timescale. The stoichiometry was easily and unambiguously determined to be 2:1 (NA:DNA), and no intermediate was observed. NMR spectra of the NA-DNA complex were recorded using $^{13}C^{15}N$-labelled DNA. The determined solution structure of the complex revealed the invasive ligands binding to the A-A mismatch and flanking G-C base pairs, causing the widowed cytosines to flip out from π-stack (Figure 2). Hydrogen-bond pairs between NA and DNA, naphthyridine-guanine and azaquinolone-adenine, are well stacked in the right-handed DNA helix, showing structural mimicry of Watson–Crick base pairing (Figure 3). This is the first observation that the small molecular ligand induced the base flipping of the nucleotide base in the Watson-Crick base pair.

Strong NA binding to the CAG-CAG triad induced formation of the NA-bound hairpin form in long $(CAG)_n$ repeats. Given the ability of NA to bind CAG repeats, a sensor in which NA was immobilized on an SPR chip was created, and its utility was assessed for diagnosis of the CAG repeat length by SPR analysis. SPR analyses of the binding of $d(CAG)_{10}$, $d(CAG)_{20}$ and $d(CAG)_{30}$ to the immobilized NA dimmer showed that signal intensities increased with repeat length. The SPR intensities of $d(CAG)_{30}$ were stronger than those of $d(CAG)_{10}$ and $d(CAG)_{20}$ at a wide range of DNA concentrations (1), suggesting that it may be possible to use the NA-immobilized SPR sensor for the rapid diagnosis of CAG repeat length.

CONCLUSION

NA-CAG-CAG complex structure determined by NMR provides the theoretical basis to design and optimize the CXG triad binding ligands. In fact, the similar structural feature has been found in the complex of naphytiridine-dimer with CGG triad (2). Currently, there is no effective therapeutic agent for treating diseases caused by triplet repeat expansion. The discovery of the small-molecular ligand NA, which binds with high affinity to repeat sites, may be a substantial substantial step toward developing effective therapeutic agents for these hereditary diseases.

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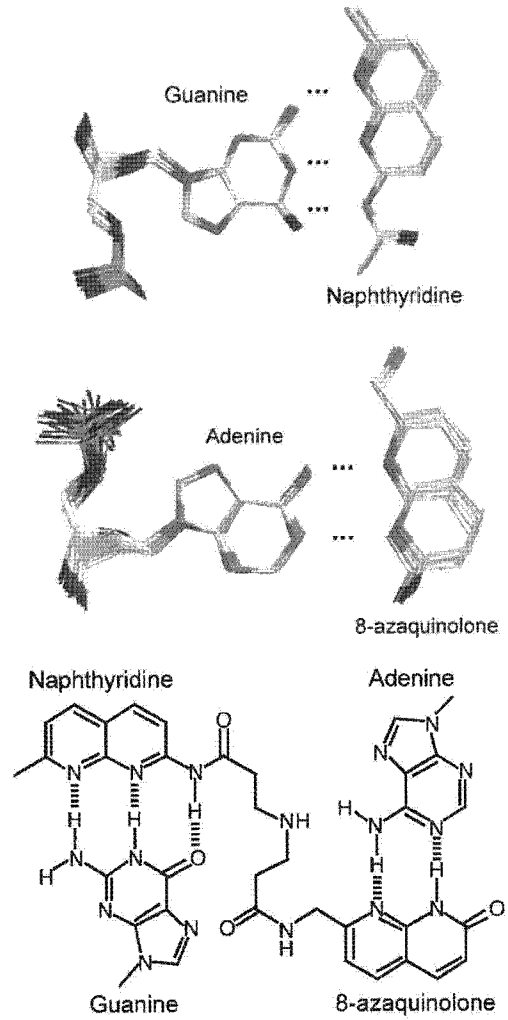

Figure 3. Superimposed NMR structures for the hydrogen bonding between guanine and naphthyridine (top) and adenine and 8-azaquinolone (middle). Naphthyridine chromophore is complementary in hydrogen bonding surface to guanine, whereas 8-azaquinolone is complementary to adenine (bottom).

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Nucleic Acids Symposium Series No. 49 51–52

NMR spectroscopic analyses of functional nucleic acids-metal interaction and their solution structure analyses

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ABSTRACT

We have studied complexations of functional nucleic acids and metal ions, by means of NMR spectroscopy. In the case of hammerhead ribozymes, they have a metal ion-binding motif in their core sequences. Upon the metallation of N7 of a guanosine in an RNA duplex modelled after hammerhead ribozymes, 20 ppm higher field shift of N7 was observed in 1-dimensional (1D) ^{15}N NMR spectra. It was found that metal ion binding to nucleobases were detectable with ^{15}N NMR spectroscopy.

INTRODUCTION

Biologically active RNA molecules utilize metal ions to fold into specific conformation or to form a catalytic center. In order to reveal their mechanisms of actions, it is important to establish methodologies for detecting metallations of nucleic acids. In the conserved core region of hammerhead ribozymes, there is a metal ion-binding motif (the A9/G10.1 site) in which N7 of guanosine (G10.1) is metallated (1-3). Therefore, the A9/G10.1 motif is suitable for establishing methodologies for the detection of nucleobase metallations. Furthermore, in order to understand the mechanism of hammerhead ribozymes, it is becoming important whether the metal ion at this motif is a catalytic center or just a structural constituent (4-6). Therefore, it is obviously important to study the interaction between metal ions and the metal ion-binding motif.

RESULTS AND DISCUSSION

We chemically synthesized series of RNA oligomers as shown below.

GA10: r(GGA*CGAG*UCC)
UGAA10: r(GGA*UGAA*UCC)

where the metallated guanosine (G10.1) and corresponding residues are in bold characters. In the case of GA10, we also synthesized stable isotope labelled RNA oligomers.

r(GGACGAGUCC) r(GGACGAGUCC)
r(GGACGAGUCC) r(GGACGAGUCC)

where underlined residues are uniformly ^{15}N- and ^{13}C-labeled guanosine residues. By using these RNA oligomers, titration experiments were performed (7-10). It was found that the chemical shift of N7 of guanosine was suitable for the detection of a nucleobase metallation, since the large chemical shift change was observed for N7(G10.1) (Table 1). More importantly, ^{15}N NMR spectroscopy (chemical shifts or *J*-coupling) seems to be applicable for other metal-nucleic acid complexes, such as mercury mediated T-T base-pair (T-Hg-T) (11).

We also examined roles of this metal ion at the motif, using an RNA oligomer with a motif of hammerhead mutants. The Mg^{2+}-titration for this mutant motif (G10.1-C11.1 to A10.1-U11.1) revealed that its affinity to Mg^{2+} was drastically reduced, although the ribozyme with this mutant motif is known to retain enzymatic activities. This observation suggests that the metal ion at the A9/G10.1 site is not a catalytic center of hammerhead ribozymes.

