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Crystallization and preliminary diffraction analysis of a group I ribozyme from bacteriophage Twort

Group I introns are catalytic RNAs that are capable of performing a variety of phosphotransesterification reactions including self-splicing and RNA cleavage. The reactions are efficient, accurate and dependent only on the presence of guanosine-nucleotide substrate and sufficient magnesium ion to stabilize the structure of the RNA. To understand how the group I intron active-site facilitates catalysis, crystals of a 242-nucleotide ribozyme bound to a four-nucleotide product RNA have been produced that diffract to 3.6 Å resolution. The space group of these crystals is $I2_12_12_1$ and the unit-cell parameters are $a = 94.6$, $b = 141.0$, $c = 210.9$ Å. A single heavy-atom derivative has been synthesized by covalent modification of the product RNA with iodine.

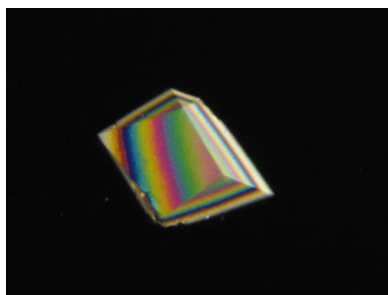
1. Introduction

A group I self-splicing intron contains an active site that allows it to excise itself from a precursor RNA and ligate the flanking exons, generating an intact mRNA, tRNA or rRNA (Cech, 1993; Kruger *et al.*, 1982; Saldanha *et al.*, 1993). This self-splicing reaction requires only sufficient magnesium ions (~ 2 mM) to fold the RNA and a guanosine-nucleoside substrate (Cech, 1993; Kruger *et al.*, 1982; Saldanha *et al.*, 1993).

The self-splicing reaction is complex, involving the orchestration of two successive transesterification reactions, but it is carried out efficiently and accurately by the group I active site. The intron possesses a binding site capable of recognizing a guanosine nucleoside and activating its 3' hydroxyl for nucleophilic attack at the 5' splice site. The 5' splice-site phosphate follows a uridine recognized in the context of a U-G wobble pair within the hairpin duplex at the 5' end of the intron (P1). Attack of the activated guanosine nucleophile at the 5' splice site liberates the 5' exon and links the guanosine substrate to the 5' end of the intron. A structural rearrangement must then occur, in which the exogenous guanosine exits the active site and is replaced by a conserved guanosine (ω G) at the 3' terminus of the intron (Been & Perrotta, 1991; Michel *et al.*, 1989). The 5' exon can then attack at the 3' splice site in a transesterification reaction that is chemically equivalent to the reverse of the first step of splicing. This reaction ligates the two exons and releases the group I intron.

Group I introns can easily be converted into *trans*-acting ribozymes. Such molecules are capable of recognizing an RNA substrate by base-pairing and activating the 3' hydroxyl of a guanosine nucleoside or ω G for nucleophilic cleavage of the substrate (Zaug *et al.*, 1988). Ribozymes such as this have been used to extensively characterize the endonuclease reaction of group I RNAs (Herschlag & Cech, 1990; Shan *et al.*, 2001).

Group I introns possess conserved core elements of secondary structure (designated P1–P9 in Fig. 1*a*) organized into three domains, P1–P2, P4–P6 and P3–P9 (Cech *et al.*, 1994). The active site is located at the junction of the P4–P6 and P3–P9 domains. To generate a picture of these widespread RNA machines, crystal structures of the P4–P6 domain from the 413-nucleotide *Tetrahymena* group I intron (Cate *et al.*, 1996; Juneau *et al.*, 2001) were solved at high resolution and the structure of a 247-nucleotide ribozyme composed of the P4–P6 and P3–P9 domains was solved at modest resolution (Golden *et al.*, 1998; Guo *et al.*, 2004). Additionally, the structure of a small

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group I intron from *Azoarcus* tRNA^{Ile} in complex with both exons was recently reported (Adams *et al.*, 2004).

