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Crystallization and X-ray diffraction analysis of an 'all-locked' nucleic acid duplex derived from a tRNA^{Ser} microhelix

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Modified nucleic acids are of great interest with respect to their nuclease resistance and enhanced thermostability. In therapeutical and diagnostic applications, such molecules can substitute for labile natural nucleic acids that are targeted against particular diseases or applied in gene therapy. The so-called 'locked nucleic acids' contain modified sugar moieties such as 2′-*O*,4′-*C*-methylene-bridged β-D-ribofuranose and are known to be very stable nucleic acid derivatives. The structure of locked nucleic acids in single or multiple LNA-substituted natural nucleic acids and in LNA–DNA or LNA–RNA heteroduplexes has been well investigated, but the X-ray structure of an 'all-locked' nucleic acid double helix has not been described to date. Here, the crystallization and X-ray diffraction data analysis of an 'all-locked' nucleic acid helix, which was designed as an LNA originating from a tRNA^{Ser} microhelix RNA structure, is presented. The crystals belonged to space group *C*2, with unit-cell parameters a = 77.91, b = 40.74, c = 30.06 Å, β = 91.02°. A high-resolution and a low-resolution data set were recorded, with the high-resolution data showing diffraction to 1.9 Å resolution. The crystals contained two double helices per asymmetric unit, with a Matthews coefficient of 2.48 Å^3 Da^{-1} and a solvent content of 66.49% for the merged data.

1. Introduction

The stabilization of nucleic acids by introducing modified nucleotides is an ongoing subject in therapeutic and diagnostic applications, as natural macromolecules such as RNA and DNA are labile towards nuclease digestion and have low thermal stability. 'Locked' nucleic acids (LNAs) and the related LNA families contain modified sugar moieties such as 2′-*O*,4′-*C*-methylene cross-linked β-D-ribofuranose, in contrast to the naturally occurring ribose/deoxyribose in RNA/DNA. LNAs were first synthesized by the groups of T. Imanishi (Obika *et al.*, 1997) and J. Wengel (Kumar *et al.*, 1998) and they bind to complementary RNA or DNA *via* standard Watson–Crick base pairing (Vester & Wengel, 2004).

It has been well documented that LNAs show a greatly increased thermostability in comparison to other modified nucleic acids. An example of a comparative study of different modifications in nucleic acids with respect to their stability has been reported for the tenascin C-binding aptamer TTA-1 after substitution with different modified nucleic acid blocks (Schmidt *et al.*, 2004). The *in vitro* thermostability was described to be in the following order using different common modifications of nucleic acids: 2′-F/2′-OMe < RNA/RNA ≤ 2′-OMe/2′-OMe < 2′-F/LNA < RNA/LNA = LNA/RNA < 2′-OMe/LNA < LNA/LNA. An explanation of the enhanced stability of LNAs has been proposed by the research groups of P. Jacobsen and J. Wengel (Petersen *et al.*, 2000) based on the investigation of an LNA–DNA duplex structure: the β-D-ribofuranose LNA is 'locked' in the 3′-*endo* conformation. This directs the phosphate backbone into a conformation with a decreased loss of entropy upon helix formation, in which the duplex favours a more efficient stacking of the nucleobases. This implicates a loss in enthalpy upon helix formation. The authors report that the formation of an LNA–DNA duplex is favoured by both enthalpy and entropy.