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Table 1 Summary of the ^{15}N, ^{13}C and ^{1}H NMR data

Metal ligand	Metal	Residue	δ(N7)[a] / ppm	δ(C8)[a] / ppm	δ(H8)[a] / ppm	$^{1}J_{N\text{-}M(II)}$[b] / Hz	Ref.
GA10[c]	Cd^{2+}	G10.1	-19.6 (3.0 eq)	+2.3 (2.5eq)	+0.38 (4.5eq)	not detected	7-9
GA10[c]	Mg^{2+}	G10.1	N.D.	+0.5 (1.0eq)	+0.12 (4.5eq)	n.a.	8-10
GA10[c]	$[Co(NH_3)_6]^{3+}$	G10.1	N.D.	< +0.1 (1.0eq)	+0.25 (1.5eq)	n.a.	8,9
GA10[c]	Na^{+}	G10.1	N.D.	< +0.1 (230 mM)[d]	+0.12 (800 mM)[d]	n.a.	8,9
RNA decamer[e]	Cd^{2+}	G10.1	~ -20 (6.0 eq)	N.D.	N.D.	N.D.	12
RNA decamer[e]	Zn^{2+}	G10.1	~ -20 (4.0 eq)	N.D.	N.D.	n.a.	12
RNA decamer[e]	Mg^{2+}	G10.1	-6.5 (10.0 eq)	N.D.	N.D.	n.a.	12
RNA decamer[e]	$[Co(NH_3)_6]^{3+}$	G10.1	< -1 (6.0 eq)	N.D.	N.D.	n.a.	12
Inosine[f]	Zn^{2+}	n.a.	-15.2 (0.7 eq)	N.D.	N.D.	n.a.	13
Inosine[f]	Hg^{2+}	n.a.	-4.8 (0.75 eq)	N.D.	N.D.	not detected	13
Guanosine[f]	Zn^{2+}	n.a.	-20.1 (1.0 eq)	N.D.	N.D.	n.a.	14
Guanosine[f]	Hg^{2+}	n.a.	-20.5 (1.0 eq)	N.D.	N.D.	N.D.	14
Guanosine (calc.)[g]	Zn^{2+}	n.a.	-14.8	N.D.	N.D.	-36.5[h]	15
Guanosine (calc.)[g]	Mg^{2+}	n.a.	-15.3	N.D.	N.D.	5.6[h]	15
DNA dodecamer[i]	Zn^{2+}	G4	N.D.	+2.5 (8.0eq)	+0.20 (8.0eq)	N.D.	16
DNA dodecamer[i]	Zn^{2+}	G3	N.D.	+1.5 (8.0eq)	+0.05 (8.0eq)	N.D.	16
d(TGGT)	$Pt(en)Cl_2$[j]	G3	N.D.	+1.1 (1.0eq)	+1.04 (1.0eq)	N.D.	17
d(TGGT)	$Pt(en)Cl_2$[j]	G2	N.D.	+0.2 (1.0eq)	+0.26 (1.0eq)	N.D.	17
UGAA10[k]	Mg^{2+}	A10.1	N.D.	N.D.	< +0.01 (1.0eq)	n.a.	8,9

not detected: *J*-coupling was not detected, although trials for its detection have been made. n.a.: not applicable. N.D.: not determined. [a] Chemical shift perturbations from the non-metalated state. Negative and positive values indicate higher- and lower-field shifts, respectively. The numbers in parentheses beside the perturbation values indicate the molar ratios of $[CdCl_2]$/[motif or nucleoside]. [b] *J*-coupling between metalated nitrogens (^{15}N) and metal ions (I=1/2), such as ^{113}Cd and ^{199}Hg. [c] r(GGACGAGUCC)$_2$. [d] Chemical shift perturbations from basal solution (40 mM Na^{+}). [e] RNA decamer: r(CGGUUGAGGC) r(GCCGAAACCG) The metal cation binding motif is underlined, and the metalated guanosine is shown in boldface. [f] Titration experiments were performed in dimethyl sulfoxide (DMSO). [g] Theoretical values from molecular orbital calculations. [h] The quadrupole moments of ^{67}Zn and ^{25}Mg are so large that experimental *J*-coupling values are not available currently. [i] DNA dodecamer: d(ATGGGTACCCAT)$_2$ [j] One $Pt(en)Cl_2$ (en = ethylenediamine) forms two covalent bonds with the two successive guanosines of d(TGGT). [k] r(GGAUGAAUCC)$_2$ [l] r(GGAGGACUCC)$_2$

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Spectroscopic analyses of DNA duplexes in the presence of mercury ions

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ABSTRACT

The DNA duplex with tandem T•T mismatches in the presence of Hg(II) has been studied by NMR spectroscopy. For this study, we synthesized decamer duplex with two successive T•T mismatches (TT10). From two-dimensional (2D) ^{1}H-^{1}H NOESY spectrum of TT10 in complex with Hg(II), we were able to trace sequential NOE walks between base protons and anomeric protons (H1'), and assigned all of them. Based on these assignments and NOESY spectra, we could assigned all the non-exchangeable protons of TT10 in the presence of Hg(II).

INTRODUCTION

Watson-Crick base pair is essential for the stability and specificity of duplex formation. Additional stable and selective base pairs would increase the capacity of DNA for information storage and might allow generation of DNAs with novel function. As one of efforts to develop such an unnatural base pair, a strategy that hydrogen bonding interactions are replaced by metal-dependent pairing of two nucleobases have recently reported. Most of this type of base pair had developed by artificial nucleobase (1-10). Recently, it was reported that thymine-thymine (T•T) mismatches in a duplex form a stable Hg(II)-mediated T•T base pair (11). Using DNA oligomer include tandem T•T mismatches, highly selective and sensitive mercury(II) sensor has developed (12). Originally, it was reported that the Hg(II)-mediated T•T base pair had been suggested by pH titration, UV, CD and NMR spectroscopic methods (13-19). However, its definitive structure has not been elucidated, to date. For this reason, T•T mismatches became interesting target for structural studies again. We have already reported resonance assignments of non-exchangeable protons in a Hg(II)-free state DNA duplex including T•T mismatches, by using NMR spectroscopy (20). In this study, we assigned non-exchangeable proton resonances in a DNA oligomer with T•T mismatches in the presence of Hg(II), as well as its exchangeable proton resonances in the absence of Hg(II).

MATERIALS AND METHODS

Two DNA decamers of 5'-CGCGTTGTCC-3' and 5'-GGACTTCGCG-3' were synthesized by the phosphoramidite method on an automated DNA/RNA synthesizer (ABI model 392). Each oligomer was purified by C18 reversed-phase column (COSMOSIL 5C18-AR-300, nakalai tesque, Japan) on HPLC system, with a linear gradient of acetonitrile. For the exchange of counter ions, each oligomer was adsorbed onto an anion-exchange column (UNO-Q; Pharmacia Biotech, Uppsala, Sweden). The column was washed with more than 10 column volumes of MILLI-Q water (MILLIPORE, USA) to wash out triethylammonium ions. The oligomer was eluted with 2M NaCl to make Na^+ the counter ion. Finally, excess NaCl was removed by a Gel filtration column (TSK-GEL G3000PW; TOSO, Japan) with MILLI-Q water as the mobile phase. The final solution contained only the oligomer and the counter ion (Na^+ ion). Each oligomer was quantitated by UV absorbance at 260 nm after digestion by nuclease P1.

		1	2	3	4	5	6	7	8	9	10	
TT10:	5' -	C	G	C	G	T	T	G	T	C	C	- 3'
	3' -	G	C	G	C	T	T	C	A	G	G	- 5'
		20	19	18	17	16	15	14	13	12	11	

Scheme 1

In order to prepare NMR solutions of a Hg(II)-bound DNA oligomer, we made solutions containing 2.0 mM TT10, 40 mM $NaClO_4$ and 4.8 mM $HgClO_4$. Then these solutions were passed through chelating resin (chelex; BIO-RAD, USA), for the removal of trace amount of paramagnetic metal cations and excess Hg(II). Solution conditions for the NMR measurements of a Hg(II)-free DNA oligomer contains 2.0 mM TT10 and 40 mM $NaClO_4$. Two-dimensional (2D) ^{1}H-^{1}H NOESY spectrum were recorded on Bruker DMX500 spectrometer, at 293 K, 8192 * 2048 complex points for a spectral width of 10000 * 10000 Hz (Hg(II)-free state), and on Bruker DMX800 spectrometer, at 293 K, 8192 * 2048 complex points for a spectral width of 8013 * 8013 Hz (Hg(II)-bound state).

