Mutual interaction between adjacent dG·dC actinomycin binding sites and dA·dT netropsin binding sites on the self-complementary d(C-G-C-G-A-A-T-T-C-G-C-G) duplex in solution

(dodecanucleotide duplex/intercalation/groove binding/adjacent complexation sites/telestability)

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ABSTRACT The Watson-Crick imino protons, the backbone phosphodiester resonances, and the antibiotic exchangeable protons have been used as markers to monitor the separate and simultaneous binding of actinomycin and netropsin to the d(C-G-C-G-A-A-T-T-C-G-C-G) self-complementary duplex in aqueous solution. We demonstrate that intercalation of actinomycin at dG(3'-5')dC sites at either end of the duplex results in a conformational perturbation at the dA·dT tetranucleotide core of the dodecanucleotide duplex. Parallel studies of the groove binding of netropsin at dA·dT sites in the interior of the duplex reveal a conformational perturbation which extends to adjacent dG·dC base pairs in the dodecanucleotide duplex. The NMR markers demonstrate that the d(C-G-C-G-A-A-T-T-C-G-C-G) duplex can accommodate actinomycin and netropsin simultaneously at adjacent dG·dC and dA·dT tetranucleotide blocks along its length with some mutual interaction between neighboring antibiotic binding sites.

There have been extensive NMR studies of the binding of antibiotics to DNA duplexes at the oligonucleotide and polynucleotide level in solution. The majority of these efforts have focussed on the binding of a single antibiotic to the DNA and have provided insights into conformational features of nucleic acid complexes with intercalating, groove-binding, and kinking agents (1). It was of interest to extend these NMR investigations to the binding of the two different antibiotics at adjacent sites on the DNA duplex and to investigate their mutual interactions along the helix.

The d(C-G-C-G-A-A-T-T-C-G-C-G) dodecanucleotide duplex (scheme I) contains more than one turn of helix so that it is sufficiently long to bind more than one antibiotic along its length. Furthermore, it contains a dA·dT tetranucleotide block flanked by dG·dC tetranucleotide blocks in either direction, providing adjacent sites for dA·dT-specific and dG·dC-specific antibiotics.

Scheme I

The antibiotic actinomycin D [scheme II, R = cyclo(Thr-DVal-Pro-Sar-MeVal)] is an intercalative agent which exhibits a sequence specificity for dG(3'-5')dC sites on double helical DNA. Intercalation of actinomycin D results in unwinding of the duplex with the pentapeptide lactone rings extending between 2 and 3 base pairs on either side of the intercalation site (2, 3).

Scheme II

Netropsin, a peptide antibiotic, exhibits a specificity for dA·dT-rich duplex regions and spans between 2 and 3 base pairs of the DNA duplex. Netropsin binds in the minor groove of the DNA, and the complex is stabilized predominantly by hydrogen bonding through the antibiotic peptide protons and electrostatic interactions through the charged ends (4, 5).

Scheme III

We have used NMR spectroscopy to investigate the complex of two actinomycins and one netropsin bound separately and simultaneously to the d(C-G-C-G-A-A-T-T-C-G-C-G) duplex in solution. The available methods permit the independent monitoring of the antibiotic resonances as well as individual base pair proton and phosphodiester resonances of the dodecanucleotide duplex upon complex formation.

EXPERIMENTAL

Synthesis. The dodecanucleotide was synthesized by the solution-phase triester method (6) by coupling the protected oligonucleotides d(C-G-C) and d(G-A-A-T-T-C-G-C-G) followed by deprotection and purification by hybridization of self-complementary strands.

Sample Preparation. Proton and phosphorus NMR spectra were run on 2.5-mg samples of the dodecanucleotide in 0.2 ml of 0.1 M phosphate/2.5 mM EDTA buffer. The actinomycin and netropsin were obtained from Merck and Farmitalia (Milan, Italy), respectively.

Spectra. The exchangeable protons in the dodecanucleotide and their antibiotic complexes were recorded in the correlation mode on a Bruker WH-360 NMR spectrometer. A typical spectrum involved 1000 scans with an acquisition time of 1 sec per scan. The chemical shifts are referenced relative to 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard.

The phosphorus NMR spectra of the dodecanucleotide and its antibiotic complexes were recorded with broad-band proton

noise decoupling on a Bruker HX-360 NMR spectrometer in the Fourier transform mode with a repetition rate of 2.7 sec. The chemical shifts are referenced relative to internal trimethylphosphate.

RESULTS AND DISCUSSION

We shall distinguish between actinomycin binding at dG·dC sites and netropsin binding at dA·dT sites on the d(C-G-C-G-A-A-T-T-C-G-C-G) duplex by monitoring the characteristic spectral changes at the Watson-Crick imino protons and the phosphodiester backbone of the dodecanucleotide on complex formation.

