Most compact hairpin-turn structure exerted by a short DNA fragment, d(GCGAAGC) in solution: an extraordinarily stable structure resistant to nucleases and heat

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ABSTRACT

The three-dimensional structure of a short DNA fragment, d(GCGAAGC) exhibiting an extraordinarily stable hairpin structure was determined by nuclear magnetic resonance spectroscopy. Two possible models were obtained by molecular modelling using distance and torsion constraints. Only one of these two models is the correct structure, which can clearly explain all the ¹H chemical shifts. d(GCGAAGC) is folded back on itself between A₄ and A₅, and all the sugars in the fragment adopt the C2'-endo conformation. This compact molecule is stabilized by regular extensive base-stacking interaction within each B-form helical strand of G₁C₂G₃A₄ and A₅G₆C₇, and by two G-C and one G₃-A₅ base pairs. The molecule is hard to differentiate into stem and loop regions, so that we classify it as a turn (*hairpin-turn*) structure exerted by a single-stranded DNA. This highly stacked structure shows high thermostability and strong resistance against nucleases contained in *E.coli* extracts and in human serum.

INTRODUCTION

Hairpin structures in DNA and RNA consisting of stem and loop regions occur naturally not only in single-stranded DNAs and RNAs but also in double-stranded DNAs and they are considered to have important biological functions. In particular, the structures and biological functions of unusually stable hairpins have been studied, in which tetra-nucleotide loops (tetra-loops), such as d(GAAA) (1), d(GTTA) (2), r(UNCG) (3–5) and r(GNRA) (6) (where N is any nucleotide and R is a purine nucleotide) are included. These structures are found in ribosomal RNAs (4, 6), hammerhead ribozymes (7), group I catalytic introns (8), RNA coliphages (9), bacteriophage T4 mRNA (3), and the replication origins of single-stranded phage DNAs (10).

In recent years, we found that a series of short DNA fragments forms an extraordinarily stable hairpin structure containing only

two G-C pairs (11). Of these fragments, d(GCGAAGC) is the shortest fragment and forms the most stable structure containing a tri-nucleotide (GAA) loop, whose melting temperature is as high as 76°C in 0.1M NaCl. The d(GCGAAGC) hairpin is much more stable than the corresponding r(GCGAAGC) hairpin ($T_m = 27 \pm 5^\circ\text{C}$ in 0.1M NaCl), although another tetra-loop DNA hairpin containing a TTCG loop is less stable than the corresponding RNA hairpin containing a UUCG loop (12). Tri-loop as well as tetra-loop hairpins occur frequently in natural DNAs and RNAs. For example, the GCGAAGC sequence has been found in replication origins of phage ϕ X174 (13) and herpes simplex virus 1 (14), in a promoter region of an *E.coli* heat-shock gene (15), and in rRNA genes. Nevertheless, there have been few structural analyses on hairpins containing tri-nucleotide loops (16–18).

Here we report a solution structure of the d(GCGAAGC) fragment determined by NMR spectroscopy, and its thermal properties as well as its modified DNA fragments. The structure was compared to those of other DNA and RNA hairpins.

On the other hand, thermally stable hairpins show high resistance against nucleases. By utilizing this property, oligonucleotides could be stabilized by adding the hairpins at their termini (19–21). We also report high resistance of the d(GCGAAGC) fragment against nucleases contained in *E.coli* extracts and in human serum.

MATERIALS AND METHODS

The oligodeoxynucleotides were synthesized on a DNA synthesizer (Applied Biosystems, 391) using standard phosphoramidite chemistry. 7-Deaza-adenosine and 7-deaza-guanosine phosphoramidite derivatives were purchased from Glen Research. The DNA fragments were purified by high-performance liquid chromatography using a reverse-phase C-18 column (SynChropak RP-P, 250×4.6mm ID) with an acetonitrile/0.1M triethylammonium acetate (pH 7.0) solvent system.

For NMR experiments, the d(GCGAAGC) fragment was treated with Dowex cation exchange resin (pyridinium form), and then with the resin (Na⁺ form) to yield the sodium salt. For all non-exchangeable proton experiments, about 1.7 μ mol of the oligomer was dissolved in 99.9 % D₂O, evaporated, and dissolved in 99.98 % D₂O. Its final concentration was 5.7mM in a buffer containing 10mM sodium phosphate and 0.15M NaCl, pD 7.0.