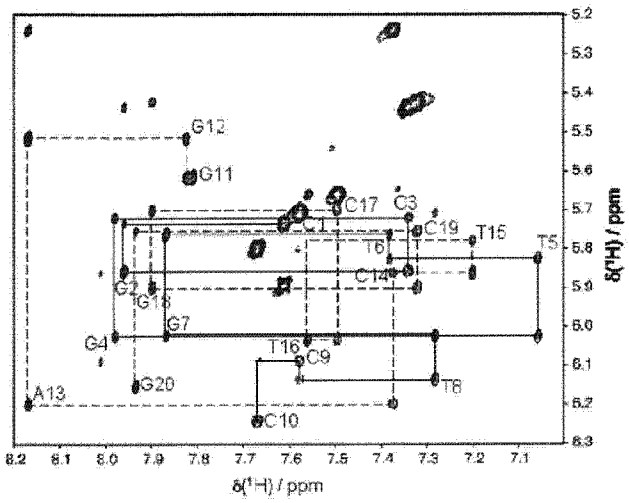

Figure 1. 2D ^{1}H-^{1}H NOESY spectra of TT10 in the presence of Hg(II). The solid and broken lines indicate the sequential NOE walks between H6/H8 and H1' resonances. Intraresidue NOE cross peaks were labeled with their residue numbers. Unlabeled strong NOEs correspond to intraresidue H5-H6 NOE cross peaks of cytidines.

RESULTS AND DISCUSSION

Sequential resonance assignments of TT10 have been carried out in the presence of Hg(II). Figure 1 shows sequential NOE connectivities between base protons (H6/H8) and anomeric protons (H1') in the NOESY spectrum. As apparently from Figure, sequential NOEs were traceable through the both strands unambiguously. Based on these assignments, we also assigned all the resonances of other non-exchangeable protons. In combination with a DQF-COSY spectrum, we assigned all H2' and H2" protons, stereospecifically. However, we were not able to perform stereospecific assignments of H5' and H5" protons. It should be mentioned that the assignments of all non-exchangeable proton resonances from NOESY spectra were consistent with each other, as well as those from the DQF-COSY spectrum.

Then we measured a 2D NOESY spectrum of Hg(II)-free TT10, as well. From the spectrum, four imino proton resonances observed around 11 ppm were found to be imino protons of T•T mismatches. From NOE connectivities of imino proton resonances, they were assigned except for those at terminal base-pairs.

In conclusion, we unambiguously assigned most of non-exchangeable proton resonances of TT10 in the presence and absence of Hg(II).

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NMR structural analysis of the G.G mismatch DNA complexed with naphthyridine-dimer

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ABSTRACT

Naphthyridine-dimer (ND) specifically recognizes G.G mismatch DNA (Figure 1). However, its detailed recognition mechanism is not clear. Here a DNA oligomer d(CTAACGGAATG) / d(CATTCGGTTAA) complexed with ND was studied by NMR. The stoichiometry of DNA to ND was determined to be 1:2 at NMR concentration (2.5 mM). Proton resonances were completely assigned including H5' and H5" using ^{1}H-^{1}H and ^{1}H-^{13}C 2D spectra of the complex. These spectra showed that four naphthyridine rings are staked in the helix and form hydrogen bonds with the four G residues in CGG / CGG region. These results indicate ND can specifically recognize the CGG / CGG sequence.

INTRODUCTION

For detecting single nucleotide polymorphism (SNP), naphthyridine-dimer (ND) has been developed and characterized as the novel sensor for the surface plasmon resonance (SPR) array (*1, 2*). ND recognizes G.G mismatches, and is applicable to various important sequences which include G.G mismatch such as the G-rich telomeric repeat sequence (*3*). Our resent studies revealed that $(CAG)_n$ triplete repeat sequences which have A.A mismatches are recognized by ND like drug, naphthyridine-azaquinolone (NA) (*4*). From ESI-TOF mass analysis, ND binds d(CTAACGGAATG) / d(CATTCGGTTAA) at two different stoichiometries, DNA:ND = 1:1 and 1:2 (*5*). The 1:2 mode is dominant when six-fold ND is present. The detailed recognition mechanisms and the reason why the stoichiometry is differet are not clear. The other key aspect is the ND linker structure which connects two naphtyridine rings. The modification of the ND linker changes binding characters such as thermo-stability and specificity to G.G mismatch (*6, 7*). Thus, the modification of the linker potentially can control the specificity to CGG / CGG sequence. In this study, using ^{1}H-^{1}H NOESY spectra of ND-DNA complex, the structural aspects of ND-DNA complex were studied to reveal the G.G mismatch recognition mechanism. Especially, the stoichiometry and the linker structure were carefully investigated.

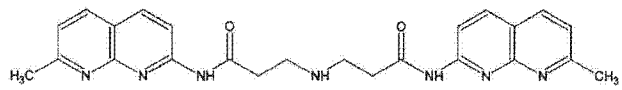

Figure 1. Naphthiridine-dimer (ND). Four nitrogen atoms of naphthiridine rings form hydrogen bonds with imino and amino protons of G. Two amide NH atoms form hydorogen bonds with carboxyl oxygen atoms of G.

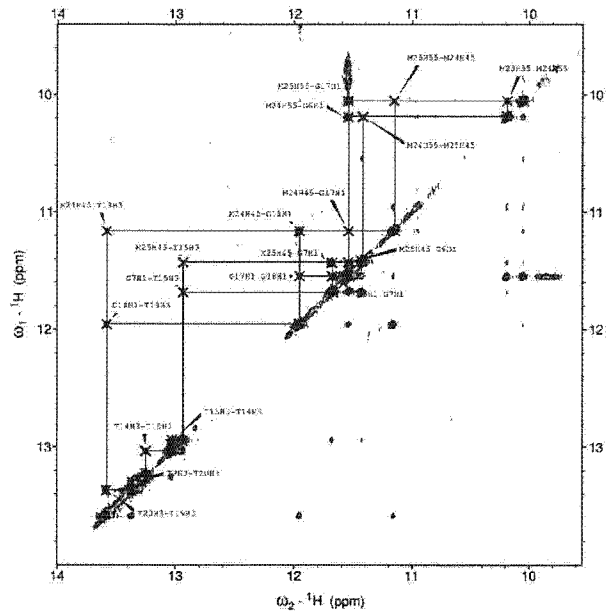

Figure 2. Sequential NOEs of d(CTAACGGAATG) / d(CATTCGGTTAA) complexed with two NDs. Imino protons of nucleic acid bases and four amide protons of NDs are shown in this region.

RESULTS AND DISCUSSION

The ND titration experiments to 2.5 mM d(CTAACGGAATG) / d(CATTCGGTTAA) duplex were carried out monitoring the imino-proton region. The peak

shifts were saturated at the ND concentration 5.0 mM. Through the titration experiments, two peak sets were detected. One was originating from free DNA, and another was from complex. These results strongly suggest that only one complex is detected, and it is 1:2 complex. In other wards, the 1:1 complex was not detected at all. The difference of the results between NMR and ESI-TOF mass experiments may be due to the sample concentration differences, 2.5 mM and 20 μM for NMR and ESI-TOF mass, respectively.

