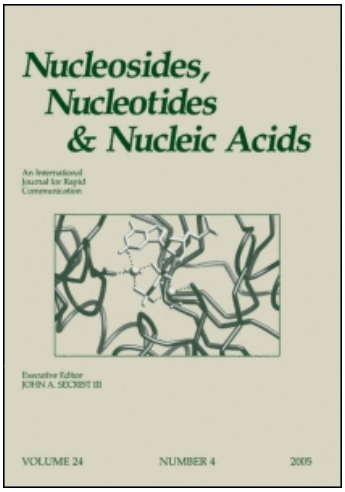

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NMR SPECTROSCOPIC STUDY OF A DNA DUPLEX WITH MERCURY-MEDIATED T-T BASE PAIRS

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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 ^{1}H resonances for mercury-free and di-mercurated duplexes, and performed titration experiments with Hg^{II} by using 1D ^{1}H 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 ^{1}H 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

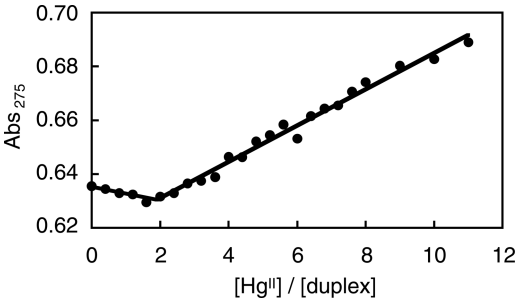

FIGURE 1 Plot of UV absorbances at 275 nm against molar equivalents ([Hg^{II}]/[duplex]). Least-squares fitted lines are shown. Possible explanations for the UV absorbance changes might be due to changes in the spectral intensity of thymidines themselves upon mercuration (0–2 eq.)[15] and putative non-specific 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 *K*d 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

