Raman spectroscopic detection of the T-Hg\textsuperscript{II}-T base pair and the ionic characteristics of mercury

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ABSTRACT

Developing applications for metal-mediated base pairs (metallo-base-pair) has recently become a high-priority area in nucleic acid research, and physicochemical analyses are important for designing and fine-tuning molecular devices using metallo-base-pairs. In this study, we characterized the Hg\textsuperscript{II}-mediated T-T (T-Hg\textsuperscript{II}-T) base pair by Raman spectroscopy, which revealed the unique physical and chemical properties of Hg\textsuperscript{II}. A characteristic Raman marker band at 1586 cm\textsuperscript{-1} was observed and assigned to the C=O stretching mode. We confirmed the assignment by the isotopic shift (\textsuperscript{18}O-labeling at O4) and density functional theory (DFT) calculations. The unusually low wavenumber of the C=O stretching suggested that the bond order of the C=O bond reduced from its canonical value. This reduction of the bond order can be explained if the enolate-like structure (N3=C4-O4) is involved as a resonance contributor in the thymine ring of the T-Hg\textsuperscript{II}-T pair. This resonance includes the N-Hg\textsuperscript{II}-bonded state (Hg\textsuperscript{II}-N3-C4=O4) and the N-Hg\textsuperscript{II}-dissociated state (Hg\textsuperscript{II}+ N3=C4-O4\textsuperscript{-}), and the latter contributor reduced the bond order of N-Hg\textsuperscript{II}. Consequently, the Hg\textsuperscript{II} nucleus in the T-Hg\textsuperscript{II}-T pair exhibited a cationic character. Natural bond orbital (NBO) analysis supports the interpretations of the Raman experiments.

INTRODUCTION

Metal-mediated nucleic acid base pairs are extensively studied molecules that are of interest because of their ability to expand the genetic code and provide new materials for nano-devices (1–15). These artificial base pairs can be made by substituting the natural nucleobases with a planar metal chelator in the DNA molecule (1–15). As an alternative, our group discovered that even the natural base, thymine, can form a stable mercury\textsuperscript{II}-mediated T-T base pair (T-Hg\textsuperscript{II}-T pair) (16–22). The RNA analogue of this molecule (U-Hg\textsuperscript{II}-U) also exists as a stable complex (23–25). The metal-mediated base pairs can only form with Hg\textsuperscript{II}, and they are used in many types of Hg\textsuperscript{II}-sensor (16,26–30). The DNA molecule itself has potential as a component of future nano-devices, and the introduction of a T-Hg\textsuperscript{II}-T pair into the sequence could enable the physical and chemical properties of such materials to be fine-tuned (31–33). Although the T-Hg\textsuperscript{II}-T pair has been extensively studied since 2004 (16,17,26–33), its precise chemical structure was only revealed by \textsuperscript{15}N NMR spectroscopy in 2007 (20–22). In the NMR analysis, the thymine was \textsuperscript{15}N-labeled at N3 and incorporated into the DNA duplex d(CGCGTGTCC)\textsuperscript{15}N and d(GGACTTCGCG) (Figure 1). In the presence of the Hg\textsuperscript{II} ion, the thymine residues formed a T-Hg\textsuperscript{II}-T pair, and \textsuperscript{15}N\textsuperscript{-15}N J-coupling across Hg\textsuperscript{II} (\textsuperscript{2}J_{NN}) was detected (Figure 1c), which provided unambiguous evidence of the formation of the N3-Hg\textsuperscript{II}-N3 bond in the T-Hg\textsuperscript{II}-T pair (20–22). This J-coupling value was theoretically examined by density functional theory (DFT)
calculations (34). Although various data (35–48) hinted at the chemical structure of the T-HgII-T pair prior to these studies, its chemical structure was conclusively determined by these studies (20–22,34).

However, despite elucidation of the precise chemical structure of the T-HgII-T base pair, the nature of the mercury atom in the base pair is unclear. To address this, we used Raman spectroscopy to measure the spectra of the T-HgII-T pair under different conditions. In the Raman spectra, we identified characteristic bands that were sensitive to irregular base-pair linkage that we assigned using site-specific 18O-labeling. These bands could also be interpreted using DFT calculations. Our analysis revealed several interesting properties of the N-HgII bonds, e.g. the bond order by natural bond orbital (NBO) analysis.