We are now investigating a 242-nucleotide ribozyme derived from an intron from the phage Twort (Landthaler & Shub, 1999). The Twort ribozyme is an active enzyme capable of binding to a four-nucleotide recognition sequence within a single-stranded RNA substrate and promoting site-specific cleavage (H. Kim, E. Chase and B. L. Golden, unpublished results). We have synthesized and crystallized the Twort ribozyme bound to a four-nucleotide product RNA. This complex captures the Twort ribozyme in a catalytic state that is distinct from that observed in the *Azoarcus* intron structure (Adams *et al.*, 2004). The Twort ribozyme complex is unreactive because the scissile phosphate is not present, while the *Azoarcus* intron was trapped by introduction of deoxyribose at the cleavage site. Thus, the two structures should provide details of the conformational changes that occur during the catalytic cycle. Crystals of the Twort ribozyme diffract X-rays to at least 3.6 Å resolution, are suitable for structure determination and can be derivatized by covalent modification of the product RNA.

2. Methods

2.1. Constructs

The gene encoding the Twort ribozyme was amplified by PCR from a subclone containing orf142-I2 from phage Twort (kindly supplied by M. Landthaler and D. A. Shub). The primers (IDT, Coralville, IA, USA) were 5'-TAGCATAAGCTTAATACGACTCACTATAGAG-CCTTTATACAGTAATGTA and 5'-CTAGCTTCTAGACTCTTCAACAATTATGTTACGGATAGGTT.

The resulting PCR product contained a T7 promoter, residues 9–252 of the orf142-I2 intron, an *EarI* restriction-endonuclease recognition site for runoff transcription reactions and *HindIII* and *XbaI* recognition sites for cloning. The second nucleotide of the ribozyme was switched from a U to an A to facilitate transcription of the RNA. The L5 loop was also converted to a GAGA tetraloop to promote crystallization. The PCR product was directionally cloned into the polylinker of pUC-19.

2.2. RNA synthesis

The plasmid template was isolated from *Escherichia coli* strain XL-1 Blue using QiaFilter plasmid-isolation kits (Qiagen). The plasmid was restricted with *EarI* restriction endonuclease and protein contaminants were removed by phenol:chloroform:isoamyl alcohol (25:24:1 by volume) extraction, chloroform extraction and ethanol precipitation.

Ribozyme was synthesized by *in vitro* transcription. Typical reactions contained 20 µg ml⁻¹ plasmid template, 0.1 mg ml⁻¹ T7 RNA polymerase (recombinant protein purified from an overexpressing strain), 2 mM each of ATP, CTP, GTP and UTP, 40 mM Tris-HCl pH 8.0, 25 mM MgCl₂, 10 mM DTT and 2 mM spermidine. Reactions were incubated at 310 K for 1–3 h. Sufficient EDTA was then added to the reactions to chelate the Mg²⁺ and to reverse the precipitation of magnesium pyrophosphate. The RNA was then recovered by ethanol precipitation.

The ribozyme was purified by denaturing 6% polyacrylamide gel electrophoresis (PAGE) using 3 mm spacers. The region of the gel containing the full-length ribozyme was identified by UV shadowing, excised and crushed by extrusion through a syringe. The RNA was extracted from the crushed gel matrix by soaking in TEN (10 mM Tris-HCl, 1 mM EDTA, 250 mM NaCl) overnight. Remaining polyacrylamide was removed from the RNA solution by centrifugation

and filtration using a 0.2 µm filter. RNA was precipitated from the TEN solution by the addition of three volumes of ice-cold ethanol and recovered by centrifugation. The RNA was then resuspended in 5 mM potassium cacodylate pH 6.0 and a Centricon concentrator was used to further exchange the buffer and to concentrate the ribozyme for crystallization. The ribozyme was stored at 253 K.

A four-nucleotide RNA, GCUU, and a modified RNA with a 5-iodouridine, GC(5iU)U, were purchased from Dharmacon Research, Inc, Lafayette, CO, USA.

2.3. Crystallization

To prepare the RNA for crystallization trials, 0.0626 mM ribozyme was mixed with 0.07 mM product RNA in a buffer containing 15 mM MgCl₂ and 5 mM potassium cacodylate. This mixture was heated at