The ^{1}H-^{1}H NOESY spectra of the 1:2 complex were recorded with 30, 200 and 300msec mixing times. TOCSY, DQF-COSY and natural abundance ^{1}H-^{13}C spectra were also recorded to complete the resonance assignment. In the imino proton region, sequential walk was completed (Figure 2). Four amide protons of ND (10 ~ 11.5 ppm) were also connected sequentially. Four H1' protons of G in the CGG / CGG region have NOE cross peaks to four corresponding amide protons of NDs. These data show four G bases form hydrogen bonds with four naphthyridine rings of ND. At the base-H1' region, the sequential walk was completed with the aromatic protons of naphtyridine rings (Figure 3). However, two H6 protons of C in the CGG / CGG were not included in this sequential walk. These results suggest the C residues in CGG / CGG region were not stacked; however, four naphthyridine rings were stacked in. Thus two NDs recognize whole CGG / CGG triplet region by hydrogen bonding and stacking.

All the protons of two ND molecules were also completely assigned. All the resonance assignments of aliphatic methylene protons of the linker region were not identical and clearly distinguished each other. This may suggest this linker region were well structured in spite of the intrinsic flexible character.

CONCLUSION

Naphthyridine-dimer (ND) can recognize the G.G mismatch in the DNA duplex. The complex between ND and d(CTAACGGAATG) / d(CATTCGGTTAA) was stable, and the stoichiometry DNA:ND was 1:2. ND intercalates into the base stacking and forms hydrogen bonds with four G residues. These results indicate ND can recognize whole CGG / CGG triplet region as well as G.G mismatch.

ACKNOWLEDGMENTS

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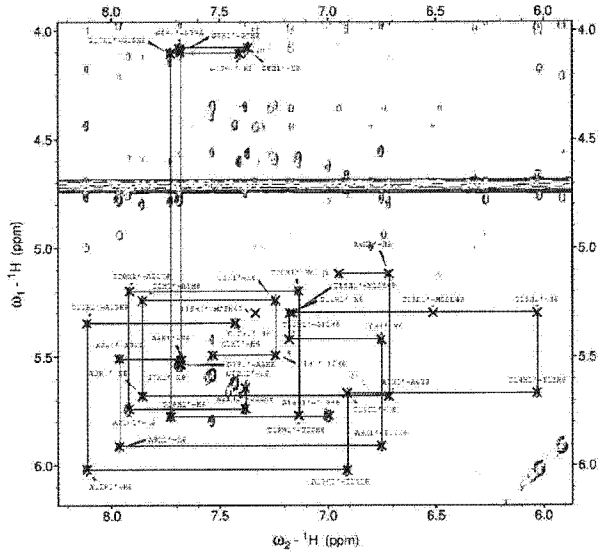

Figure 3. Sequential NOEs of d(CTAACGGAATG) / d(CATTCGGTTAA) complexed with two NDs. Base-sugar H1' region is shown. The NOE cross peaks at 4.1 ppm are from H1' protons of mismatched G residues in CGG / CGG region.

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NMR SPECTROSCOPIC STUDY OF A DNA DUPLEX WITH MERCURY-MEDIATED T-T BASE PAIRS

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□ *Recently, we reported that T-T mismatches can specifically recognize Hg^{II} (T-Hg^{II}-T pair formation). In order to understand the properties of the T-Hg^{II}-T pair, we recorded NMR spectra for a DNA duplex, d(CGCG**TT**GTCC) • d(GGAC**TT**CGCG), with two successive T-T mismatches (Hg^{II}-binding sites). We assigned ^1H resonances for mercury-free and di-mercurated duplexes, and performed titration experiments with Hg^{II} by using 1D ^1H NMR spectra. Because of the above mentioned assignments, we could confirm the existence of mono-mercurated species, because individual components gave independent NMR signals in the titration spectra.*

Keywords ^1H NMR; Hg^{II}-mediated T-T base pair; Titration; Equilibrium

INTRODUCTION

It has been recently demonstrated that metal-mediated base pairs can be formed by replacing natural nucleobases with artificial metal chelators.[1–11] In addition, we demonstrated that a natural nucleobase, thymine, is also able to form a mercury-mediated T-T pair (T-Hg^{II}-T pair formation), and because of this property, DNA duplexes with T-Hg^{II}-T pairs could be used

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as a mercury sensor.[12–15] Although data suggesting Hg^{II}-recognition have been reported,[16–28] no definitive conclusion had been made regarding the recognition mode of Hg^{II} until our previous study.[15] The above mentioned DNA duplexes, which include metal-mediated base pairs, could be potentially useful for nanotechnology devices because of the controllability of the secondary and tertiary structures of the DNA molecules.[29,30]

NMR spectroscopy is a suitable method for investigating the physicochemical properties of a DNA duplex with T-Hg^{II}-T pairs, because it provides structural information on target molecules and their solution equilibria.[13–15,25–28,31–36] Therefore, here we carried out NMR spectroscopic studies on the complex formed by Hg^{II} and a DNA duplex, d(CGCG**TT**GTCC) • d(GGAC**TT**CGCG), since, within the sequences we examined, this duplex gave the most clear spectra. This is the first NMR study of the DNA duplex containing consecutive metal ions.

EXPERIMENTAL SECTION

Sample Preparation

Two DNA decamers, 5′-CGCGTTGTCC-3′ and 5′-GGACTTCGCG-3′, were synthesized by using a phosphoramidite method on an automated DNA/RNA synthesizer model 392 (Applied Bio-systems, Foster City, CA). Each oligomer was purified on a C18 reverse-phase column (Cosmosil 5C18-AR-300; Nakalai Tesque, Kyoto, Japan) in a high performance liquid chromatography (HPLC) system, with a linear gradient of 5–50% acetonitrile (30 min), 0.1M triethylammonium acetate as a basal buffer, and a flow rate of 3.0 mL/min. For the exchange of counter ions, each oligomer was adsorbed onto an anion-exchange column (UNO-Q; BIO-RAD, Hercules, CA). The column was washed with more than 10 column volumes of Milli-Q water (Millipore, Billerica, MA) to wash out the triethylammonium ions. The oligomer was then eluted with 2M NaCl so that Na^+ became the counter ion. Finally, excess NaCl was removed by desalting on a gel filtration column (TSK-GEL G3000PW; TOSOH, Tokyo, Japan) with Milli-Q water as the mobile phase. The final solution contained only the oligomer and the counter ion (Na^+). Each oligomer was quantitated by ultraviolet (UV) absorbance at 260 nm after digestion with nuclease P1.

Titration Experiments with UV Spectra

UV spectra were recorded for a solution containing 5 μM of the DNA duplex (d(CGCGTTGTCC) • d(GGACTTCGCG)), 20 mM Na-MOPS buffer (pH 7.0), in the presence of various concentrations of $Hg(OAc)_2$. UV absorbances at 275 nm were plotted against the molar equivalents.

Two-Dimensional NMR Measurements

Typical solutions for two-dimensional (2D) NMR measurements contained 2.0 mM DNA duplex, 100 mM $NaClO_4$, 1.0 mM Na-cacodylate buffer pH 6.0, with or without 4.8 mM $Hg(ClO_4)_2$, in D_2O. Two-dimensional ^1H-^1H NOESY spectrum without $Hg(ClO_4)_2$ were recorded on a JEOL ECA600 spectrometer, at 296 K, with 2048 * 1024 complex points for a spectral width of 5402 * 5402 Hz, and 16 scans were averaged. Two-dimensional ^1H-^1H COSY spectrum without $Hg(ClO_4)_2$ was recorded on a JEOL ECA600 spectrometer, at 296 K, with 1024 * 1024 complex points for a spectral width of 5402 * 5402 Hz, and 16 scans were averaged. We used an absolute value COSY spectrum for the mercury-free duplex due to a severe cross-peak overlap. Two-dimensional ^1H-^1H NOESY spectrum with $Hg(ClO_4)_2$ was recorded on a Bruker DRX800 spectrometer, at 293 K, with 8192 * 2048 complex points for a spectral width of 8013 * 8013 Hz, and 16 scans were averaged. Phase-sensitive ^1H-^1H COSY spectrum with $Hg(ClO_4)_2$ was recorded on a Bruker DRX800 spectrometer, at 296 K, with 8192 * 1024 complex points for a spectral width of 8013 * 8013 Hz, and 16 scans were averaged.