Watson-Crick imino protons

The imino proton spectrum of the dodecanucleotide duplex in aqueous 0.1 M phosphate at 27°C is presented as curve A in Fig. 1. The terminal imino proton of dG·dC base pair 1 has broadened out due to fraying (7) at the ends of the duplex at this temperature. The remaining five resonances have been previously assigned to imino protons of individual base pairs in the dodecanucleotide sequence with the dA·dT imino resonances (base pair 5 at 13.79 ppm and base pair 6 at 13.67 ppm) resonating to the low field side of the dG·dC imino resonances (base pair 2 at 13.10 ppm, base pair 3 at 12.945 ppm, and base pair 4 at 12.74 ppm).

Actinomycin Complex. There are two dG(3'-5')dC sites in the d(C-G-C-G-A-A-T-T-C-G-C-G) sequence which can result in actinomycin intercalation between base pairs 2 and 3 within each dG·dC tetranucleotide block at either end of the duplex. The imino proton spectrum of the complex with two actinomycins per dodecanucleotide duplex at 27°C is presented as curve B in Fig. 1. The dG·dC spectral region is quite complicated. The upfield-shifted envelope centered at ≈ 12.4 ppm is assigned (7, 8) to base pairs 2 and 3 at the intercalation site. The

FIG. 1. The 360-MHz correlation proton NMR spectra (12–14.5 ppm) of the dodecanucleotide duplex at pH 6.80 (curve A), the complex of two actinomycins per dodecanucleotide at pH 6.75 (curve B), the complex of one netropsin per dodecanucleotide at pH 6.89 (curve C), and the complex of two actinomycins and one netropsin per dodecanucleotide at pH 6.75 (curve D) in 0.1 M phosphate/2.5 mM EDTA in $H_2O/{}^2H_2O$, 4:1, 27°C. The upfield shifts at the guanosine H-1 imino protons on actinomycin binding are designated by an asterisk. The downfield shift at one thymidine H-3 imino proton on netropsin binding is designated by an arrow.

FIG. 2. Temperature dependence of the thymidine H-3 proton chemical shifts at base pairs 5 and 6 (0.1 M phosphate/2.5 mM EDTA in $H_2O/{}^2H_2O$, 4:1). (A) Dodecanucleotide duplex at pH 6.80 ($\circ$) and the complex of two actinomycins per dodecanucleotide at pH 6.75 ($\bullet$). (B) The complex of one netropsin per dodecanucleotide at pH 6.89 ($\circ$), and the complex of two actinomycins and one netropsin per dodecanucleotide at pH 6.75 ($\bullet$).

resonance at ≈ 12.84 ppm is assigned to base pair 4.

There are small upfield shifts of 0.15 and 0.05 ppm at the thymidine H-3 resonances of dA·dT base pairs 5 and 6 respectively (Fig. 1, curve A) on addition of two equivalents of actinomycin (Fig. 1, curve B) to the dodecanucleotide duplex, with the corresponding temperature-dependent chemical shifts plotted in Fig. 2A. The above observation that actinomycin binding at the dG·dC tetranucleotide regions perturbs adjacent dA·dT base pair regions in the dodecanucleotide duplex is consistent with studies by Wells and coworkers (9) who demonstrated that the binding of actinomycin influenced the properties of the dG·dC binding region as well as the dA·dT nonbinding region in the $(dC_{15}dA_{15})\cdot(dT_{15}dG_{15})$ complementary block copolymer.

Netropsin Complex. The imino proton spectral region of the complex of one netropsin per d(C-G-C-G-A-A-T-T-C-G-C-G) duplex in 0.1 M phosphate at 27°C is presented as curve C in Fig. 1. We observed a large selective downfield shift in the dA·dT region with one of the thymidine H-3 resonances at 13.67 or 13.79 ppm in the dodecanucleotide (Fig. 1, curve A) shifting to 14.25 ppm in the complex (Fig. 1, curve C). This spectrum remained unchanged on addition of a second equivalent of netropsin, demonstrating that the dodecanucleotide duplex contains only one binding site for the peptide antibiotic in solution.

The temperature-dependent chemical shifts of the thymidine H-3 resonances of the netropsin-dodecanucleotide complex in 0.1 M phosphate are plotted in Fig. 2B. It should be noted that netropsin removed the 2-fold symmetry of the dodecanucleotide duplex. This is manifested in an 0.02-ppm chemical shift separation for both dA·dT base pair imino protons and for two dG·dC base pair imino protons in the complex between 15°C and 45°C.