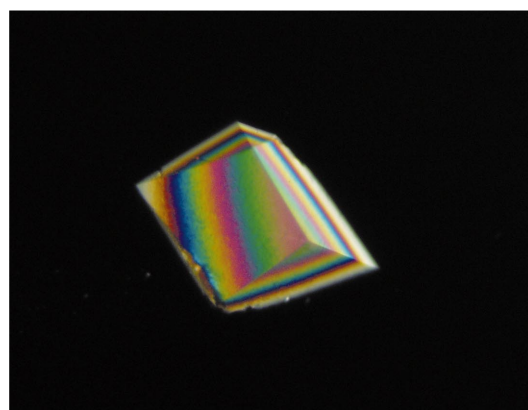
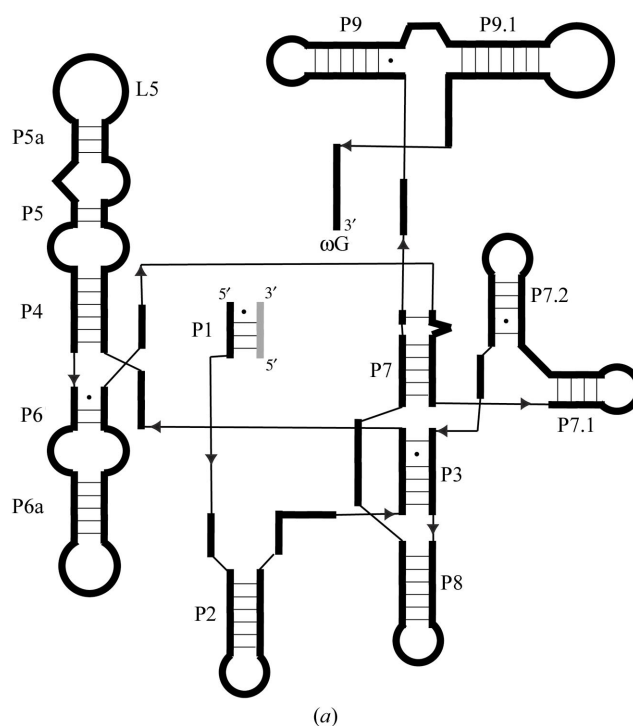


Figure 1

(a) Twort ribozyme–product complex. Secondary-structure diagram of the Twort ribozyme (black) bound to a four-nucleotide product (gray). The 5' and 3' ends of the chains are indicated. Thin lines indicate Watson–Crick base pairs and G–U wobble base pairs are indicated by dots. (b) An orthorhombic crystal of the Twort ribozyme–product complex. This crystal is 0.2–0.3 mm in each dimension.

323 K for 5 min and allowed to cool to room temperature on the benchtop to allow proper folding of the ribozyme.

Crystallization conditions were determined by screening using the hanging-drop method. Preliminary crystallization conditions were identified using a 24-condition sparse-matrix designed for screening large RNAs (Golden & Kundrot, 2003). Crystals were produced by mixing the RNA with 50 mM potassium cacodylate pH 6.5, 0.5 mM spermine and 1.8 M Li₂SO₄. Well solutions were generated by mixing one volume of the above precipitant with 0.5–0.75 volumes of saturated Li₂SO₄. Crystals take ~10 d to grow at 291 K.

Derivative crystals were grown using a product RNA with 5-iodouridine modification at the third nucleotide. Since 5-iodouridine is photosensitive, the RNA was protected from ambient light during crystallization and data collection.

2.4. Data collection

Prior to data collection, crystals were stabilized in a solution containing 30 mM MgCl₂, 50 mM potassium cacodylate pH 6.5, 1 mM spermine and 15% (w/v) xylitol that was saturated with Li₂SO₄. Following stabilization, the crystals were flash-frozen in liquid nitrogen. Native data were collected at 100 K at beamline BM-8 at the Advanced Photon Source. Derivative data were collected using a rotating-anode generator. Data were processed using *HKL2000* (Otwinowski & Minor, 1997). *CNS* was used to merge and scale native and derivative data and to calculate a difference Patterson map (Brünger *et al.*, 1998). Data-collection statistics are given in Table 1.