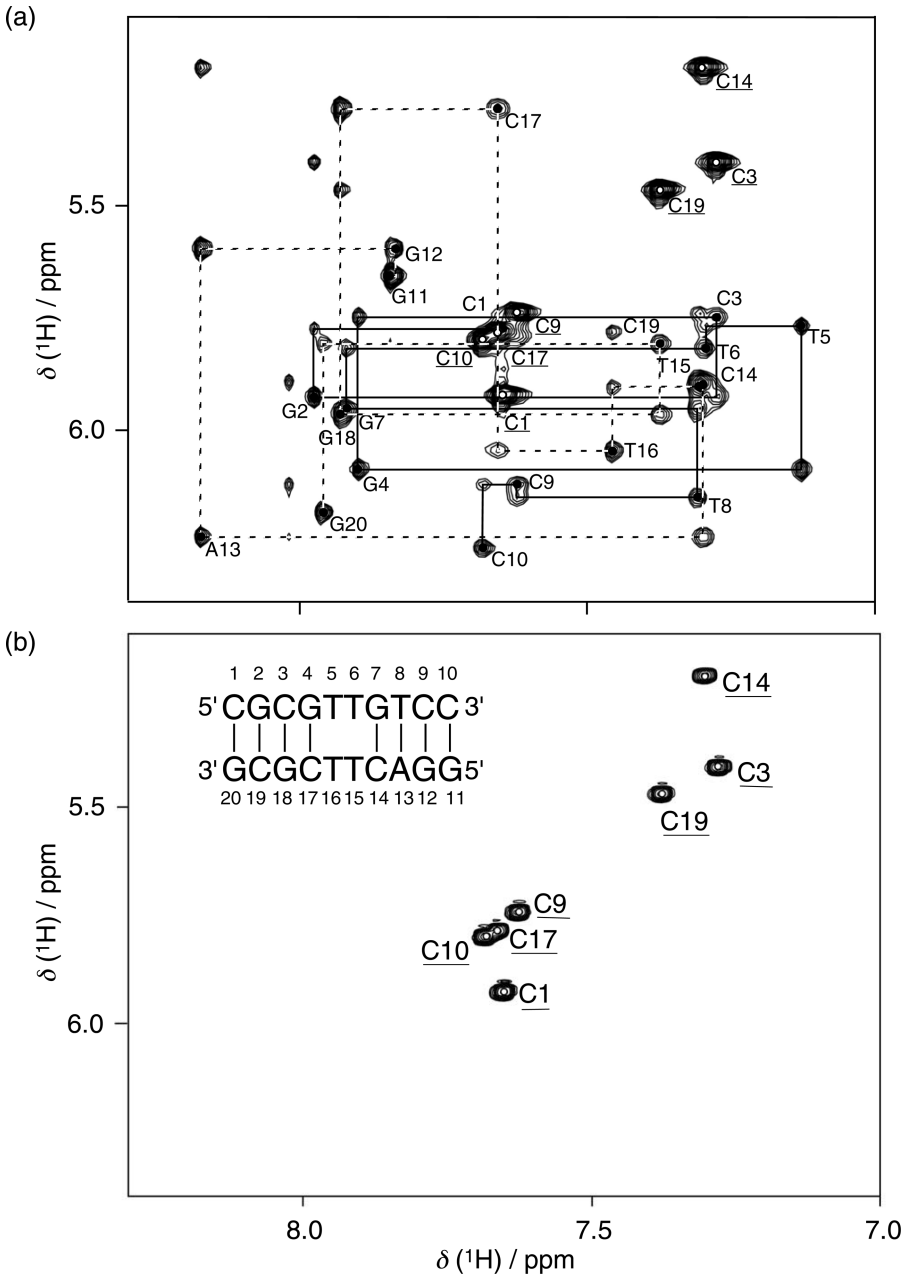

FIGURE 2 NOESY and absolute value COSY spectra for the mercury-free DNA duplex. (a) NOESY spectrum with sequential NOE walks between anomeric (H1′) and base protons (black line: C1-C10; dotted line: G11-G20). Intra-residue cross-peaks are presented with the corresponding residue numbers (closed circles).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