MATERIALS AND METHODS

DNA synthesis

DNA oligomers (CGCGTTGTC 1 and GGACTTCGC 2) and non-labeled thymidylyl (3′–5′) thymidine (TpT) were synthesized by the phosphoramidite method (Figure 1a and b), and purified using a reversed-phase column (COSMOSIL 5C18-AR-300; Nakalai Tesque, Kyoto, Japan). The solutions containing non-labeled TpT were evaporated under vacuum several times to remove unwanted triethylammonium acetate buffer and acetonitrile. The DNA oligomers 1 and 2 were further purified using an anion-exchange column (UNO Q-6; BIO-RAD, CA, USA) to exchange the triethylammonium counter ion with sodium. Excess NaCl was removed using a gel filtration column (TSK-GEL G3000PW; TOSOH, Tokyo, Japan) with MILLI-Q water (MILLIPORE, MA, USA) as the mobile phase. Each oligomer was quantitated by UV absorbance at 260 nm after digestion with nuclease P1 (Yamasa, Choshi, Japan). Hg(ClO4)2 (Wako, Osaka, Japan) was used as the HgII source. 18O-labeled TpT at the O4 position (18O4-labeled TpT) was synthesized by the procedure shown in Supplementary Scheme S1; further details are described in the Supplementary Data.

Raman spectroscopy

To prepare the HgII-DNA complex, a solution of 2.0 mM DNA duplex 1 and 2 and 4.8 mM Hg(ClO4)2 were made, and excess HgII cations were removed using a chelating resin (Chelex 100, BIO-RAD) as described previously (20). The resulting solution was concentrated to yield the HgII-DNA complex at a final concentration of 2.0 mM for measurement purposes. To prepare the HgII-free DNA duplex, NaClO4 was added to the 2.0 mM DNA duplex 1 and 2 solution to enable the final concentration of the ClO4– ion to be adjusted to 9.6 mM.

The Raman spectra of TpT were recorded using a 10 mM TpT solution containing 0–1.75 molar equivalents of Hg(ClO4)2 as a simple model for the system. The pH of the solution was adjusted to 6.5 by direct titration with HCl or NaOH. The Raman spectra of thymidine 5′-monophosphate (5′-TMP) were recorded under various conditions. Each sample was sealed in a glass capillary and excited with the 514.5 nm line of a Coherent Innova 70 Ar+ laser. The Raman scattered light was collected with a camera lens, dispersed on a Jasco NR-1800 triple spectrometer, and detected with a liquid-nitrogen-cooled CCD detector. The temperatures of the samples were maintained at 295 K. Raman scattering from the solvent was subtracted from each spectrum.
DFT calculations

The geometry of 1-methylthymine, and two possible patterns of the T-Hg II-T complexes (Supplementary Figure S1) were optimized by Gaussian 03, rev. D02 (49) at the B3LYP/6-31+G(d,p) level of theory with the polarizable continuum model (PCM) of water solvent. The core electrons of the mercury atom were treated using the MWB60 relativistic pseudo-potential, while the valence electrons were treated using the MWB60 basis set. For fully optimized structures, vibrational analysis was performed and the back-scattered Raman intensities were calculated at the same level of theory. The calculated line spectra were weighted by the temperature factor (50,51) and convoluted with Lorentzian band shapes, using 5 cm⁻¹ full width at half height.¹⁸O isotope effects were evaluated using the same force field and different oxygen masses.

The two possible topologies of the T-HgII-T complex shown in Supplementary Figure S1a and b produced almost the same spectra (Supplementary Figure S2), and the topology shown in Supplementary Figure S1a was used for comparison with experiment. Natural charges and bond orders were calculated at the B3LYP/6-31+G(d,p)/PCM(water) level with the NBO 5.0 program linked to Gaussian (49).

