden, B., Kundrot, C., Cech, T. & Doudna, J. D. (1996). *Science*, **273**, 1678–1685.
Cotton, F. A. & Wilkinson, G. (1988). *Advanced Inorganic Chemistry*. New York: John Wiley & Sons.
Dai, X., De Mesmaeker, A. & Joyce, G. F. (1995). *Science*, **267**, 237–240.
Doudna, J. A., Grosshans, C., Gooding, A. & Kundrot, C. E. (1993). *Proc. Natl Acad. Sci. USA*, **90**, 7829–7833.
Georgopapadakou, N. H. & Scott, A. I. (1977). *J. Theor. Biol.* **69**, 381–384.
Kim, S. H., Quigley, G. J., Suddath, F. L., McPherson, A., Sneden, D., Kim, J. J., Weinzierl, J. & Rich, A. (1973). *Science*, **179**, 285–288.
Lorsch, J. R. & Szostak, J. W. (1994*a*). *Nature (London)*, **371**, 31–36.
Lorsch, J. R. & Szostak, J. W. (1994*b*). *Biochemistry*, **33**, 973–982.
Morgan Conn, M., Prudent, J. R. & Schultz, P. G. (1996). *J. Am. Chem. Soc.* **118**, 7012–7013.
Pley, H. W., Flaherty, K. M. & McKay, D. B. (1994). *Nature (London)*, **372**, 68–74.
Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. & Klug, A. (1974). *Nature (London)*, **250**, 546–551.
Shen, L. X. & Tinoco, I. Jr (1995). *J. Mol. Biol.* **247**, 963–978.
Scott, W., Finch, J. & Klug, A. (1995). *Cell*, **81**, 991–1002.
Wilson, C. & Szostak, J. W. (1995). *Nature (London)*, **374**, 777–82.