Interaction of Mercury(II) with the DNA Dodecamer CGCGAATTCGCG. A ^1H and ^{15}N NMR Study

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Abstract: The interaction between the oligonucleotide $[d(CGCGAATTCGCG)]_2$ and Hg(II) has been studied by ^1H and natural abundance ^{15}N NMR spectroscopy. The titration of the dodecamer with $Hg(ClO_4)_2$ was monitored by ^1H NMR spectroscopy in aqueous solution (pH 7) at 292 K. The titration pattern is consistent with a transition to a new conformer of the dodecamer induced by Hg(II). At intermediate stages of the titration, the ^1H signals from the new conformer coexist with those of the original one, indicating slow exchange between the two forms on the NMR time scale. In the imino region of the ^1H spectrum, the thymine N3H resonances disappear upon Hg(II) addition, showing that Hg(II) interferes with the Watson–Crick hydrogen bonds of the A·T base pairs. At about five Hg(II) ions added per dodecamer duplex, all original ^1H signals have vanished, i.e., the conformational transition is completed. Addition of KCN in 6-fold excess to Hg(II) restores the original ^1H spectrum of the dodecamer. The ^1H resonance assignment of the Hg(II) form of the dodecamer has been carried out by means of two-dimensional (DQF-COSY/NOESY) and one-dimensional (TOE) NMR techniques. Comparison of chemical shifts and NOESY cross peaks of the original and Hg(II) form of the dodecamer indicate that it remains in a B-DNA like conformation. The most prominent changes are found for the AT tract, while the ^1H spectrum of the terminal $d(CGC)_2$ parts of the duplex is hardly affected by the presence of Hg(II). Comparison of the rotational correlation times of the two forms rules out the possibility of a Hg(II)-induced duplex to hairpin transition. Hg(II)-induced ^{15}N chemical shifts and cross peak splitting patterns of the ^1H–^{15}N HMQC spectra confirm that Hg(II) interacts solely with the AT tract of the duplex. The data suggest that four Hg(II) ions form covalent bonds with the four A·T base pairs, involving A5/A6 NH_2 through loss of protons and T7/T8 O4 on opposite strands. The insertion of Hg(II) ions produces a "bulge" in the AT region of the duplex.

Introduction

Recently, we have shown that certain divalent transition-metal ions, Mn(II) and Zn(II), bind preferentially to guanine residues in short oligonucleotides (10–12 base pairs) in a sequence-selective manner.[1,2] These metals are expected to have affinities for the nucleobases as well as the phosphate groups. The denaturation and renaturation of DNA in the presence of different divalent metal ions have been studied through the melting behavior of DNA monitored by UV spectroscopy.[3] The results were interpreted in terms of varying preferences for binding to the bases or phosphate of the nucleotides among the different metal ions, e.g., renaturation on cooling of DNA in the presence of certain metal ions was explained by metal-binding to the bases. "Hard" metal ions like Mg(II) are mainly involved in phosphate binding, while many 3d transition-metal ions, e.g., Co(II) and Ni(II), exhibit significant affinities for both phosphate and bases. The observed effects on DNA in the presence of Cd(II) and especially Cu(II) were explained in terms of even stronger preference for the nucleobases. Further along this trend of increasing base affinity, we find Hg(II) which is known to bind reversibly to DNA.[4] In a study by Thomas, it was concluded from ultraviolet absorption spectroscopy that Hg(II) binds to weakly basic nitrogene sites on the purine and pyrimidine bases.[5] Further studies by UV spectroscopy and potentiometric methods revealed that Hg(II) interacts most strongly with AT-rich DNA, and, irrespective of base composition, initial Hg(II) addition is accompanied by the loss of about two H^+ ions per Hg(II) ion bound and that the formation of a "first" complex is completed at $r = 0.5$ ($r = [Hg(II)]/[nucleotide]$).[6] The reaction of Hg(II) with native DNA can be reversed by means of complexing agents like CN^- or Cl^-. For instance, the transforming activity of Hg(II)-treated pneumococcal DNA is restored after the removal of Hg(II).[7] Katz proposed a chain slippage mechanism for Hg(II) complexes with DNA, where thymines in alternating $d(AT)_n$ polymers cross-link the complementary strands through the formation of N3–Hg–N3 bonds, thus corrupting the conventional Watson–Crick hydrogen bonds.[8] An alternative to the chain slippage model, is the cross-linking by Hg(II) of the original base pairs, with proton loss on the amino groups of adenine and cytosine in addition to proton loss on thymine N3 and guanine N1.[9] Early UV spectroscopic studies of the interaction of Hg(II) with monomer nucleosides (and DNA polymers)[10] and UV spectroscopy combined with blocking of amino groups of the nucleosides by formaldehyde[11] supported the assumption that Hg(II) binds N3 (or O2/O4) on thymine, N1 (or O6) on guanine, and NH_2 on cytosine and adenine. The relative affinities of Hg(II) to the monomer nucleosides were found to decrease in the order T > G ≫ A,C.[10]