The binding of the peptide antibiotic in the center of the

dodecanucleotide duplex resulted in a chemical shift perturbation extending to the imino protons of adjacent dG·dC base pairs and a loss of the 2-fold element of symmetry at (positions 5 and 6) and adjacent to (positions 3 and 4) the dA·dT tetranucleotide complexation site (curves A and C in Fig. 1 and Fig. 2). This conclusion is supported by earlier NMR investigations which demonstrated that netropsin at low binding ratios induces a conformational change in poly(dA·dT) which is propagated to adjacent antibiotic-free base pair regions (10).

Actinomycin and Netropsin Complex. These resolvable dodecanucleotide imino proton markers which shift in opposite directions on addition of intercalation and groove-binding agents permit study of the simultaneous binding of actinomycin at the dG·dC-rich segments at either end and of netropsin at the dA·dT-rich central region of the d(C-G-C-G-A-A-T-T-C-G-C-G) duplex. The imino proton spectrum of the dodecanucleotide complex with two equivalents of actinomycin and one equivalent of netropsin at 27°C is presented as curve D in Fig. 1. The dG·dC imino proton spectral region (12.3–12.9 ppm) in the dodecanucleotide complex containing the intercalating and groove binding agents is similar to that of the complex with the intercalating agent alone (Fig. 1, curve B). This observation is consistent with intercalation of actinomycin at either end of the dodecanucleotide duplex in the presence of bound netropsin.

The two resolved dA·dT imino protons between 13.6 and 14.3 ppm in the dodecanucleotide complex with two actinomycins and one netropsin (Fig. 1, curve D) resonate in the same spectral region as the corresponding resonances in the complex with netropsin only (Fig. 1, curve C). Thus, netropsin binds in the groove in the center of the duplex in the presence of bound actinomycin at either end of the duplex. We observed differences in the temperature-dependent thymidine imino proton chemical shifts in the netropsin·dodecanucleotide complex in the absence and presence of actinomycin (Fig. 2B) which suggest mutual interaction between adjacent actinomycin and netropsin binding sites along the helix.

The premelting transition localized at the dA·dT base pairs 5 and 6 in the center of the dodecanucleotide duplex was maintained in the actinomycin complex and in the netropsin complex, and when both antibiotics were bound simultaneously (Fig. 2).

The NMR studies that monitor specific protons on individual base pairs in the dodecanucleotide sequence (Fig. 1) and the optical and hydrodynamic methods (spectral titration, thermal denaturation, and analytical buoyant density centrifugation) that monitor average properties of block copolymers and natural and superhelical DNAs (9, 11) both suggest that actinomycin and netropsin can bind at adjacent sites on the nucleic acid duplex.

Phosphodiester backbone

The phosphorus NMR spectrum of the d(C-G-C-G-A-A-T-T-C-G-C-G) duplex at ambient temperature exhibited partially resolved resonances between 4.05 and 4.51 ppm upfield from standard trimethylphosphate (Fig. 3, curve A). We are unable to assign these resolved resonances to individual phosphodiesters in the sequence.

Actinomycin Complex. We observed phosphorus resonances centered at 1.64 and 2.54 ppm downfield from the main cluster on addition of two equivalents of actinomycin to the dodecanucleotide duplex (Fig. 3, curve B). These downfield-shifted phosphorus resonances are assigned to the phosphodiesters on partner strands facing the benzenoid and quinonoid edges of the phenoxazone ring (scheme II) in the tightly bound intercalative complex (7, 12).

Netropsin Complex. The phosphorus spectrum of the com-

FIG. 3. The proton noise decoupled 145.72-MHz ^{31}P spectra (1–6 ppm upfield from standard trimethylphosphate) of the dodecanucleotide duplex at pH 7.50 (curve A), the complex of two actinomycins per dodecanucleotide at pH 7.85 (curve B), the complex of one netropsin per dodecanucleotide at pH 7.70 (curve C), and the complex of the two actinomycins and one netropsin per dodecanucleotide at pH 7.80 (curve D) in 0.1 M phosphate/2.5 mM EDTA in $^2\text{H}_2\text{O}$ at ambient temperature. The downfield phosphorus shifts at the actinomycin intercalation sites are designated by asterisks. The upfield phosphorus chemical shift at the netropsin groove binding site is designated by an arrow.

plex of one netropsin per dodecanucleotide duplex exhibited new resonances at 4.8 and 4.9 ppm upfield from the main cluster between 4.0 and 4.5 ppm (Fig. 3, curve C). We assign these upfield shifted resonances to the phosphodiesters at the netropsin binding site (13) in the dA·dT tetranucleotide core of the dodecanucleotide duplex.