All the NMR experiments were performed on a Bruker AMX-500 spectrometer (500 MHz for ¹H and 202 MHz for ³¹P) at a probe temperature of 25°C. For one dimensional experiments, free induction decays (FIDs) of 16K data points were accumulated and spectra of 32K real data points were obtained. Before Fourier transformation, a line broadening of 0.2 or 2.0 Hz was applied for ¹H or ³¹P experiments, respectively. Spectral width was 6024 or 3048 Hz for the ¹H or ³¹P experiments, respectively. For two dimensional experiments, 512 FIDs of 2K data points were accumulated with the time proportional phase incrementation for phase sensitive detection of the t1 dimension (22), and spectra of 2K $\times$ 1K real data points were obtained. Before two-dimensional Fourier transformation, a $\pi/2$ -shifted sine-bell window function was applied for each dimension. We performed NOESY experiments (22) with mixing times of 100 and 200 ms, a HOHAHA experiment (23) with a mixing time of 203 ms, a DQF-COSY experiment (22, 24) with ³¹P decoupling for both dimensions. A ³¹P-¹H COSY experiment (25) was carried out without presaturation of ¹H resonances.

For gel-mobility analysis, each oligomer was labeled at the 5'-terminus with [γ -³²P]ATP (Amersham) using T4 polynucleotide kinase (Boehringer), and directly electrophoresed on 20% polyacrylamide gel containing 7M urea, 0.045M Tris-borate and 1mM EDTA (pH 8.0) at 30 or 70°C (11).

The melting profiles were obtained at 260 nm with a Gilford spectrophotometer, Response II, using 1 A₂₆₀U/ml of oligomer in 50mM sodium cacodylate and 0.1M NaCl (pH 7.0). Tm's were calculated by the first derivatives of the melting curves.

Assessment of oligomers' stability against nucleases was performed by the followed procedure. *E. coli* Q13 ribosomes and S100 fractions were prepared according to the published procedure (26), and the ribosomes were treated with bentonite (19, 27). Human serum was purchased from Miles Inc. Each ³²P-labeled oligomer (approximately 35 pmol) was incubated with *E. coli* 70S ribosomes (0.1 pmol) and S100 fractions (0.001 OD₂₆₀ unit = 1 μ g) in 50mM Tris-HCl (pH 7.8), 50nM KCl, 12.5mM Mg(OAc)₂, and 6mM 2-mercaptoethanol at 37°C for 70 min, or with 5 μ l of human serum at 37°C for 90 min. The mixture was added to the same volume of a dye solution containing 10M urea and electrophoresed on 20% polyacrylamide gel electrophoresis containing 7M urea, 0.045M Tris-borate (pH 8.0) and 1mM EDTA at 70°C. The result was analyzed with a Bio-imaging analyzer BAS2000 (Fuji Film).

RESULTS AND DISCUSSION

NMR analysis

All of the non-exchangeable protons of d(GCGAAGC) were assigned by conventional methods (28) using DQF-COSY (22), HOHAHA (23) and NOESY (22) spectra. The assignments of H3', H4' and H5'/H5'' protons were confirmed by a ³¹P-¹H COSY experiment (25). Table 1 lists the assigned ¹H and ³¹P resonances for the oligomer. The A₄H4' signal was observed as

Table 1. ¹H and ³¹P chemical shifts of the non-exchangeable proton of the d(GCGAAGC) hairpin

	H1'	H2'	H2''	H3'	H4'	H5',5''	H8/H6	H2/H5	P
G1	6.00	2.55	2.73	4.86	4.25	3.77	7.98		-4.27
C2	6.09	1.68	2.36	4.85	4.28	4.11	7.06	5.07	-4.83
G3	5.42	2.71	2.59	4.95	4.50	4.14	8.12		-4.55
A4	6.03	2.36	2.31	4.64	2.19	3.14	3.45	8.13	-4.69
A5	6.35	2.95		4.90	4.40	3.87	4.05	8.18	-4.99
G6	5.61	2.63		4.96	4.44	4.22	4.35	8.05	-3.76
C7	6.31	2.20	2.32	4.50	4.17	4.11	4.28	5.39	

Conditions are 0.15M NaCl and 10mM sodium phosphate, pD 7.0, 25°C proton shifts are measured relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate, phosphorus shifts are relative to trimethyl phosphate.