Recently, Gruenwedel and Cruikshank have shown that the exposure of native calf thymus DNA to increasing amounts of $Hg(ClO_4)_2$ produces profound changes in its CD spectrum at $0.01 < r < 1.0$ as well as a decrease in the endonucleolytic DNA cleavage rate by staphylococcal nuclease at $0.08 < r < 0.5$.[12] In agreement with the results of Dove and Yamane,[7] these changes

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can be reversed upon removal of Hg(II) by NaCN. These observation are explained by interstrand cross-linking of DNA bases by Hg(II), thus assisting its complete renaturation. The changes (inversions) in the CD spectrum of DNA upon addition of Hg(II) is explained by a transition of the secondary structure of DNA from B-form ($r < 0.01$) through C-form DNA ($0.01 < r < 0.12$) to *Z*-form DNA ($0.12 < r \leq 1.0$). In contrast to Hg(II), CH_3Hg^{II} will accelerate the endonucleolytic cleavage of DNA. The changes induced by CH_3Hg^{II} are not reversible upon removal by NaCN. This is explained by CH_3Hg^{II}-binding to DNA without making cross-links, thus producing single-stranded DNA irreversibly. The reactions of Hg(II) with DNA constituents have been studied by ^{1}H NMR spectroscopy.[13-16] Young *et al.* observed a loss of thymine imino protons (T NH3) from poly(dA-dT) upon the addition of Hg(II) as monitored by ^{1}H NMR in H_2O solution. However, there were no indications of denaturation of the "Hg(II) complex" of poly(dA–dT) even when the sample was heated to 65° as monitored by ^{1}H NMR and UV spectroscopy. In contrast, on addition of Hg(II) the UV spectrum of poly(dA)·poly(dT) display hyperchromism, indicating some loss of secondary structure.[15] Moreover, the changes of the UV spectrum of poly(dA-dT) suggest that there is one particular binding mode below $r = 0.25$, at the same ratio as the T NH3 resonances have completely vanished from the ^{1}H NMR spectra. These results were interpreted as due to genuine Hg(II) binding at T N3, thus supporting Katz' chain slippage model for cross-linking of two DNA strands by Hg(II).[8]

Changes in ^{1}H NMR chemical shifts of monomer thymine base protons upon the addition of Hg(II) were small, while those of the thymines of poly(dA-dT) displayed larger shift effects. These observatiosn indicate that the latter effects are mainly due to changes in the secondary structure and that intrinsic shift changes due to the covalent binding of Hg(II) to the nucleobase ring system are negligible.[15] In a complementary binding study of Hg(II) to poly(dA-dT) and poly(dA)·poly(dT) (and its single strand components) by means of UV and CD spectroscopy, Sarker *et al.* found evidence for stronger binding to thymine than to adenine.[17] Buncel *et al.* found evidence from ^{1}H and ^{13}C NMR for mixed-ligand Hg(II) complexes of nucleosides in DMSO, i.e., T–Hg–G is formed in addition to its more stable homonucleoside counterparts; significant ^{13}C chemical shifts were interpreted in terms of Hg(II) binding at T N3 and G N1.[16] Buchanan and Stothers have utilized ^{15}N NMR spectroscopy for probing metal–nucleoside interactions and changes in ^{15}N chemical shifts of three nucleosides where taken as evidence for the binding of Hg(II) to G N1, A N7, and T N3.[18]

