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Crystallization and preliminary crystallographic study of triple-helical DNA

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Single crystals of d(CTCCT$_S$CCGCGCG)·d(CGCGCGGAG) have been grown by the vapor-diffusion method using 2-methyl-2,4-pentanediol as a precipitant. The crystals are tetragonal, space group $P4_2$, with unit-cell parameters $a = b = 53.8$, $c = 43.1$ Å, and diffract to 1.8 Å resolution at a synchrotron X-ray beamline. In the crystal, the asymmetric unit contains one copy of the construct. The two halves of the structure are related by non-crystallographic twofold symmetry. These observations are consistent with the conclusion that the sequences of the 12-mer and 9-mer oligonucleotides form a duplex DNA at one end and a triplex DNA at the other end.

1. Introduction

Formation of triple-stranded nucleic acid was first reported by Felsenfeld *et al.* (1957) using the 1:2 stoichiometric binding of $(rA)_n$:$(rU)_n$. Recent interest in DNA triplexes is growing owing to their possible biological roles and biochemical and therapeutic applications (Chan & Glazer, 1997). Subsequent biochemical studies have revealed details of the DNA triplex and led to the improved design of 'antisense' oligonucleotides.

Structural information on DNA triplexes in solution has been obtained from NMR studies where a single oligonucleotide forms intramolecular triplet base pairs (Radhakrishnan & Patel, 1994; Tarkoy *et al.*, 1998). In contrast to the earlier X-ray fiber diffraction studies, these NMR results show that the DNA triplexes are B-form DNA but differ from the canonical B-conformation. However, parallel structural information from X-ray crystallography is extremely limited, and to date no crystal structure of a DNA triple helix has been determined. Although distinct from the DNA triplex, crystal structures of the peptide–nucleic acid (PNA; Betts *et al.*, 1995) and of some mismatched triplet base pairs (Van Meervelt *et al.*, 1995; Vlieghe *et al.*, 1996) by crystal packing have been observed.

We have been attempting to crystallize a DNA triplex but our original crystals displayed only a fiber-type X-ray diffraction pattern (Liu *et al.*, 1994), perhaps because of disorder in the crystal packing. Therefore, sequences (Fig. 1) were designed so that they could form both double- and triple-helical portions and so that the duplex parts would be flexible enough to have intermolecular interactions that enhance crystal packing. In this paper, we report the crystallization and preliminary X-ray crystallographic analysis of the sequences above. Formation of the triplex under the crystallization conditions was confirmed in solution by measuring UV melting temperatures as a function of pH.

2. Synthesis and purification of oligonucleotides

Two oligonucleotides, a 12-mer d(CTCCT$_S$C-CGCGCG) and a 9-mer d(CGCGCGGAG), were synthesized on an ABI Model 392 automated DNA synthesizer using a solid-phase cyanoethylphosphoramidite method on a 10 μ*M* scale. Since preliminary X-ray diffraction studies show that replacing one of the two non-bridging phosphate O atoms between T5 and C6 in the 12-mer with a S atom results in better diffracting crystals, the C6 in the 12-mer was synthesized in the form of a phosphorothioate. Selective sulfurization at C6 was carried out manually by passing 4 ml of 1% sulfurizing reagent, 3*H*-1,2-benzodithiole-3-

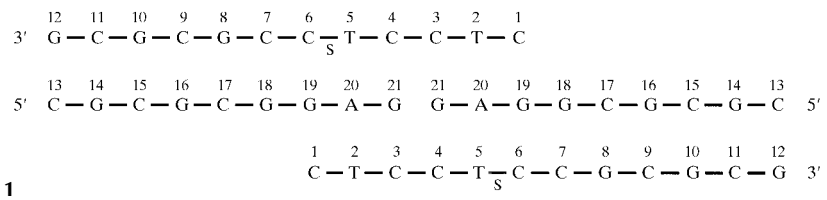

Figure 1
The sequences of oligonucleotides which form a triplex in the central part and duplexes at the two ends.

one-1,1-dioxide (Glen Research Corporation) in acetonitrile, through the column for 2 min (Lyer *et al.*, 1990). In addition, a brominated 12-mer, which was derivatized by replacing two thymine bases with bromouracil, was synthesized by directly incorporating 5-bromouracil bases for a multiwavelength anomolous dispersion (MAD) experiment. Standard synthetic procedures were used, except that the deprotection with ammonium hydroxide was carried out at room temperature for 24 h. The average coupling efficiency for the oligonucleotides ranged from 93 to 95% (dimethoxytrityl cation assay). The oligonucleotides were then purified on a 20% denaturing polyacrylamide gel containing 7.6 *M* urea, 0.09 *M* Tris/boric acid buffer pH 8.3 and 0.02 *M* EDTA. The main bands were extracted with triethylammonium bicarbonate buffer and desalted with Sep-Pak columns. Molar extinction coefficients were determined by phosphate analysis as described previously (Muraoka *et al.*, 1980). The molar extinction coefficients for the oligonucleotides are 9700 absorbance units (M^{-1} cm^{-1}) for the 9-mer, 8350 for the 12-mer and 8080 for the brominated 12-mer (measured at 254 nm for the first and 260 nm for the latter two).