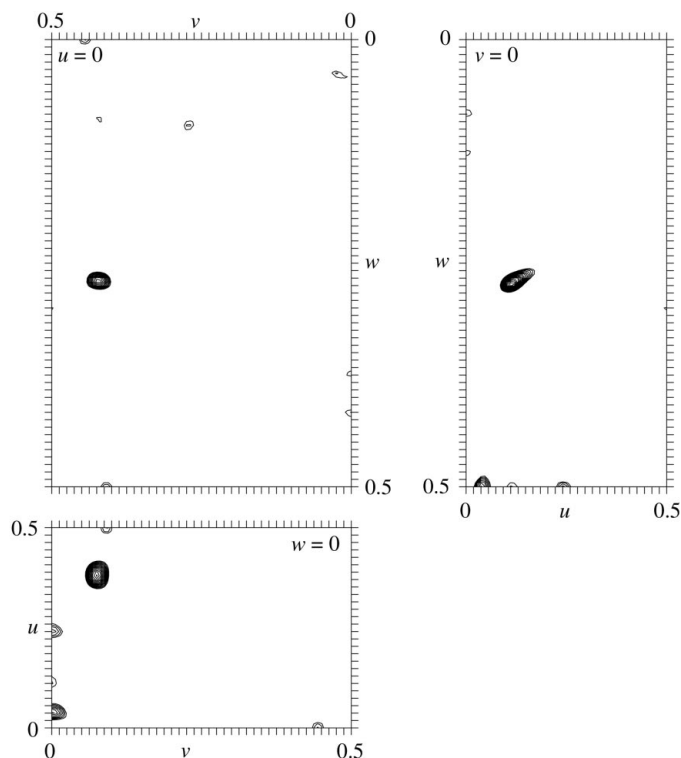


Figure 2

An iodine derivative. An isomorphous difference Patterson map was calculated using data between 15 and 5.5 Å and the Harker sections were plotted (contoured at a lower level of 4σ and at 0.5σ increments). The peaks correspond to a single iodine site, suggesting that there is a single molecule in the asymmetric unit. The relative positions of the peaks are consistent with space group *I*₂*1*₂*1*.

Table 1

Data-collection statistics.

Values in parentheses are for the last shell.

	Native	Iodine
Space group	<i>I</i> ₂ <i>1</i> ₂ <i>1</i>	<i>I</i> ₂ <i>1</i> ₂ <i>1</i>
Source	NE-CAT	Cu <i>K</i> α
Unit-cell parameters (Å)	<i>a</i> = 94.6, <i>b</i> = 141.0, <i>c</i> = 210.9	<i>a</i> = 94.6, <i>b</i> = 141.2, <i>c</i> = 211.3
Resolution limit (Å)	20.0–3.6 (3.73–3.6)	20–4.5 (4.66–4.5)
Observed reflections	61934	25486
Unique reflections	15937	8238
Completeness (%)	98.0 (99.7)	94.8 (97.8)
<i>R</i> _{sym} † (%)	3.8 (37.2)	7.1 (44.4)
<i>I</i> /σ(<i>I</i>)	34.7 (3.8)	26.4 (3.5)
Mosaic spread	0.816	0.377

$$\dagger R_{\text{sym}} = \frac{\sum_{hkl} |I(hkl)_{\text{obs}} - \langle I(hkl) \rangle|}{\sum_{hkl} I(hkl)_{\text{obs}}}$$

3. Results

We have obtained crystals of a group I ribozyme derived from a self-splicing intron from the bacteriophage Twort (Fig. 1*b*). Analysis of a dissolved crystal by denaturing PAGE indicates the crystals are composed of full-length ribozyme (not shown). The presence of the product analog was confirmed by ³²P labeling of material from a washed crystal (data not shown). The space group of these crystals appears to be *I*222 or *I*₂*1*₂*1*, with unit-cell parameters *a* = 94.6, *b* = 141.0, *c* = 210.9 Å. The Matthews coefficient *V*_M is calculated to be 4.4 Å³ Da⁻¹ if one molecule comprises the asymmetric unit. This value is similar to that observed for crystals of the *Tetrahymena* group I catalytic core (*V*_M = 4.9 Å³ Da⁻¹; Golden *et al.*, 1997) and seems to be typical for crystals of large RNAs.

A single iodine derivative was synthesized by cocrystallization of the ribozyme with an iodinated product analog. The difference Patterson map reveals strong peaks (~10σ) on the Harker sections (Fig. 2). These data resolve the space-group ambiguity, revealing it to be *I*₂*1*₂*1*, and the number of peaks in the map is consistent with a single molecule in the asymmetric unit. The quality of the derivative data indicated that the derivative is isomorphous and highly occupied. This result demonstrates the utility of covalent iodine modifications for structural analysis of large (80 kDa) RNAs.

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