Figure 1. A portion of the NOESY (28) spectrum showing the connectivity between sugar proton H2'/H2'' and aromatic proton H8/H6 resonances of the d(GCGAAGC) fragment.

far up-field as 2 ppm as compared with H4' signals of other residues, indicating that the proton is located close to a base moiety, so that a ring current shift occurs.

Fig. 1 shows the sequential connectivity through NOE's between H8/H6 and H2'/H2''. The strong H8/H6-H2'-H2'' NOE cross-peaks could be connected along the G₁C₂G₃A₄ and A₅G₆C₇ moieties in the fragment, but no NOE's were observed between the A₄H2'/H2'' and A₅H8 residues.

The coupling constants $J_{H1',H2'}$ and $J_{H1',H2''}$ show that all residues take the C2'-endo sugar pucker conformation. The coupling constants $J_{H5',P}$, $J_{H5'',P}$ and $J_{H4',P}$ show that the conformations around the C5'-O5' bond for residues except for A₅ adopt the *trans* form. For residue A₅, $J_{H5'',P}$ was as large as 20 Hz, indicating that the torsion angle of H5''-C5'-O5'-P is around 180° (29) and, therefore, the conformation around the C5'-O5' bond adopt the *gauche*⁺ form. Only for A₅ could the coupling constant $J_{H4',P}$ not observed, which also indicates that the conformation around the C5'-O5' bond does not adopt the

Figure 2. Model A and B of the d(GCGAAGC) fragment derived from molecular modeling. The views from the major groove (a) and the left side views (b) are shown. The adenine base of A₅ in both model is colored red, and the H4' proton of A₄ is colored cyan. The sharp turn region is colored green.

trans form. By the phosphate decoupled DQF-COSY spectrum, the coupling constant $J_{H4',H5''}$ of A₄ was found to be as large as 10 Hz, indicating that the conformation of H4'-C4'-C5'-H5'' is *trans* and, therefore, the conformation of C3'-C4'-C5'-O5' is also *trans*.

At least one exchangeable proton was detected at 13.04 ppm as a broad signal in the ¹H NMR spectrum recorded in 90% H₂O at 25°C. This corresponds to the imino proton signal of a G residue involved in a G-C base pair.

These results suggest that the fragment is folded back between A₄ and A₅, and each of the G₁C₂G₃A₄ and A₅G₆C₇ moieties forms a highly homogeneous helical structure like that of B-form DNA.

Structural determination

Determination of the structure of the fragment was started from a single-stranded DNA-B type linear structure; a series of simulated annealing calculations was performed using the

Figure 3. a. Stereoviews of the final structure of the d(GCGAAGC) fragment. The view from the major groove is shown. G₃ and A₅ which form a non-Watson-Crick G₃-A₅ base pair are colored red. The sharp turn region is colored green. **b.** Stick model of the structure. G₁C₂G₃A₄ moiety is colored blue and A₅G₆C₇ moiety is green.

program X-PLOR (30) with 44 intra- and 26 inter-residue distance constraints derived from NOEs, 6 distance constraints for fixing the G₁-C₇ and C₂-G₆ base pairs, and 37 torsion constraints, in which 21 were for sugar pucker, 6 for rotation around the O5'-C5' bond, one for rotation around the C4'-C5' bond of A₅, and 9 for fixing the B-DNA structure of the G₁C₂-G₆C₇ stem.

The distance of each proton pair was calculated from the corresponding cross-peak volume in the NOESY spectrum by the relation; $r_{ij} / r_{ref} = (\text{Volume}_{ref} / \text{Volume}_{ij})^{-6}$, where we choose the distance between H5 and H6 protons of cytidines as the reference. In structure determination calculations, the distances were treated with a range of $\pm 0.5 \text{ \AA}$, because NOEs

Figure 4. a. Schematic diagram of the d(GCGAAGC) hairpin structure (open circles, C2'-endo sugar; boxes, base with anti conformation; boldface box, A₅ base wobbling around the anti- and high-anti conformations; stippled circles, phosphate; dotted lines, hydrogen bond; black boxes, stacking). b. Schematic representation of the G₃-A₅ base pair in the fragment.

may contain indirect effects. On the other hand, in the calculations, the dihedral angles were treated with a range of ± 30 degree.