Actinomycin and Netropsin Complex. Because the phosphorus markers for intercalation and groove-binding antibiotics shift in opposite directions, it should be possible to monitor the simultaneous binding of both antibiotics at adjacent sites on the dodecanucleotide duplex.

The phosphorus spectrum of the dodecanucleotide complex with two equivalents of actinomycin and one equivalent of netropsin at ambient temperature is presented as curve D in Fig. 3. These are broad resonances at 1.72 and 2.64 ppm in the dodecanucleotide complex with the intercalating and groove binding agent, similar to what was observed in the complex with the intercalating agent alone (Fig. 3, curve B). A phosphorus resonance is observed at 4.72 ppm in the dodecanucleotide complex with both antibiotics, similar to the upfield shift from the main cluster observed in the complex with netropsin alone (Fig. 3, curve C). These results are consistent with the simultaneous binding of netropsin at the dA·dT-rich center of the duplex and actinomycin at the dG·dC-rich ends of the dodecanucleotide duplex.

It should be noted that the upfield-shifted phosphorus is centered at 4.84 ppm in the netropsin·dodecanucleotide complex (Fig. 3, curve C) and moves to 4.72 ppm in the complex with both antibiotics (Fig. 3, curve D) so that there is mutual interaction between the actinomycin and netropsin binding sites along the helix.

Netropsin exchangeable protons

Netropsin contains exchangeable amide and guanidinium protons which resonate between 7.5 and 9 ppm in the absence of nucleic acid in H_2O solution. We did not observe any exchange-

FIG. 4. The 360-MHz correlation proton NMR spectra (7.5–11 ppm) of the complex of one netropsin per dodecanucleotide at pH 6.89 (curve A) and the complex of two actinomycins and one netropsin per dodecanucleotide at pH 6.75 (curve B), in 0.1 M phosphate/2.5 mM EDTA in $\text{H}_2\text{O}/^2\text{H}_2\text{O}$, 4:1, at 27°C. The netropsin exchangeable protons that are intermolecularly hydrogen bonded to the dA·dT minor groove base pair edges are designated by arrows.

able resonances between 9 and 10 ppm in the proton spectra of the dodecanucleotide duplex and its complex with two equivalents of actinomycin. By contrast, the spectrum of the dodecanucleotide complex with one equivalent of netropsin exhibits exchangeable resonances at 9.10, 9.25, and 9.70 ppm (Fig. 4, curve A). These downfield-shifted resonances are assigned to the antibiotic peptide protons, the guanidinium-exchangeable protons or both participating in the intermolecular hydrogen bonds with the dA·dT base pair edges in the minor groove on complex formation (13, 14).

The complex of two actinomycins and one netropsin with the dodecanucleotide duplex exhibits exchangeable resonances at 9.11 and 9.76 ppm (Fig. 4, curve B), similar to the complex with netropsin alone (Fig. 4, curve A). These data demonstrate that netropsin interacts with the dA·dT tetranucleotide core of the d(C-G-C-G-A-A-T-T-C-G-C-G) duplex in the presence of bound actinomycin at the flanking dG·dC tetranucleotide regions.

SUMMARY

We have investigated the simultaneous binding of netropsin at the dA·dT-rich tetranucleotide core and of actinomycin at the dG·dC-rich tetranucleotide ends of the self-complementary d(C-G-C-G-A-A-T-T-C-G-C-G) duplex in solution by high-res-

olution NMR spectroscopy. The Watson-Crick dG·dC imino protons shift to high field on intercalation of actinomycin and the dA·dT imino protons shift to low field on groove binding of netropsin so that these markers can be used to demonstrate that the dodecanucleotide duplex simultaneously accommodates the intercalating and groove-binding agents at adjacent sites on the helix. This conclusion is reinforced by phosphorus spectra showing downfield shifts in the phosphodiester backbone on intercalation of actinomycin and upfield shifts on groove binding of netropsin to the dodecanucleotide duplex.

The structural basis of the mutual interaction between adjacent intercalation and groove-binding antibiotic complexation sites cannot be quantitated on the basis of the available chemical shift parameters of the dodecanucleotide complex. The imino proton chemical shifts are sensitive to changes in the hydrogen bonding and base pair overlaps whereas the phosphorus chemical shifts probe the O—P torsion or O—P—O bond angles, or both, of the phosphodiester backbone.

The 360-MHz correlation spectra of nucleic acid exchangeable protons in H_2O solution were recorded at the Mid Atlantic Regional Facility at the University of Pennsylvania Medical School (funded by National Institutes of Health Grant RR542).

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