NMR spectra of duplex 1•2 and TpT

For the DNA duplex 1•2, 1D ¹H NMR spectra were recorded as Hg II-free, Hg II-bound and Hg II-removed forms. We found that the Hg II atoms can be removed even at temperatures below 100°C (Supplementary Figure S3). NMR spectra were also recorded for TpT to verify the complex formation of TpT with Hg II (Supplementary Figures S4 and S5), and the T-Hg II-T pair formations were confirmed (Supplementary Figures S3–S5).

RESULTS

Raman spectroscopic characterizations of the T-Hg II-T pair

The Raman spectra of the DNA duplex 1•2 in the presence and absence of Hg(ClO₄)₂, are shown in Figure 2. Many small changes were observed in the duplex, in particular, those at 1704, 1576, 1487, 1422, 1372, 1172 and 749 cm⁻¹, caused by the complexation with Hg II. In previous NMR studies (20–22), we confirmed that Hg II exclusively binds to the T-T mismatch sites in the same DNA duplex 1•2 to form two successive T-Hg II-T base pairs, based on the observation of 2-bond ¹⁵N–¹⁵N J-coupling (Figure 1). Therefore, we can attribute the observed Raman spectral changes to the same Hg II-binding.

Because the changes in the Raman spectra of the DNA duplex were small and were overlapped by the stronger Raman bands from the sugar-phosphate backbone and bases other than T, we used the Raman spectra of the thymidylyl (3’–5’) thymidine (TpT) for detailed studies. The spectra acquired in the presence and absence of Hg(ClO₄)₂ are shown in Figure 3. Clearly, the bands present at 1664 and 749 cm⁻¹ were significantly affected by the addition of Hg(ClO₄)₂, which is consistent with the duplex results.
Raman band at 749 cm\(^{-1}\): evidence for the formation of the T-Hg\(^{II}\)-T base pair

The Raman band at 749 cm\(^{-1}\) was perturbed upon the complexation of thymine with Hg\(^{II}\) for both the duplex 1\bullet 2 and TpT (Figures 2 and 3). We next investigated the Raman band of TpT and its Hg\(^{II}\)-complex in H\(_2\)O and D\(_2\)O (Figure 4). Upon the addition of Hg\(^{II}\) to TpT, the Raman band at 749 cm\(^{-1}\) was suppressed (Figure 4c and d). For the Hg\(^{II}\)-free TpT in D\(_2\)O, it is shifted by 13 cm\(^{-1}\) (Figure 4a and b). At this stage, the vibrational mode for the Raman band at 749 cm\(^{-1}\) was found to include the contribution from the imino proton of the thymine base.

To examine if the imino proton contributed to this band, we recorded the Raman spectra of deprotonated thymidine 5'-monophosphate (5'-TMP) under strong basic conditions. The spectra obtained for 5'-TMP showed that the Raman band at 749 cm\(^{-1}\) disappeared under strong basic conditions (Supplementary Figure S6). Thus, its absence upon the addition of Hg\(^{II}\) indicates deprotonation of N3 due to the Hg\(^{II}\)-binding. Furthermore, the spectral change that occurred at around 749 cm\(^{-1}\) in the DNA duplex 1\bullet 2 may be similarly explained (Supplementary Figure S7). In summary, we have demonstrated the T-Hg\(^{II}\)-T pairing by the Raman spectra.

Raman bands around 1664 cm\(^{-1}\) of TpT as a probe for thymine–Hg\(^{II}\) interaction

We observed that the Raman bands around 1664 cm\(^{-1}\) from TpT altered upon Hg\(^{II}\)-binding. As the broad Raman band at 1664 cm\(^{-1}\) includes contributions from both the C2=O2 and C4=O4 stretches of thymine (52,53) and these carbonyl groups are in close proximity to Hg\(^{II}\), the Raman spectral features in this region may be useful for probing the thymine-Hg\(^{II}\) interactions. To examine the spectral changes around 1664 cm\(^{-1}\) in detail, Hg\(^{II}\)-titration experiments of TpT were performed (Figure 5). As the concentration of Hg\(^{II}\) was increased, a shoulder band at 1685 cm\(^{-1}\) lost intensity and a new band at 1586 cm\(^{-1}\) emerged (Figure 5). This strongly suggests that at least one or both of the carbonyl groups C2=O2 and C4=O4 in the T-Hg\(^{II}\)-T pair were affected by Hg\(^{II}\)-binding. In addition, if the newly emerged Raman band at 1586 cm\(^{-1}\) is assigned to the stretching mode of the carbonyl groups, it appears that quite a large perturbation occurred to the C=O double bond(s) in thymine upon Hg\(^{II}\)-complexation.