Two groups, consisting of 40 and 23 structures, which satisfied all the constraints were obtained from 100 trial structures; the root mean square deviations of the non-hydrogen atom coordinates within each group were 1.24 and 1.13Å, respectively. There was none in the remaining 37 structures, which satisfied all NMR constraints. The final structure of each group was obtained by molecular dynamics and energy minimization calculations from each average structure with the program AMBER (31). In molecular dynamics calculation, constraints similar to those used in X-PLOR calculation were used. Fig. 2 shows two types of models A and B of the final structures of the d(G₁C₂G₃A₄A₅G₆C₇) fragment.

For the structure belonging to the 40-structure group (model A, Fig. 2), the A₅ base is stacked with the G₆ base and a G₃-A₅ base pair is formed as shown in Fig. 4b. In contrast, for the structure belonging to the 23-structure group (model B, Fig. 2), the adenine base of A₅ is located in the major groove.

Judging from the fact that the resonance of the H4' of A₄ was observed at more than 2 ppm up-field compared to those of other H4' protons (Table 1), model A appears to be reasonable. As shown in Fig. 2, the H4' of A₄ in model A is located close to the adenine base of A₅ and the large up-field shift can be explained by the ring current on the base. However, model B cannot explain the large up-field shift, because H4' is located too far from any nucleotide base (Fig. 2).

As a result of the final minimization calculation with the program AMBER without NMR constraints, serious distance

Figure 5. Electrophoretic patterns of ³²P-labeled d(GCGAAGC), its 7-deaza-replaced derivatives and d(CGACGAG) fragments in 20% polyacrylamide gel containing 7M urea at 30°C. The T_m values of these fragments are also shown. No melting temperature of d(CGACGAG) was observed from 20–100°C. The bases marked with asterisks were replaced with the corresponding 7-deaza derivatives.

violations of A₅H1'-G₆H5'/5" and A₅H8-A₅H2'/2" occurred in model B. Thus, we judged this model B to be an artifact of calculation.

The C₂H2' signal was also found as far up-field as 1.68 ppm. The more than 0.5 ppm up-field shift is explained by the ring current on the G₃ base in both models A and B, because C₂H2' is spatially located just under the G₃ base.

We obtained evidence for the existence of the G₃-A₅ base pair (Fig. 4b) from the gel-mobility shift assay and thermal properties of the modified d(CGAAGC) fragments in which adenine or guanine was replaced by 7-deazaadenine or 7-deazaguanine, respectively. An extraordinarily stable hairpin shows a higher mobility in polyacrylamide gel electrophoresis, even in a gel containing 7M urea, due to its compactly folded structure (11). As shown in Fig. 5, only the replacement of the A₅ base with 7-deazaadenine changes the mobility of the fragments to the normal mobility which a single-stranded DNA fragment, such as d(CGACGAG), exhibits. Thus, the N7 of A₅ is important for its folding. The T_m's of the fragments containing the modified A₅ base also showed a reduction by 12–13°C (see Fig. 5).

Structural analysis

The characteristics of the final structure (model A) are shown in Fig. 3 and schematically in Fig. 4. The final structure is composed of two B-form strands, G₁C₂G₃A₄ and A₅G₆C₇, with two G-C and one G-A pairs bound to each strand. As shown in Fig. 4b, the mode of hydrogen bonding in the G-A pair is similar to that found in a stable RNA hairpin containing a G(A/C)AA loop (6) and in a DNA duplex (32, 33), but is different from that found in another stable DNA hairpin containing a GTTA loop (2).

Only the three torsion angles between A₄ and A₅ residues, C3'-O3'-P-O5' (*trans*), P-O5'-C5'-C4' (*gauche*⁺) and O5'-C5'-C4'-C3' (*trans*), are different from those of the typical B-form structure. Thus, the sharp turn is feasible by the rotation

Figure 6. ^1H NMR spectra in the H1'/H5 region of d(GCGAAGC) at 25°C (a), 35°C (b), 45°C (c), 55°C (d), and 65°C (e) in D_2O containing 0.15M NaCl and 10mM sodium phosphate buffer (pD 7.0).

around these three bonds of $\text{A}_4\text{O}3'\text{-P}$, $\text{A}_5\text{O}5'\text{-C}5'$ and $\text{A}_5\text{C}5'\text{-C}4'$ (Fig. 3).

Almost all nucleotides take an anti-glycosidic angle conformation. However, the strong NOE's observed between $\text{A}_5\text{H}8$ and $\text{A}_5\text{H}1'/2'/2''$ suggested that the A_5 base does not adopt any single conformation and probably wobbles around anti- and high anti-conformations. We noted that model A and model B are not interconvertible and, thus, this ' A_5 base wobbling' does not mean that model A is exchanged with model B.