Titration Experiments with NMR Spectra

Solutions for 1D ^1H NMR measurements contained 2.0 mM DNA duplex, 100 mM $NaClO_4$, 2.0 mM Na-cacodylate buffer (pH 6.0), and various concentrations of $Hg(ClO_4)_2$ in H_2O/D_2O (95:5). We selected an Na-cacodylate system as a buffer, since cacodylate does not precipitate with Hg^{II} under the conditions that we used. In spite of the buffer usage, pH became approximately 4 at the final titration point. However, we confirmed that spectral patterns of the di-mercurated DNA oligomers at pH 4 was essentially the same as those at pH 6. Titration experiments with 1D ^1H NMR spectra were recorded on a JEOL ECA600 spectrometer, at 296 K, with 32,768 complex points for a spectral width of 15,024 Hz, and processed using an exponential function to give line broadening of 3 Hz.

RESULTS AND DISCUSSION

Titration Experiments with UV Spectra

In order to confirm whether the synthesized DNA duplex was suitable for titration experiments with NMR spectra, we examined the affinity of Hg^{II} for the DNA duplex and determined how many Hg^{II} were able to bind to the DNA duplex. For this purpose, we performed titration experiments of the DNA duplex with Hg^{II}, by using UV absorbances at 275 nm (Figure 1). It was found that the titration curve was sharply kinked at 2 molar equivalents of Hg^{II} to a DNA duplex, indicating that 2 molar equivalents of Hg^{II} bound

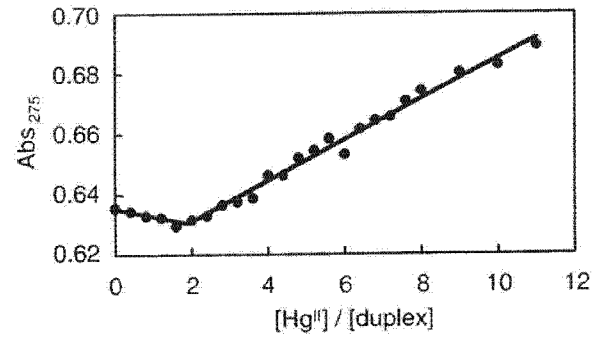

FIGURE 1 Plot of UV absorbances at 275 nm against molar equivalents ([Hg^{II}]/[duplex]). Least-squares fitted lines are shown. Possible explanations for the UV absorbance changes might be due to changes in the spectral intensity of thymidines themselves upon mercuration (0–2 eq.)[15] and putative non-specific interactions of Hg^{II} with DNA oligomers (2–11 eq.).[19,39] Titration NMR spectra also suggested this non-specific binding, since the methyl proton resonance of T8 in di-mercurated DNA duplex (white star) shifted most extensively during titrations, although T8 was not a mercurated site (Figure 4).

to one DNA duplex. This finding is plausible because the DNA duplex contained two T-T mismatches (the putative Hg^{II}-binding site) per duplex. More importantly, the sharp kink in the titration curve indicated that the concentrations of Hg^{II} and the DNA duplex were much higher than the *Kd* value for the Hg^{II}-DNA complexation, indicating that Hg^{II} had very high affinity for this DNA duplex. Therefore, the DNA duplex used here is suitable for physicochemical studies such as structural and thermodynamic studies.

Resonance Assignments for the Mercury-Free DNA Duplex

Before the titration experiments, resonance assignments of the DNA duplex d(CGCGTTGTCC) • d(GGACTTCGCG) needed to be carried out. Therefore, we recorded 2D ^{1}H-^{1}H NOESY and 2D ^{1}H-^{1}H COSY spectra for the duplex in D_2O (Figure 2). In the cross section between H8/H6/H2 and H1′/H5, H5-H6 correlations of cytidines were identified from the COSY spectrum (Figure 2b). Due to a severe cross-peak overlap, we used a conventional absolute value COSY spectrum (Figure 2b). With reference to this information, we were able to assign all base protons and anomeric protons. It was found that sequential NOE walks between base protons and anomeric protons could be traced though both strands (Figure 2a).

We next extended these assignments to other sugar proton resonances, with reference to the abovementioned assignments. In the cross section between base protons and H2′/H2″, their sequential NOE walks were traceable through both strands. H3′ and H4′ resonances were assigned by using the NOE cross-peaks with intra-residue H1′ resonances.[37] Stereo-specific assignments of H2′/H2″ resonances were performed by using cross-peak intensities of H2′-H3′ and H2″-H3′, estimated from the COSY spectrum.[37] Methyl proton resonances (Me) of thymidines were assigned by using intra-residue H6-Me NOE cross-peaks. Finally, H5′ and H5″ resonances were assigned by