Raman spectra of \(^{18}\)O-labeled TpT

To determine which of the C2=O2 and C4=O4 groups of thymine was more affected by the Hg\(^{II}\)-complexation, \(^{18}\)O-labeled TpT at the O4 position (\(^{18}\)O4-labeled TpT) was synthesized (Figure 1b). In Figure 6, the Raman

![Figure 4. Raman spectra of TpT (650-830 cm\(^{-1}\)). (a) TpT alone in H\(_2\)O. (b) TpT alone in D\(_2\)O. (c) The Hg\(^{II}\)-TpT complex in H\(_2\)O. (d) The Hg\(^{II}\)-TpT complex in D\(_2\)O.](image)

![Figure 5. Hg\(^{II}\)-titration experiments of TpT by Raman spectroscopy. The molar equivalencies represented by each color are as follows: black: 0.0 eq., indigo: 0.8 eq., blue: 1.2 eq., light blue: 1.3 eq., green: 1.5 eq. and light green: 1.75 eq.](image)
spectra of $^{18}$O$_4$-labeled and non-labeled TpT in the presence and absence of Hg(ClO$_4$)$_2$ are shown, with the main contributions provided by DFT calculations. The 1586 cm$^{-1}$ (non-labeled) band was specifically shifted to 1570 cm$^{-1}$ for the $^{18}$O$_4$-labeled TpT (Figure 6), which clearly indicates that the band originates from the vibrational mode associated with the C=O carbonyl group.

**DFT calculations of the Raman spectra**

To find why the experimental wavenumber of the newly emerged Raman band at 1586 cm$^{-1}$ (TpT) was exceptionally low for a C=O stretching mode, DFT calculations of the Raman spectra for the T-Hg$^{II}$-T base pair were performed (Figure 7). The DFT calculations reproduced the band at 1586 cm$^{-1}$ (assignment details: Figures 6 and 7). The normal mode analysis also confirms that this band comes from the C=O stretching vibration (Figure 8). A closer look revealed that this band was actually composed of two normal modes (Figure 8), namely, the in-phase and out-of-phase combinations of the C=O stretching vibration of thymine bases in the T-Hg$^{II}$-T pair. Both modes involve vibration of all carbonyl groups in the T-Hg$^{II}$-T pair, but the major contribution comes from C4=O.

The calculated Raman spectra of 1-methylthymine and the T-Hg$^{II}$-T pair whose O4 atoms were substituted with $^{18}$O are also shown in Figure 7a and c. In line with our experimental evidence, the theoretical C4=O stretching bands were shifted toward low-wavenumbers (Figure 7c and d).

The shoulder band observed at 1685 cm$^{-1}$ in Hg$^{II}$-free TpT (non-labeled compound) was assigned to the C2=O$_2$ stretching. Upon $^{18}$O$_4$-labeling, this band moved to 1660 cm$^{-1}$ and overlapped with that of the C5=C6 stretching (Figure 6).

**DISCUSSION**

Although the wavenumber of 1586 cm$^{-1}$ is exceptionally low for the C4=O carbonyl stretching compared to normal carbonyl stretching, the $^{18}$O-isotope shift of the Raman bands demonstrated that the main contribution to the characteristic 1586 cm$^{-1}$ band comes from the C4=O stretching mode. The DFT calculations further supported this assignment (Figure 8). The band at 1586 cm$^{-1}$ was also observed by Morzyk-Ociepa and Michalska (48), who tentatively assigned it (based on the DFT calculations) using a deprotonated 1-methylthymine anion as a hypothetical model of the T-Hg$^{II}$-T pair. In contrast, we have simulated the Raman spectra of the T-Hg$^{II}$-T pair using a more realistic system that includes the heavy metal Hg$^{II}$ (1-methylthymine-Hg$^{II}$ (2:1) complex). Consequently, our DFT calculations further revealed that this band was composed of the collective