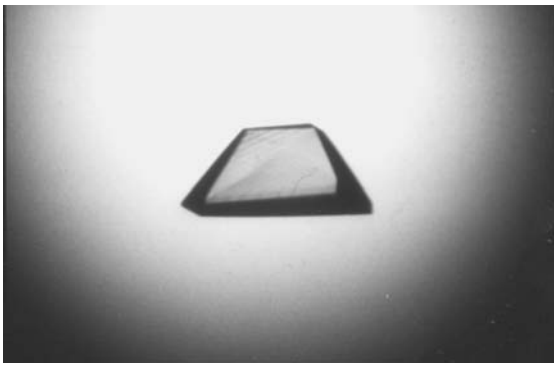

Figure 2
A crystal of DNA triplex with the sequence shown in Fig. 1.

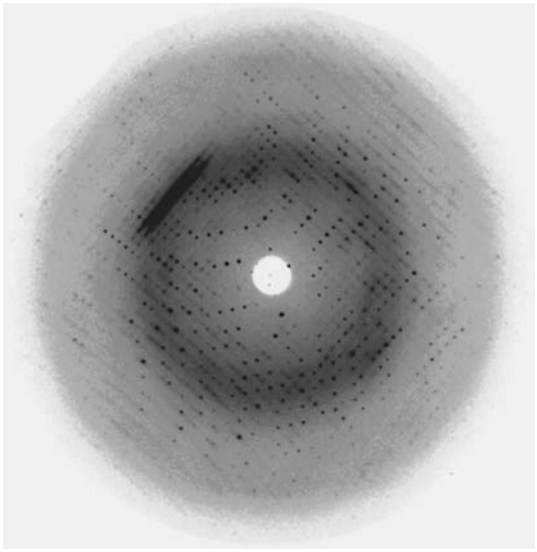

Figure 3
An oscillation photograph of a DNA triplex crystal. The diffraction pattern was obtained at 95 K on beamline X9B at the Brookhaven National Synchrotron Light Source.

3. Crystallization

Prior to crystallization, equimolar amount of both strands were mixed and the strand concentration was adjusted with water to 1.26 m*M*. The hanging-drop method was used for screening crystallization condition against various buffer solutions and salts. Typically, a small volume (2 µl) of triplex solution was mixed with an equal volume of solution containing various additives. Three or four drops with each additive solution were placed on the same siliconized cover slide and equilibrated at 293 K against a reservoir (800 µl) containing MPD and NaCl. The best crystals were grown with an additive solution containing 0.04 *M* sodium cacodylate (pH 5.04), 0.2 *M* NaCl, 0.1 *M* $MgCl_2$, 0.01 *M* spermine.4HCl, 12% MPD and a reservoir solution containing 47% MPD and 0.17 *M* NaCl. Crystals (Fig. 2) appeared after 4 d and grew to a maximum size of 0.3 × 0.25 × 0.25 mm within two weeks. Measurement of UV melting curves of oligonucleotides in solution indicated that the triple helix would be intact under the crystallization conditions.

4. X-ray diffraction experiments and analysis

The crystals were flash-frozen in liquid propane and stored in liquid nitrogen for data collection. Initial X-ray data to 2.2 Å resolution were collected at 95 K on a R-AXIS IIc imaging-plate system mounted on a Rigaku RU-200 rotating-anode X-ray generator operating at 50 kV and 100 mA. All diffraction data were integrated with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). The crystals are tetragonal with unit-cell dimensions $a = b = 53.8$, $c = 43.1$ Å. Analysis of data, including systematic absences, suggested that the space group is $P4_2$ with a dimer of the duplex in the asymmetric unit. These observations are consistent with our solution studies showing that the 5′-terminal CTC bases of the 12-mer would form triplet base pairs with the complementary bases of the 9-mer in another duplex.

Two thymine bases in the 12-mer were replaced with bromouracil bases for a multiwavelength anomolous dispersion (MAD) experiment. MAD data were collected to 1.8 Å resolution at beamline X9B at the Brookhaven National Synchrotron Light Source with three different wavelengths near or at the *K* absorption edge of bromine (Fig. 3). Various difference Patterson maps show four bromine sites per asymmetric unit, as we expect. Structure determination has been described elsewhere (Rhee *et al.*, 1999).

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