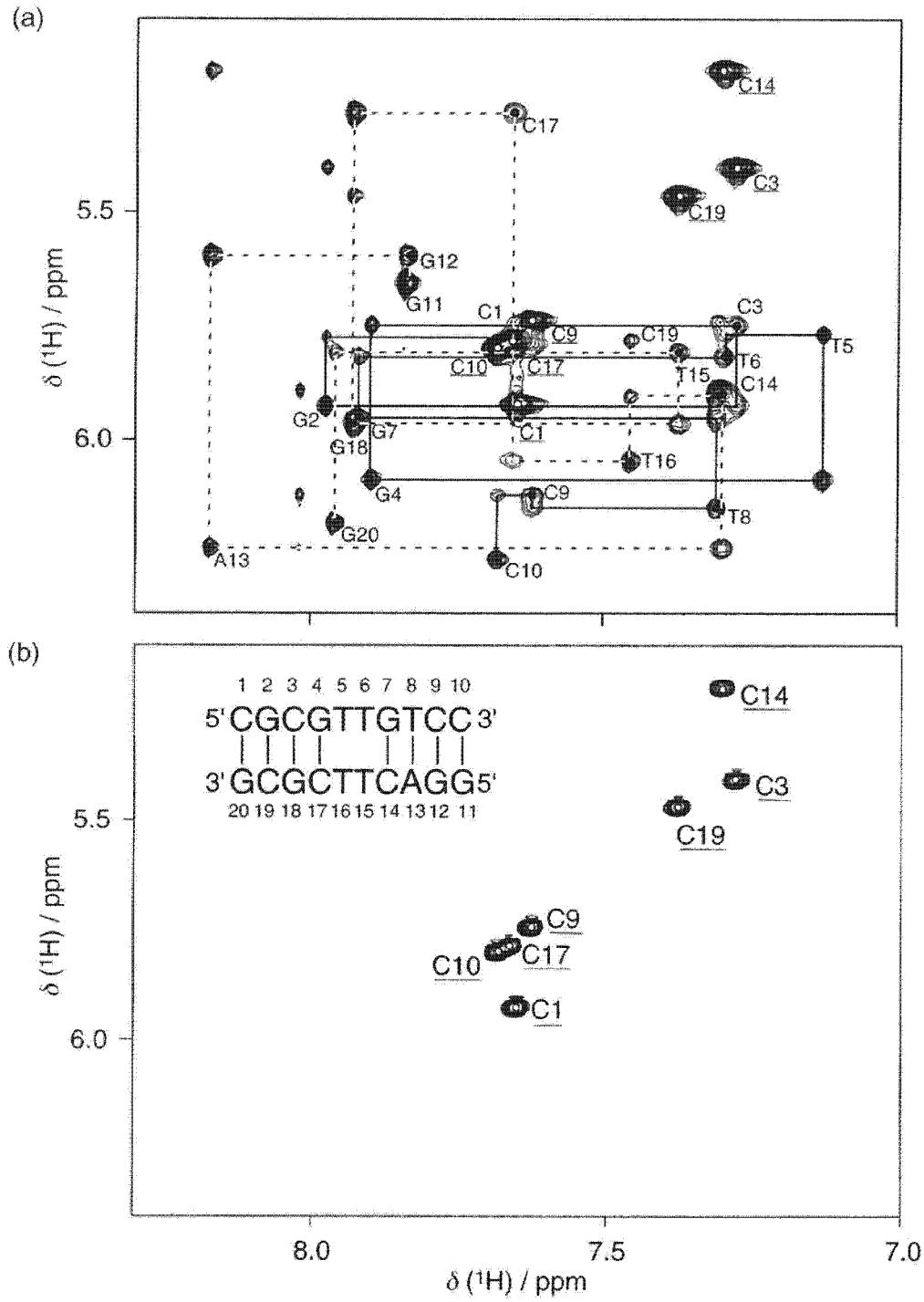

FIGURE 2 NOESY and absolute value COSY spectra for the mercury-free DNA duplex. (a) NOESY spectrum with sequential NOE walks between anomeric (H1′) and base protons (black line: C1-C10; dotted line: G11-G20). Intra-residue cross-peaks are presented with the corresponding residue numbers (closed circles). (b) COSY spectrum with H5-H6 cross-peaks of cytidines labeled with their residue numbers.

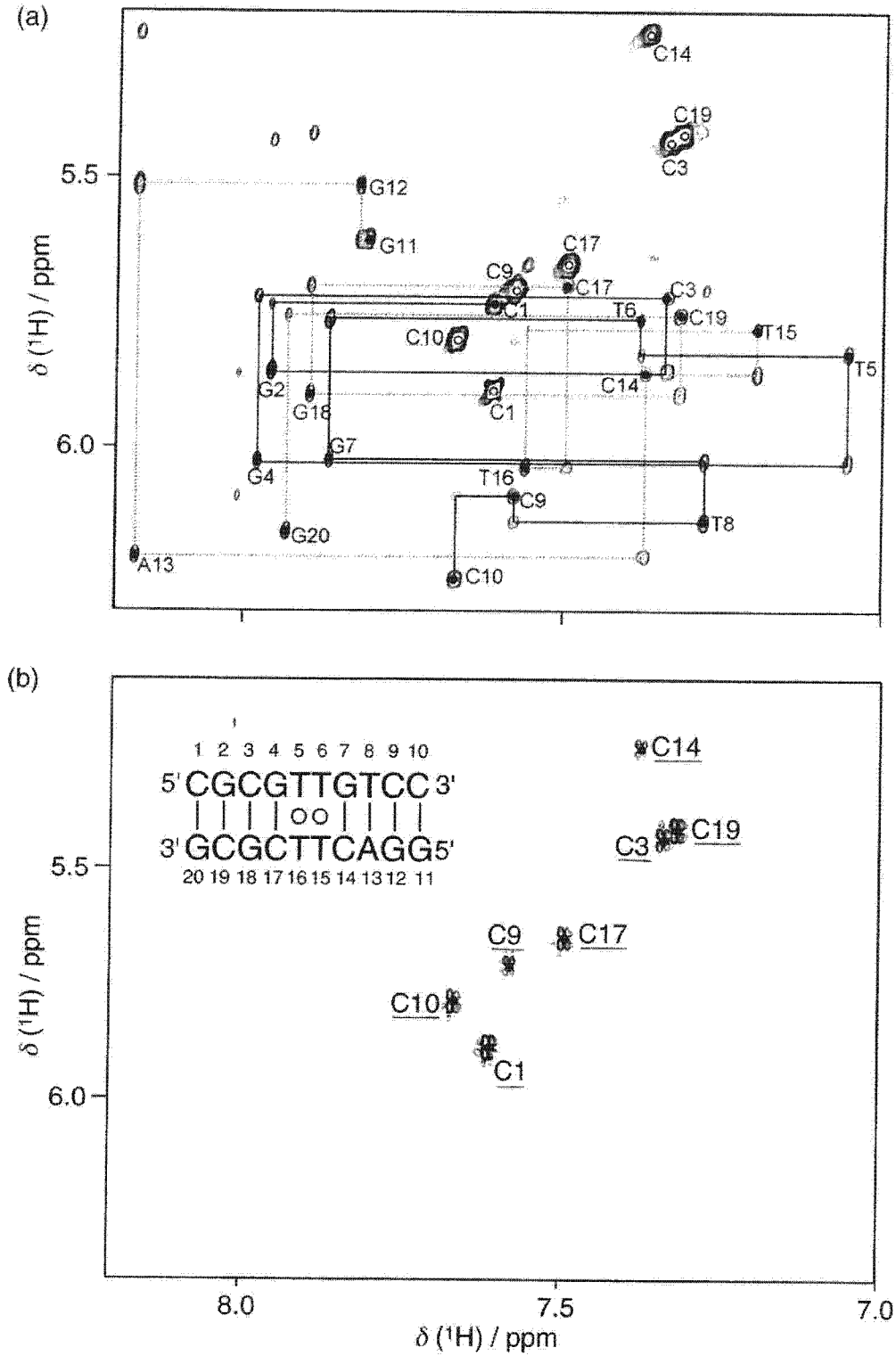

FIGURE 3 NOESY and phase-sensitive COSY spectra for the di-mercurated DNA duplex. (a) NOESY spectrum with sequential NOE walks between anomeric (H1′) and base protons (black line: C1-C10, dotted line: G11-G20). Intra-residue cross-peaks are presented with the corresponding residue numbers (closed circles). (b) COSY spectrum with H5-H6 cross-peaks of cytidines labeled with their residue numbers.

TABLE 1 Chemical Shift Table for the Mercury-Free DNA Duplex[a]

	H2/H5/Me[b]	H6/H8[c]	H1′	H2′	H2″	H3′	H4′	H5′/H5″[d]
C1	5.93	7.65	5.78	2.00	2.47	4.72	4.08	3.75/3.75
G2	n.a.	7.98	5.93	2.69	2.75	4.98	4.37	4.01/3.73
C3	5.41	7.28	5.75	1.92	2.40	4.85	4.20	4.15/4.10
G4	n.a.	7.90	6.09	2.66	2.78	4.95	4.41	4.13/4.10
T5	1.66	7.13	5.77	1.96	2.47	4.76	4.15	4.40/4.13
T6	1.54	7.30	5.81	2.04	2.44	4.84	4.15	-/-
G7	n.a.	7.92	5.95	2.63	2.79	4.94	4.33	4.12/4.09
T8	1.35	7.31	6.15	2.21	2.57	4.91	4.27	4.24/4.18
C9	5.74	7.62	6.12	2.24	2.52	4.86	4.20	4.24/4.15
C10	5.80	7.68	6.26	2.30	2.30	4.58	4.06	4.20/4.18
G11	n.a.	7.85	5.66	2.48	2.67	4.82	4.17	3.67/3.67
G12	n.a.	7.83	5.60	2.69	2.77	5.02	4.38	-/-
A13	8.02	8.17	6.24	2.69	2.86	5.04	4.49	4.25/4.21
C14	5.20	7.31	5.90	2.25	2.49	4.68	4.30	4.09/-
T15	1.65	7.31	5.91	1.95	2.43	4.74	4.06	4.21/-
T16	1.50	7.46	6.05	2.01	2.43	4.82	4.05	4.17/-
C17	5.78	7.66	5.29	2.38	2.43	4.89	4.19	4.04/3.99
G18	n.a.	7.93	5.97	2.61	2.78	5.02	4.37	3.99/-
C19	5.47	7.37	5.81	1.97	2.38	4.86	4.19	4.21/4.15
G20	n.a.	7.96	6.19	2.64	2.39	4.70	4.21	4.10/-

[a]Chemical shifts are given in ppm.