![Figure 6](image1.png)  
*Figure 6. Raman spectra of (a) $^{18}$O$_4$-labeled TpT, (b) TpT, (c) $^{18}$O$_4$-labeled Hg$^{II}$-TpT complex (Hg$^{II}$/TpT = 1.75) and (d) Hg$^{II}$-TpT complex (Hg$^{II}$/TpT = 1.75). Normal modes for Hg$^{II}$-free 1-methylthymine (non-labeled and $^{18}$O-labeled ones) are shown in Supplementary Figure S10. A rough assignment based on the theoretical spectra (Figure 7) and the normal mode analyses (Figure 8 and Supplementary Figure S10), the main contributors to the experimental Raman bands around 1664 cm$^{-1}$ were assigned as follows. (a) $^{18}$O$_4$-labeled Hg$^{II}$-free TpT: 1660 cm$^{-1}$ C2=O$_2$ stretching and C5=C6 stretching; 1630 cm$^{-1}$ C4=O4 stretching. (b) Hg$^{II}$-free TpT: 1685 cm$^{-1}$ C2=O$_2$ stretching; 1664 cm$^{-1}$ C5=C6 stretching; 1655 cm$^{-1}$ C4=O4 stretching. (c) $^{18}$O$_4$-labeled Hg$^{II}$-TpT complex: 1652 cm$^{-1}$ C2=O$_2$; C5=C6; 1570 cm$^{-1}$ C4=O4 stretching. (d) Hg$^{II}$-TpT complex: 1654 cm$^{-1}$ C2=O$_2$; C5=C6; 1586 cm$^{-1}$ C4=O4 stretching. The assignment of the Raman bands for Hg$^{II}$-free TpT was principally the same as in reference (48).

![Figure 7](image2.png)  
*Figure 7. The high-wavenumber range of theoretical Raman spectra. (a) $^{18}$O$_4$-labeled 1-methylthymine; (b) non-labeled 1-methylthymine; (c) $^{18}$O$_4$-labeled 1-methylthymine-Hg$^{II}$ (2:1) complex; and (d) non-labeled 1-methylthymine-Hg$^{II}$ (2:1) complex. Throughout the calculations, 1-methylthymine was used as a model of thymidine. Major contributors to the Raman bands around the C=O stretching region in the theoretical spectra are as follows: (b), 1712 cm$^{-1}$; C2=O$_2$ stretching; 1686 cm$^{-1}$; C5=C6 stretching; 1570 cm$^{-1}$ C4=O4 stretching. (d), Hg$^{II}$-TpT complex: 1654 cm$^{-1}$ C2=O$_2$ stretching and C5=C6 stretching; 1586 cm$^{-1}$ C4=O4 stretching. The assignment of the Raman bands for Hg$^{II}$-free TpT was principally the same as in reference (48). Asterisks indicate an apparent wavenumber due to the band overlap.*
vibrational modes from all 'four' carbonyl groups in the T-HgII-T base pair (Figure 8).

From a Raman spectral perspective, a lowering of the wavenumber of a carbonyl stretching mode indicates a reduced bond order of the C=O bond. Hence, the resonance effect shown in Figure 9 might be responsible for this phenomenon. Within resonance contributors, the enolate-like structures 6 and 7 in Figure 9 would be responsible for the reduced bond order. As a result, all the resonance effects shown in Figure 9 give the resonance hybrid 8 an average structure. This interpretation is consistent with the observation of the Raman band around 1588 cm\(^{-1}\) for the TpT at pH 12.4, which originates from the deprotonated thymine base at N3 and the resulting enolate-like structure of the thymine bases (Supplementary Figure S8).
In addition, the same resonance effect should also reduce the effective bond order of the N-HgII bond, because in the idealized resonance contributors 2–4, the N-HgII bond is dissociated which makes the character of the N-HgII bond ionic and weaker. The N-HgII bond is thermally cleavable even below 100°C (Supplementary Figures S3), which is indicative of an ionic character. This interpretation is consistent with our previous 15N-NMR study of T-HgII-T pairs. In that study, large down-field shifts of the 15N resonances of N3 were observed upon the complexation of thymine with HgII (20–22), and this chemical shift change was explained by the (partially) ionic character of the N-HgII bond, based on the theory of 15N chemical shifts (21,22,54,55). However, the N-HgII bond is formally a covalent bond and, therefore, we have demonstrated that the N-HgII bonds in the T-HgII-T pair are labile covalent bonds with a significant degree of ionic character.