The structure and conformation of locked nucleic acids has been well investigated and has been analyzed extensively using single or multiple LNA-substituted natural nucleic acids or heteroduplexes such as LNA–DNA or LNA–RNA complexes. Structural studies using β-D- or α-L-LNA/DNA mix-mers hybridized to RNA or DNA showed the following. β-D-LNA (LNA/DNA mix-mer)–RNA duplexes adopt the A-type conformation, whereas α-L-LNA (LNA/DNA mix-mer)–DNA helices adopt the B-type conformation (Petersen *et al.*, 2002; Vester & Wengel, 2004). Investigations using fully modified LNA strands associated with RNA or DNA forming a heteroduplex showed that the β-D-ribofuranose LNA binds to RNA adopting the A-RNA conformation and the binding of β-D-ribofuranose to DNA induces a mixed N- and S-type sugar puckering (Nielsen *et al.*, 2004), whereas the α-L-ribofuranose LNA binds to DNA in a B-DNA type conformation (Nielsen *et al.*, 2002). In summary, the β-D-ribofuranose LNA substitutions induce an A-type nucleic acid conformation and the 'locked' 3′-*endo* conformation seems to increase the thermostability of the duplex to a great extent.

An understanding of the LNA tertiary structure is of great interest in order to explain the enhanced thermostability of these modified nucleic acids. The application of nucleic acids in diagnostic and therapeutic medicine, in gene therapy and in drug design is an ongoing research field and opens a broad field of new medications. Nevertheless, the nuclease-sensitivity and the low stability of nucleic acids is a great problem which often prevents successful applications. Approaches involving the application of modified nucleic acids which possess enhanced thermostability and nuclease resistance will facilitate the use of such molecules in therapy and diagnostics. LNAs are known to be very stable nucleic acids and the structure of an 'all-locked' nucleic acid duplex will provide insight into the detailed local geometric parameters, which may help in finding further explanations for their increased stability. LNAs have a great potential for use in drug development and for application in diagnostics and therapy (Kaur *et al.*, 2007; Petersen & Wengel, 2003).

To our knowledge, the structure of an 'all-locked' nucleic acid homoduplex has not yet been described. The question of how the conformation of the modified LNA sugar influences the phosphate backbone and the stacking of base pairs in a completely 'all-locked' β-D-ribofuranose nucleic acid led us to the idea of undertaking a comparative X-ray structure analysis. We have recently solved the 1.2 Å resolution crystal structure of an *Escherichia coli* $tRNA^{Ser}$ microhelix, which resembles the aminoacyl stem of the tRNA (Eichert *et al.*, 2009). We therefore decided to focus our interest on crystallizing an 'all-locked' LNA duplex with a sequence corresponding to this RNA. We designed the helix as a completely 'all-locked' nucleic acid by maintaining the base sequence of the RNA. Here, we present the crystallization and preliminary X-ray diffraction analysis of the LNA homoduplex. These data could lead to the first X-ray structure of an 'all-locked' nucleic acid duplex which can be directly compared with the corresponding RNA structure.

2. Materials and methods

2.1. Crystallization of the 'all-locked' LNA $tRNA^{Ser}$ microhelix

The sequence of the 7-mer LNA helix was derived from the *E. coli* $tRNA^{Ser}$ aminoacyl stem microhelix which has been crystallized previously (Eichert *et al.*, 2009) and originated from the tRNA isoacceptor with database ID RS 1661 (Sprinzl & Vassilenko, 2005). The LNA was designed to contain exclusively locked nucleic acid building blocks by maintaining the base sequence of the RNA for further comparative studies, except for the U to T exchange in standard LNA synthesis. Since we have previously evaluated the quality of commercially available LNAs in functional studies of aptamers (Schmidt *et al.*, 2004) and crystallization experiments (Förster *et al.*, 2006), we again employed locked oligonucleotides from commercial sources for the present study. Chemically synthesized single strands with sequences 5′-LNG-LNG-LNT-LNG-LNA-LNG-LNG-3′ and 5′-LNC-LNC-LNT-LNC-LNA-LNC-LNC-3′ were purchased from IBA (Göttingen, Germany) at HPLC purification grade. No further purification was undertaken as we routinely crystallize chemically synthesized oligonucleotides after HPLC purification. For hybridization, both LNA single strands were annealed in distilled water, heated to 363 K and subsequently cooled to room temperature within 3–4 h. The resulting LNA duplex was concentrated in a speed vac (SpeedVac SC 110, Savant, Minnesota, USA) to a final concentration of 0.5 m*M*. This sample was used for all subsequent crystallization setups.