[b]Chemical shifts for H2 of adenosine, or H5 of cytidine, or the methyl proton of thymidine.

[c]Chemical shifts for H8 of the purine residues or H6 of the pyrimidine residues.

[d]Chemical shifts for H5′ and H5″. Stereospecific assignments were not carried out.

n.a.: not applicable (Assignments were not applicable due to the lack of the corresponding protons.) -: not assigned, due to signal overlaps.

using the residual NOE cross-peaks with base protons. Thus, we assigned 164 nonexchangeable proton resonances out of 173 expected resonances (Table 1). These assignments were fully consistent within the NOESY and COSY spectra.

Resonance Assignments for the Di-Mercurated DNA Duplex

Next, we performed resonance assignments for the Hg^{II}-DNA (2:1) complex as described for the mercury-free complex above, except that the phase-sensitive COSY spectrum was used. The NOESY and COSY spectra are presented in Figure 3. Basically, resonance assignments were performed as described above. In addition, by using the phase-sensitive COSY spectrum, *J*-coupling values for H1′-H2′/H2″ could be read. Therefore, stereospecific assignments of H2′ and H2″ resonances were performed with reference to these *J*-coupling values, in combination with the H3′-H2′/H2″ cross-peak in the COSY spectrum (data not shown).[37] The resulting resonance assignments are listed in Table 2. Thus, we assigned all 173 expected resonances

TABLE 2 Chemical Shift Table for the Di-Mercurated DNA Duplex[a]

	H2/H5/Me[b]	H6/H8[c]	H1′	H2′	H2″	H3′	H4′	H5′/H5″[d]
C1	5.89	7.61	5.74	1.94	2.38	4.69	4.05	3.70/3.70
G2	n.a.	7.96	5.86	2.66	2.69	4.96	4.33	3.96/4.07
C3	5.44	7.34	5.72	2.01	2.43	4.85	4.18	4.13/4.18
G4	n.a.	7.98	6.03	2.58	2.82	4.95	4.39	4.09/4.12
T5	1.56	7.06	5.82	1.89	2.51	4.75	4.12	4.09/4.27
T6	1.80	7.38	5.76	2.22	2.27	4.82	3.99	4.08/4.10
G7	n.a.	7.87	6.02	2.64	2.81	4.92	4.35	3.97/4.08
T8	1.26	7.28	6.14	2.20	2.54	4.90	4.23	4.15/4.17
C9	5.71	7.58	6.09	2.21	2.48	4.82	4.16	4.05/4.11
C10	5.80	7.67	6.24	2.27	2.27	4.56	4.04	3.93/4.15
G11	n.a.	7.81	5.62	2.45	2.62	4.79	4.13	3.62/3.62
G12	n.a.	7.82	5.52	2.67	2.73	4.99	4.34	4.02/4.10
A13	8.01	8.17	6.20	2.66	2.84	5.03	4.46	4.16/4.21
C14	5.24	7.38	5.86	2.29	2.50	4.67	4.28	4.07/4.23
T15	1.72	7.20	5.78	1.72	2.27	4.62	3.92	4.04/4.23
T16	1.64	7.56	6.03	2.25	2.50	4.84	4.16	3.91/3.98
C17	5.66	7.50	5.70	2.11	2.38	4.82	4.08	4.10/4.21
G18	n.a.	7.90	5.90	2.61	2.71	4.97	4.34	4.00/4.10
C19	5.42	7.32	5.76	1.90	2.32	4.81	4.15	4.07/4.09
G20	n.a.	7.94	6.15	2.36	2.61	4.67	4.17	4.05/4.04

[a]Chemical shifts are given in ppm.

[b]Chemical shifts for H2 of adenosine, or H5 of cytidine, or the methyl proton of thymidine.

[c]Chemical shifts for H8 of the purine residues or H6 of the pyrimidine residues.

[d]Chemical shifts for H5′ and H5″. Stereospecific assignments were not carried out.

n.a.: not applicable (Assignments were not applicable due to the lack of the corresponding protons.)

(Table 2). We confirmed that these assignments were consistent within the NOESY and COSY spectra.

From NOESY spectra in the presence (Figure 3a) and absence (Figure 2a) of Hg^{II}, the DNA oligomers were found to be in a duplex form, irrespective of whether the DNA oligomers captured Hg^{II} or not. This is because sequential NOE walks were traceable through the strands in both conditions (Figures 2a and 3a).

Titration Experiments with NMR Spectra

We have already reported the results of primitive titration experiments of this DNA duplex with Hg^{II},[15] performed with reference to imino proton resonances, which were directly exchanged with Hg^{II}. From these experiments, we were able to monitor the direct exchange of imino protons of T-T mismatches with Hg^{II}.[15] However, in order to detect each duplex species (mercury-free, mono-mercurated and di-mercurated DNA duplexes), resonances of non-exchangeable protons (i.e., methyl protons) are superior to those of imino protons (exchangeable protons), because exchangeable

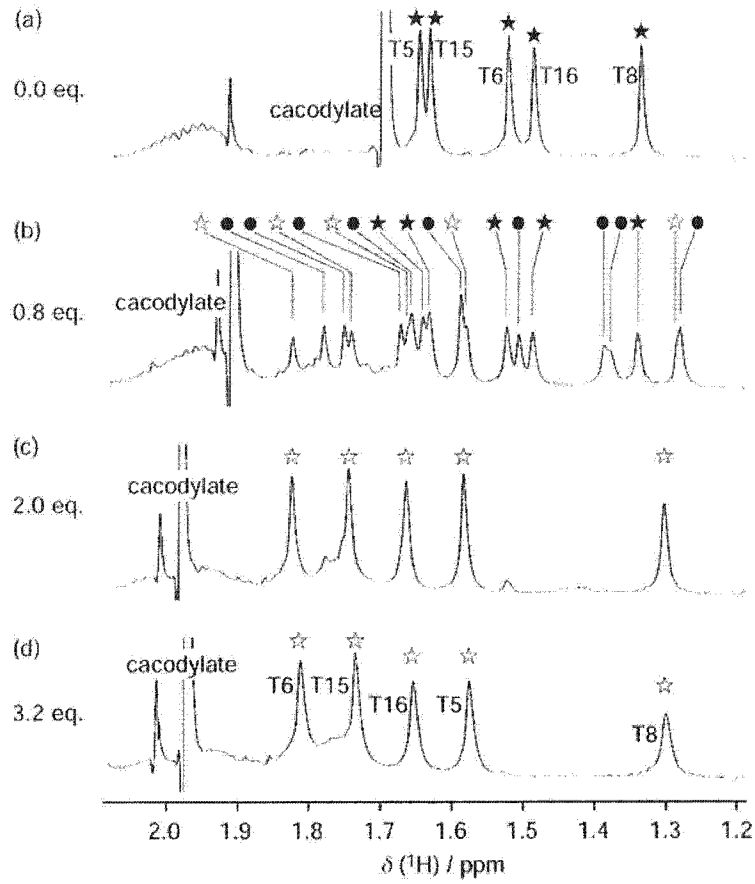

FIGURE 4 Titration experiments using 1D ^{1}H NMR spectra. Molar equivalents ([Hg^{II}]/[duplex]) are presented on the left of each panel. Resonance assignments for methyl protons of thymidines in the mercury-free and di-mercurated duplexes are presented in the top and bottom panels, respectively. Resonances are labeled with black stars (mercury-free duplex), black circles (mono-mercurated duplex), or white stars (di-mercurated duplex), with respect to their origins. Small minor peaks in panel (c) were not assigned currently.[38] Slight signal broadening under Hg^{II}-excess conditions is found to be due to a contamination of trace amount of para-magnetic metal ions in the $Hg(ClO_4)_2$ reagent.

proton resonances often disappear due to chemical exchanges during structural transitions.