Next, we considered if there was any relationship between the ionicity of the N-HgII bond and the geometry of the T-HgII-T base pair calculated by the DFT method (Figure 10). The N-HgII-N linkage in the calculated geometry was essentially linear and HgII binding to O4 seems to be weak. The calculated inter-atomic distances between the Hg atom and the keto-oxygen atoms ranged from 3.13 Å to 3.16 Å, whereas those between Hg and N3 were 2.14 Å (Figure 10a). These structural features are consistent with those observed in the crystal structure of the 1-methylthymine-HgII complex (43) (Figure 10b), namely the inter-atomic distances N3-HgII: 2.04 Å; O4-HgII: 2.98 Å; O2-HgII: 3.05 Å. This means that HgII binding to O4 is not necessarily required for the reduction of the C4=O4 bond order.

We further characterized the bond order and the natural charge within the T-HgII-T base pair theoretically (Figure 10c–e; Supplementary Table S1). The calculated results show that the bond order of C4=O4 for the T-HgII-T pair is reduced to 1.17, from 1.21 for a neutral 1-methylthymine base (Figure 10c and d). The bond order of the N3-HgII bond becomes 0.22, which is much less than the bond order of N3–H3 (0.50; Figure 10c and d). This is consistent with our interpretation that the N–HgII bond is less covalent and rather more ionic than N–H and N–C bonds.

Consequently, the HgII atom in the T-HgII-T pair becomes cationic with a calculated natural charge of +1.45 (Figure 10e). This cationic property seems to be related to the theoretical assumption made by Voityuk (31) that the lowest unoccupied molecular orbital (LUMO) is continuously distributed around the HgII nuclei in tandem T-HgII-T base pairs. Even the LUMO orbital in a single T-HgII-T base pair possesses the same character, giving thus good pre-requisite to overlap with the density of neighboring LUMO in a consecutive T-HgII-T step (Supplementary Figure S9). This is because the HgII nucleus is the most electro-deficient part in the T-HgII-T base pairs, and may accept an additional electron. Hence, the cationic nature of the HgII nucleus is an intrinsic property of the T-HgII-T base pair.

Thus, we realistically simulated the Raman spectra, natural charges and bond orders of the T-HgII-T pair, using a model system comprising the heavy metal of HgII (1-methylthymine-HgII (2:1) complex), which is indicative of an ionic character. This interpretation is consistent with our previous 15N-NMR study of T-HgII-T pairs. In that study, large down-field shifts of the 15N resonances of N3 were observed upon the complexation of thymine with HgII (20–22), and this chemical shift change was explained by the (partially) ionic character of the N-HgII bond, based on the theory of 15N chemical shifts (21,22,54,55). However, the N-HgII bond is formally a covalent bond and, therefore, we have demonstrated that the N-HgII bonds in the T-HgII-T pair are labile covalent bonds with a significant degree of ionic character.

In summary, we assigned the observed Raman band at 1586 cm\(^{-1}\) to a carbonyl stretching vibration, with the

![Figure 10. Results of the DFT calculations.](image-url)
main contribution from the C4=O4 stretching mode. The low wavenumber shift of this carbonyl vibration, measured upon adding Hg\(^{II}\), is associated with the reduced bond order of the C4=O4 bond. This is due to the partial enolization of the thymine bases in the T-Hg\(^{II}\)-T base pair. This effect promotes a partial ionic character of the N-Hg\(^{II}\) bond and makes the Hg\(^{II}\) atom in the T-Hg\(^{II}\)-T base pair cationic. Based on the strong agreement between the experimental and theoretical data, we conclude that the Hg\(^{II}\) atom in the T-Hg\(^{II}\)-T base pair is cationic, and that the Hg–N3 bond is less covalent and rather more ionic than N–H and N–C bonds.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Methods, Supplementary Table 1, Supplementary Scheme 1, Supplementary Figures 1–10 and Supplementary Reference [56].

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