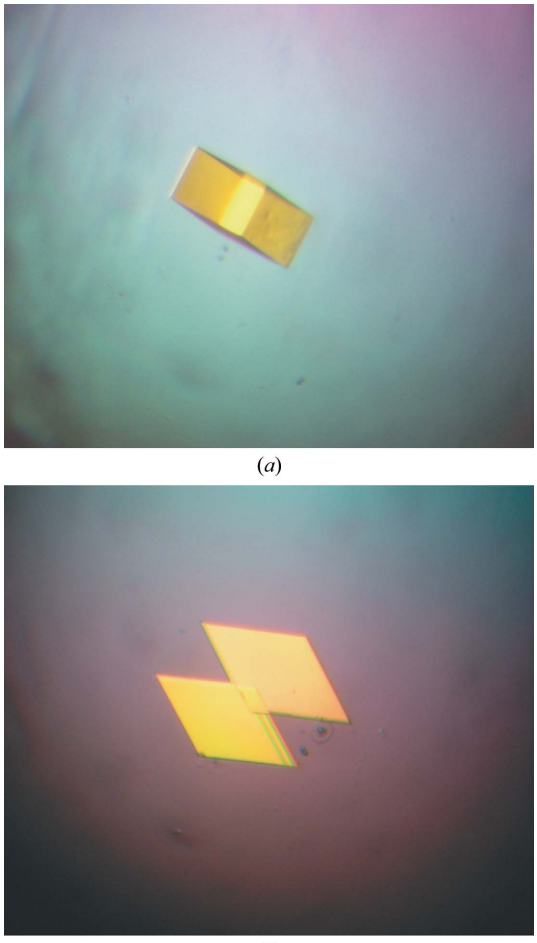
For the initial crystallization screening experiments, we used two different crystallization kits. The first was the Natrix Nucleic Acid Crystallization Kit (HR2-116; Hampton Research, California, USA) consisting of 48 different conditions. Within this screen, each solution was used in a 100 µl reservoir well and 1 µl of the reservoir solution was added to the LNA in the droplet for crystallization experiments as follows. Setups were prepared at 294 K using the sitting-drop vapour-diffusion technique with CrystalQuick Lp plates from Greiner Bio-One (Germany). 1 µl of a 0.5 m*M* unbuffered solution of LNA in distilled water was mixed with 1 µl reservoir solution and equilibrated against 100 µl reservoir solution; the plates were directly covered with a VIEWseal foil (Greiner Bio-One, Germany). As a second screen, we used the Nucleic Acid Mini Screen from Hampton Research (HR2-118; Hampton Research, California, USA) with 24 different conditions using Linbro plates (ICN Biomedicals Inc., Ohio, USA). In contrast to the first screen, 40%(*v*/*v*) MPD (2-methyl-2,4-pentanediol) in distilled water pH 7.4 was used as the reservoir solution in all setups. Here, the hanging-drop vapour-diffusion technique was applied, with the crystal droplets hanging on cover slides. For crystallization trials, 1 µl of a 0.5 m*M* unbuffered solution of LNA in distilled water was used and combined with 1 µl crystallization solution from the screen. Equilibration took place at 294 K against 1 ml 40%(*v*/*v*) MPD in distilled water pH 7.4 as described above.

Crystals appeared after 3–4 d using the following conditions from the second crystallization screen: 40 m*M* sodium cacodylate pH 5.5, 20 m*M* cobalt hexammine, 80 m*M* sodium chloride, 20 m*M* magnesium chloride, 10%(*v*/*v*) MPD with equilibration against 35%(*v*/*v*) MPD in distilled water pH 7.4. Optimization of crystal growth was performed by variation of the aqueous MPD concentration in the reservoir between 30 and 42%. The best crystals appeared within the range 33–41%(*v*/*v*) MPD in Linbro plates using the hanging-drop vapour-diffusion technique.