Therefore, we monitored methyl proton resonances of thymidines in the presence of various concentrations of $Hg(ClO_4)_2$ (Figure 4). This is because methyl proton resonances are observed in a region that is independent from other resonances and T-T mismatches are the target sites for Hg^{II}. From the titration spectra, it was found that most duplexes were converted into the di-mercurated species with 2 molar equivalents of Hg^{II} to the duplex (Figure 4c).[38] This observation is consistent with the results of titration experiments using UV spectra (Figure 1). During the course of the titrations, independent signals from mercury-free and di-mercurated DNA duplex species were observed simultaneously (Figure 4b). More interestingly, in addition to these signals, we observed unassigned signals other than those from the mercury-free and di-mercurated species (Figure 4b). This is a clear

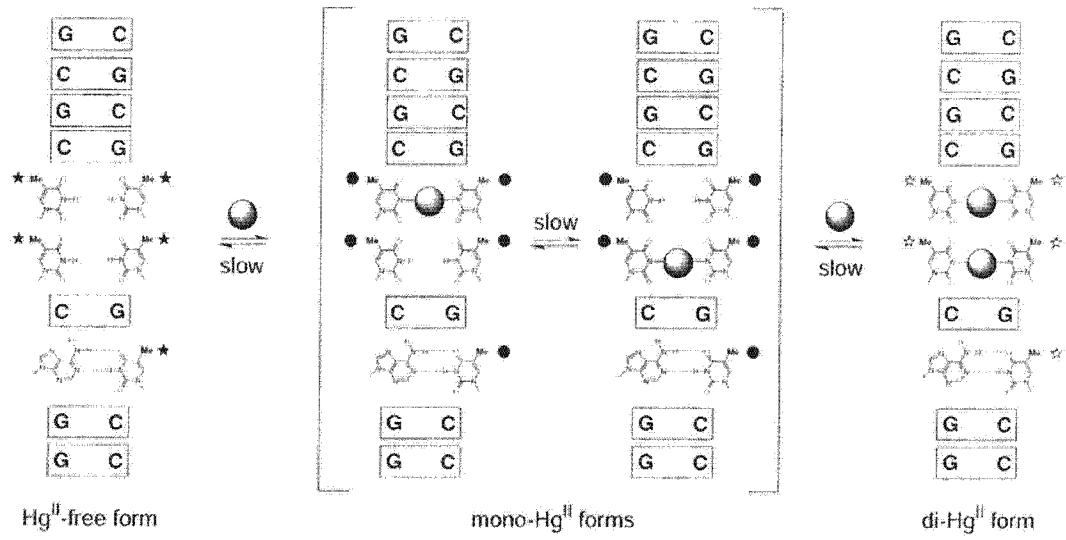

FIGURE 5 Equilibrium system of mercury-free, mono-mercurated and di-mercurated duplexes. Methyl groups are labeled with black stars (mercury-free duplex), black circles (mono-mercurated duplex), or white stars (di-mercurated duplex), as shown in Figure 4.

indication of the existence of transient species. Furthermore, this indicates that the Hg^{II}-association and dissociation processes were so slow that individual species in solution gave independent signals. Thus, this DNA duplex-Hg^{II} complex is an interesting system for the physicochemical studies of nucleic acid-metal complexations, because of the observation of transient species.

A possible candidate for the transient species is two DNA duplexes in complex with a single Hg^{II} at one of the T-T mismatches (Figure 5). If this is the case, there would be two mono-Hg^{II} species and, in total, four species of DNA duplexes possible, namely one Hg^{II}-free form, two mono-Hg^{II} forms and one di-Hg^{II} form (Figure 5). Therefore, 20 methyl proton signals should be observed (four species each with five methyl groups) under Hg^{II}-unsaturated conditions. At least 19 signals were identified at 0.8 molar equivalents of Hg^{II} to one DNA duplex (Figure 4b). It should also be mentioned that we could easily identified the ^1H resonances of the transient species because of the rigid assignments of methyl proton resonances of the mercury-free and di-mercurated DNA duplexes. Consequently, we unambiguously determined the existence of a transient species, and were able to account for the NMR spectra based on the structural transition between mercury-free, mono-mercurated and di-mercurated DNA duplexes. In our previous paper, we observed transient signals of imino protons, as well.[15] Accordingly, it was reconfirmed that these transient signals of imino protons arose from two kinds of mono-Hg^{II} species.

CONCLUDING REMARKS

We performed NOE-based resonance assignments for the mercury-free and di-mercurated DNA duplexes, and assigned most of the

non-exchangeable proton resonances except for several H5′/H5″ resonances. Then, we performed titration experiments using 1D ^{1}H NMR spectra and found that the Hg^{II} association and dissociation processes were so slow that species in solution gave independent ^{1}H resonances. Because of the resonance assignments of the mercury-free and di-mercurated species, we could unambiguously identify ^{1}H resonances from transient species, most likely two mono-mercurated species. The titration NMR spectra were consistent with the structural transitions between the mercury-free, mono-mercurated and di-mercurated DNA duplexes.

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38. In Figure 4c, unassigned small signals were observed, and there are two possibilities for this result. One is the possibility that the unassigned signals are those from a transient species, and other signals of the transient species are overlapped with those from di-mercurated species. The other is that unassigned signals arise from an unknown minor conformer. Currently, we can not determine which hypothesis is plausible.
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Letter to the Editor

^{1}H, ^{13}C and ^{15}N resonance assignments of the VAP-A: OSBP complex

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The 25-hydroxycholesterol (25-OHC) traffic by oxysterol binding protein (OSBP) is mediated by complex formation of OSBP with VAMP-associated protein-A (VAP-A). VAP-A is an endoplasmic reticulum (ER) integral membrane protein that contains a cytoplasmic major sperm protein (MSP) domain. There is a FFAT (referring to two phenylalanines [FF] in an acidic tract) motif in OSBP that is recognized by the MSP domain of VAP-A. Thus, the interaction facilitates localization of OSBP to the ER, where newly synthesized 25-hydroxycholesterol (25-OHC) efficiently binds to OSBP (Wyles et al., 2002). As a first step toward understanding the mechanism of 25-OHC traffic mediated by VAP-A and OSBP, we performed NMR studies of the complex composed of a VAP-A MSP domain (5–128) and an OSBP peptide (345–379) containing the FFAT motif. 2D, 3D and 4D NMR experiments were performed with the complex composed of ^{13}C- and ^{15}N-labeled VAP-A and non-labeled OSBP, ^{13}C- and ^{15}N-labeled OSBP and non-labeled VAP-A, and ^{15}N-labeled VAP-A and ^{15}N-labeled OSBP. All resonances of the backbone nuclei (^{1}HN, ^{15}N, $^{13}C\alpha$ and $^{13}C'$) of the complex were assigned with the exception of N34 C′ of VAP-A. Furthermore, more than 90% of the side chain ^{1}H and ^{13}C resonances of the complex were also assigned. T367-G373 of OSBP showed minor peaks possibly derived from a minor conformation. BMRB deposit with accession No. 7025.
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