Whereas H1' sugar protons of G_1 , C_2 , G_6 and C_7 gave sharp peaks, those of G_3 , A_4 and A_5 gave broad bands at 25°C as shown in Fig. 6, probably because of a small structural fluctuation in the loop region caused by wobbling of the A_5 base. This possibility was confirmed by the fact that these broad peaks sharpened at 45°C (Fig. 6).

Another DNA hairpin containing a TTCG loop is less stable than the corresponding RNA hairpin containing a UUCG loop, because the loop in the DNA hairpin reveals no specific internucleotide interactions (12). However, the d(GCGAAGC) hairpin shows extremely high stability compared to the corresponding RNA hairpin of the r(GCGAAGC) and r(GCGAAAGC) fragments (11), and other stable RNA hairpins (34, 35). In the stable RNA hairpins containing UNGC and GNRA loops, some nucleotides in the loops adopt the $\text{C}2'\text{-endo}$ conformation and others the $\text{C}3'\text{-endo}$ conformation. The coexistence of two different conformations leads to incomplete base-base stackings in the loops (4, 6), although a non-Watson-Crick base pair exists in the loop region. In contrast, all nucleotides in the d(GCGAAGC) fragment adopt the $\text{C}2'\text{-endo}$ conformation, and easily form strong base-base stackings with neighboring nucleotides except between A_4 and A_5 . The $\text{G}_3\text{-A}_4$ stacking may also contribute to stabilize the adjacent $\text{G}_3\text{-A}_5$ base pair, since the formation of a G-A base pair is usually affected by the properties of the neighboring bases (33-35). We therefore suggest that the extensive base-base stackings in d(GCGAAGC) are responsible for the extraordinary thermal stability of the

b

5'- ^{32}P -labeled fragment	recovery yield (%)	
	<i>E. coli</i> extracts	human serum
d(GCGAAGC)	62	57
d(GCGAAAGC)	23	50
d(CGACGAG)	6	14

Figure 7. Resistance of the d(GCGAAGC), d(GCGAAAGC), and d(CGACGAG) fragments against nucleases contained in *E. coli* ribosomes and S100 fractions, and in human serum. a: the autoradiogram of gel-electrophoretic patterns at 60°C, b: the recovery yields of the remaining intact fragments calculated from their band densities. Distinct difference in the mobility of these ^{32}P -labeled fragments could be slightly observed in gel-electrophoresis at 60°C.

structure. As shown in Fig. 3b, the structure appears as if the two strands of double helix are connected between A_4 and A_5 . Since the structure is hard to differentiate into stem and loop regions, we classify it as a DNA turn (*hairpin-turn*) structure in which the turn occurs at the single phosphate intervening between A_4 and A_5 .

Resistance of the d(GCGAAGC) fragment against nucleases

It is known that the thermally stable DNA hairpins tend to be resistant against nucleases (1, 19). Recently, it turned out that some oligodeoxynucleotides tagged with hairpin structures at their termini are endowed resistance against nucleases (19-21). This new method is useful for stabilization of antisense oligomers (20, 21).

We expected that the highly stacked structure of the d(GCGAAGC) fragment would show high resistance against nucleases, and examined the nuclease sensitivities of this fragment and its sequence variants. The d(GCGAAGC), d(GCGAAAGC), and d(CGACGAG) fragments were digested with nucleases intrinsically contained in *E. coli* ribosomes and S100 fractions, and in human serum, and the degradation rates of these fragments were analyzed by gel-electrophoresis. In Fig. 7, the patterns of the autoradiogram as well as the recovery yields of the remaining intact fragments clearly demonstrate that the d(GCGAAGC) hairpin is the most stable of these fragments, much better than

the d(GCGAAAGC) hairpin, which has been thought to be the most stable hairpin (1) and used as a self-stabilized oligomer (19, 20).

Thus, d(GCGAAAGC) would be useful as a self-stabilizing sequence, from this point of view it is expected that it may have some important roles in natural DNAs. The GCGAAGC sequence is known to occur in some promoter regions for transcription(14) and replication origins(13) in double-stranded DNA, suggesting that this *hairpin-turn* sequence in those regions prefers to form a cruciform structure, which is recognized by a DNA-binding protein (36).

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