2.2. Acquisition of X-ray diffraction data and data-processing statistics

Following our previous experience with nucleic acid crystals grown in MPD, the crystals were directly flash-frozen in the crystallization solution. In the presence of roughly 20%(*v*/*v*) MPD, which is the estimated concentration in the droplet after equilibration, no further cryoprotectant reagent was needed. X-ray diffraction data were recorded at the Elettra Synchrotron (Trieste, Italy) on beamline XRD1 at a wavelength of 1.000 Å. Two data sets were collected: a high-resolution data set in the resolution range 80.0–1.90 Å and a subsequent low-resolution data set from 80.0 to 2.70 Å resolution. The crystallographic data were processed and merged using the programs

DENZO and *SCALEPACK* from the *HKL*-2000 package (Otwinowski & Minor, 1997). The Matthews coefficient and solvent content was calculated according to Matthews (1968). Molecular-replacement calculations were calculated with the program *Phaser* (McCoy *et al.*, 2005) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) using different nucleic acids as models, such as an artificially constructed LNA model that was built of LNA building blocks which correspond to the sequence of the LNA helix or the natural tRNA^{Ser} microhelix.



(*a*)

(*b*)

Figure 1
Representative crystals of the 'all-locked' LNA duplex with the base sequence originating from the *E. coli* tRNA^{Ser} microhelix. The crystals show approximate dimensions of 0.2 × 0.2 × 0.1–0.05 mm. Pictures of two different setups are shown, with equilibration using 33%(*v*/*v*) MPD (*a*) or 35%(*v*/*v*) MPD (*b*). Details are described in the text.

3. Results and discussion

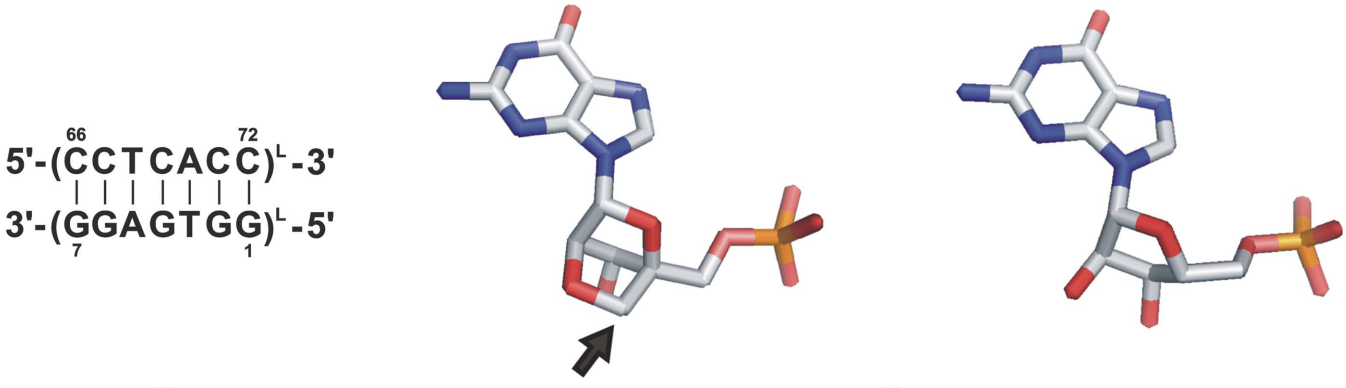
3.1. Crystallization

We focused on crystallizing a locked nucleic acid duplex containing exclusively LNA nucleotides derived from a tRNA^{Ser} microhelix, the structure of which we have solved recently (Eichert *et al.*, 2009), in order to perform a comparative structure analysis between LNA and RNA. The LNA 7-mer duplex, with a base sequence corresponding to that of the *E. coli* tRNA^{Ser} microhelix isoacceptor RS 1661 (Sprinzl & Vassilenko, 2005), crystallized in 40 m*M* sodium cacodylate pH 5.5, 20 m*M* cobalt hexammine, 80 m*M* sodium chloride, 20 m*M* magnesium chloride, 10%(*v*/*v*) MPD with equilibration against various concentrations of aqueous MPD. The crystal used in the measurement was equilibrated against 40%(*v*/*v*) MPD. Representative crystals had approximate dimensions of 0.2 × 0.2 × 0.1–0.05 mm and are shown in Fig. 1.