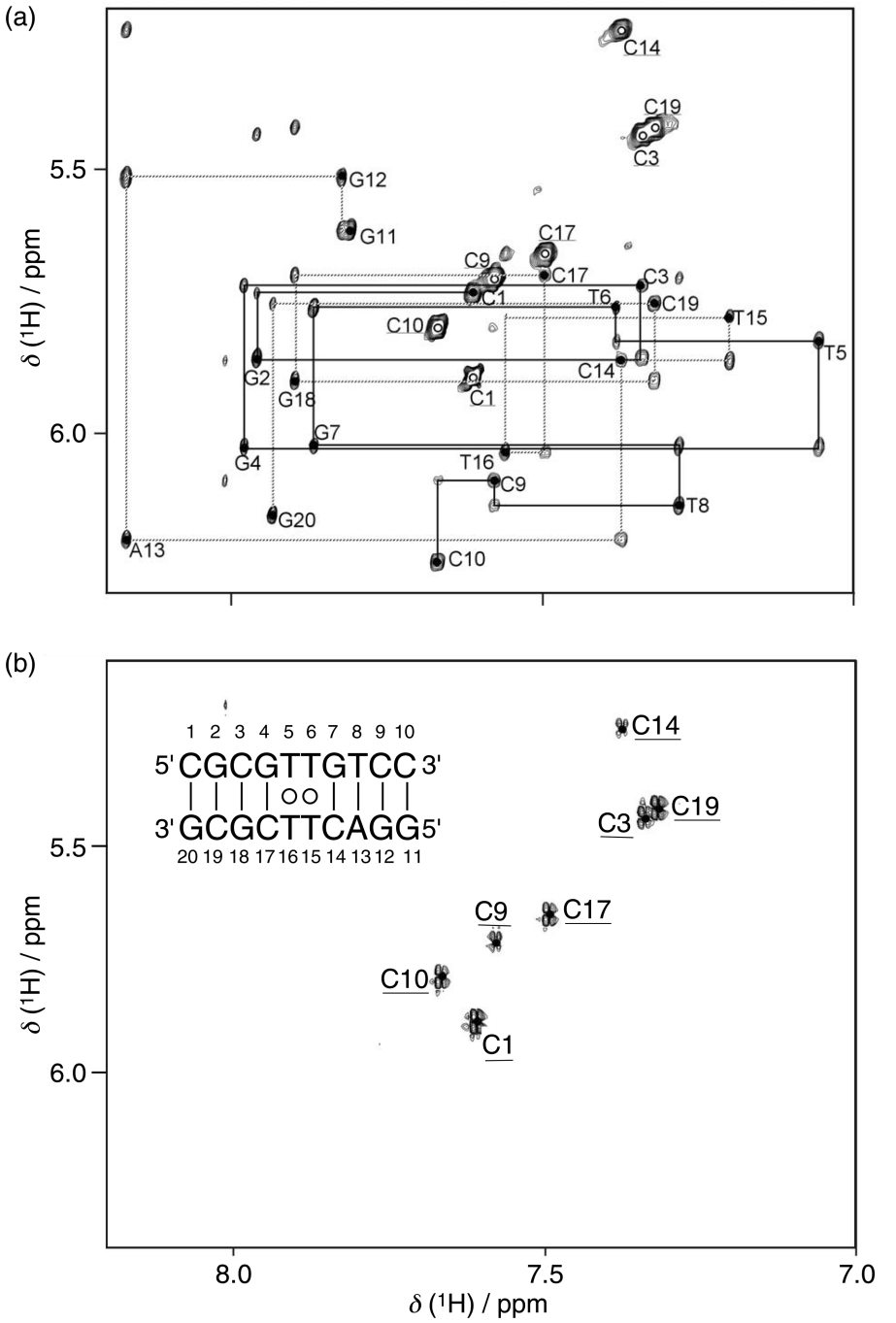

FIGURE 3 NOESY and phase-sensitive COSY spectra for the di-mercurated DNA duplex. (a) NOESY spectrum with sequential NOE walks between anomeric (H1′) and base protons (black line: C1-C10, dotted line: G11-G20). Intra-residue cross-peaks are presented with the corresponding residue numbers (closed circles). (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	2.73	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

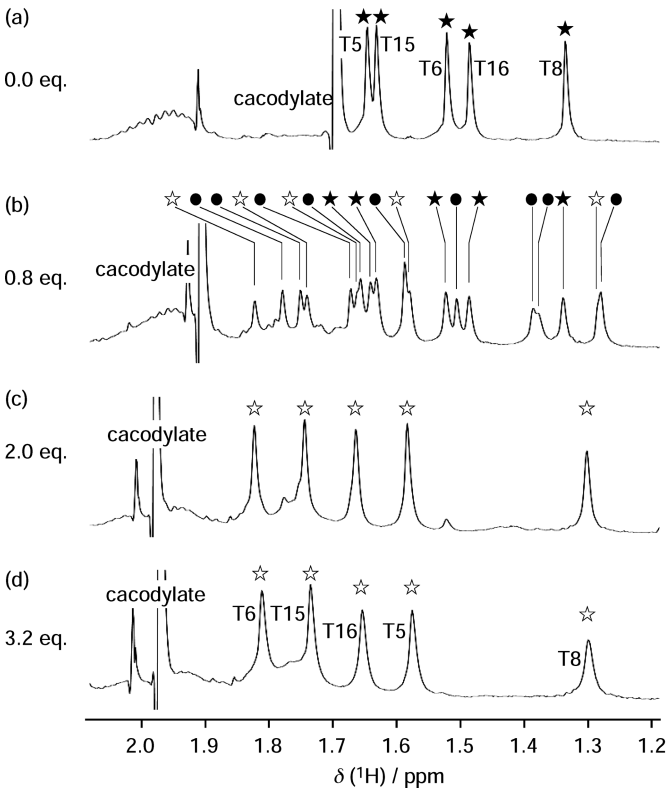

FIGURE 4 Titration experiments using 1D ^{1}H NMR spectra. Molar equivalents ([Hg^{II}]/[duplex]) are presented on the left of each panel. Resonance assignments for methyl protons of thymidines in the mercury-free and di-mercurated duplexes are presented in the top and bottom panels, respectively. Resonances 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

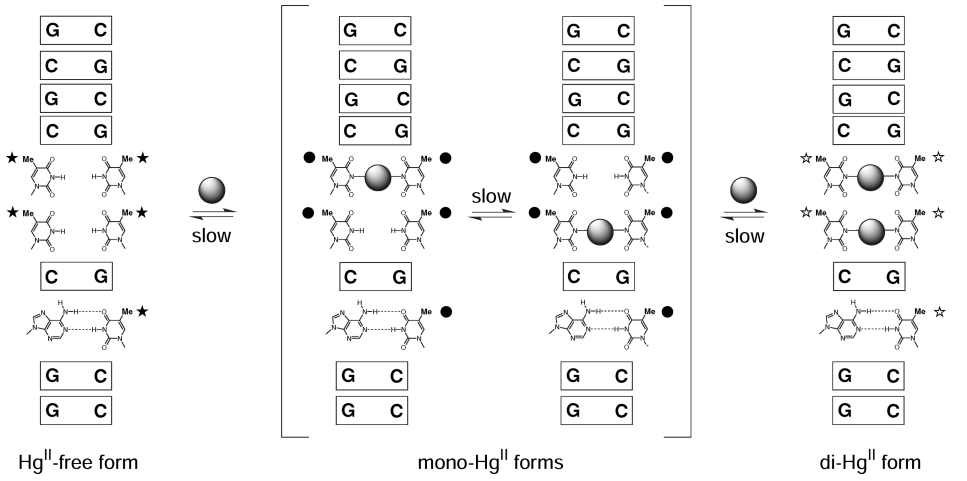

FIGURE 5 Equilibrium system of mercury-free, mono-mercurated and di-mercurated duplexes. Methyl groups are labeled with black stars (mercury-free duplex), black circles (mono-mercurated duplex), or white stars (di-mercurated duplex), as shown in Figure 4.

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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