3.2. Crystallographic data

The 'all-LNA' duplex (Fig. 2) crystallized in space group *C*2 with two helices per asymmetric unit. We collected two data sets. The high-resolution data contained diffraction data to 1.9 Å resolution with high completeness and a low *R* value. The low-resolution data were collected from 80 to 2.7 Å resolution. The data sets were merged and the following crystallographic statistics were calculated (Table 1). The unit-cell parameters were $a = 77.91$, $b = 40.74$, $c = 30.06$ Å, $\beta = 91.02^{\circ}$, with a Matthews coefficient of 2.48 Å^3 Da^{-1}, which corresponds to a solvent content of 66.5% and two LNA duplexes per asymmetric unit. The overall R_{merge} value was 7.3% (21.7% for the last resolution shell; 1.93–1.90 Å) and the overall completeness was 98.0% (97.2% for the highest resolution shell). The structure was solved by molecular replacement and we are presently running refinement calculations.

By determining the structure of an 'all-locked' β-D-ribofuranose LNA homoduplex, we wish to contribute to a more detailed understanding of the increased thermostability of locked nucleic acids. Such an understanding will enhance specific drug design and the application of these molecules in diagnostics and gene therapy. The



(*a*) (*b*)

Figure 2
(*a*) The aminoacyl stem from *E. coli* tRNA^{Ser} was used for the design of an 'all-locked' nucleic acid which was crystallized in this study. The sequence is presented in the figure and the numbering corresponds to the tRNA^{Ser} acceptor stem taken from the tRNA database (Sprinzl & Vassilenko, 2005). (*b*) A guanosine nucleotide is shown as an LNA with the 2′-*O*,4′-*C* methylene-bridged β-D-ribofuranose (indicated by an arrow; left) and shown as RNA containing the natural ribose with the 2′,3′-*cis*-diol group (right).

Table 1
X-ray diffraction data and processing statistics for the 'all-LNA' tRNA^{Ser} microhelix crystal.

A high-resolution and a low-resolution data set were recorded and the data were merged; details are described in the text. The statistics of the merged data are shown in the table. Values in parentheses are for the highest resolution shell.

Beamline	XRD1, Elettra
Wavelength (Å)	1.000
Space group	*C*2
Unit-cell parameters (Å, °)	a = 77.91, b = 40.74, c = 30.06, β = 91.02
V_M (Å^3 Da^{-1})	2.48
Duplexes per ASU	2
Solvent content (%)	66.5
Measured reflections	35694
Unique reflections	7382
Resolution range (Å)	80.0–1.90 (1.93–1.90)
Completeness (%)	98.0 (97.2)
Multiplicity	4.8 (3.8)
R_{merge}† (%)	7.3 (21.7)
Average $I/\sigma(I)$	19.7 (1.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed individual and mean intensities of a reflection with indices hkl, respectively, $\sum_i$ is the sum over the individual measurements of a reflection with indices hkl and $\sum_{hkl}$ is the sum over all reflections.

tRNA^{Ser} microhelix serves as a model structure for the investigation of the detailed local geometric parameters of an 'all-locked' nucleic acid compared with those of the natural RNA, as we have already solved the corresponding RNA structure (Eichert *et al.*, 2009). A comparative study between the natural 'all-RNA' duplex and the modified 'all-LNA' duplex should provide new insights into LNA conformation and stability, as to our knowledge this will be the first X-ray structure of an 'all-locked' nucleic acid helix. These extremely stable modified nucleic acids have potential for use in medical applications and gene therapy in the future (Kaur *et al.*, 2007; Petersen & Wengel, 2003).

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