Recently, Jia *et al.* have demonstrated the power of multinuclear (^{1}H, ^{31}P, and ^{13}C) NMR spectroscopy in gaining insight into the nature of complexes of single diamagnetic metals ions, like Zn^{2+}, with oligonucleotides.[19]

In the present study we will, in an analogous manner, try to extend ^{15}N NMR studies of monomer nucleotide/Hg(II) complexes to oligomers by applying natural abundance ^{1}H–^{15}N HMQC correlation spectroscopy. The results from these experiments supplement 1D and 2D ^{1}H NMR data.

The DNA oligomer chosen for our studies, [d(CGCGAATTCGCG)]$_2$, has been the subject of thorough investigations in the solid state by X-ray crystallography[20] as well as in solution by NMR methods.[21-23] The dodecamer contains the recognition sequence d(GAATTC) of the restriction enzyme *Eco*RI. The interactions of this dodecamer with Zn(II) and Mn(II) have previously been investigated by NMR methods in our laboratory.[1,2]

Experimental Section

Sample Preparations. The DNA dodecamer [d(CGCGAATTCGCG)]$_2$ was obtained from Dr. B. R. Reid (University of Washington, Seattle, WA). It was synthesized using the solid-phase phosphite triester technique.[21] The synthetic DNA was purified by chromatography in distilled water on a 120-cm Sephadex G-25 column and lyophilized to dryness. The palindrome dodecamer readily forms a duplex, and therefore the subsequent annealing step normally used for non-palindrome oligomers was unnecessary. As judged from ^{1}H NMR spectra, Figures 1 and 2, impurities giving rise to signals at 8.46, 3.36, 3.23, 3.13, 2.57, and 0.63 ppm, amount to less than 10%. The impurities probably originate from incomplete removal of various protecting groups used during the DNA synthesis.

During the NMR investigations three different DNA samples were used. Two concentrated solutions contained about 330 and 370 OD_{260} units of DNA dodecamer, samples A and B, and a third one only 23 OD_{260} units, sample C. Assuming that the dodecamer adopts a single stranded random coil conformation in aqueous nonbuffered solutions, the UV absorbances correspond to ca. 3.7, 4.1, and 0.26 mM of duplex form in a 0.4-mL solution, where $\epsilon_{260} = 1.107 \times 10^5$ M^{-1} cm for one strand of our oligomer.[24] The amounts of DNA were also estimated by weighing of DNA samples A and B after drying and by addition of an intensity standard into the NMR tube after the final NMR measurements were completed. These methods gave higher concentrations of DNA, ca. 4.8 and 5.4 mM for samples A and B, respectively. These results indicate that a large fraction of DNA has an ordered structure at the measuring conditions employed, cf. $\epsilon_{260} \approx 8.2 \times 10^4$ M^{-1} cm per strand for the oligomer in duplex form.[24]

The DNA sample A (4.8 mM) was dissolved in 0.4 mL of buffer containing 170 mM sodium phosphate (pH 7.0) and 200 mM $NaClO_4$. Samples B (5.4 M) and C (0.34 mM) were dissolved in 0.4 mL of buffers containing 10 mM sodium phosphate (pH 7.0). The buffers were made from analytical reagents from E. Merck. The solutions were centrifugated to eliminate any solid particles and finally transferred to 5-mm NMR tubes. The DNA samples were repeatedly lyophilized to dryness from 99.9% D_2O and then once from 99.96% D_2O. Finally, the samples were dissolved in 0.4 mL of 99.996% D_2O. The samples were lyophilized directly in the NMR tubes to avoid the critical transfer step of the highly deuterated solvent. No internal chemical shift standard was used. The pH of the deuterated solutions was not corrected for isotope effects. After completing the experiments on the Hg(II)-free solution of DNA samples A and B that required D_2O as a solvent, the samples were lyophilized to dryness and redissolved in H_2O. Then the exchangeable proton signals of the DNA oligomer were observed after successive additions of aliquots of $Hg(ClO_4)_2$ to the solutions. Finally, the Hg(II)-containing solution of sample A was repeatedly lyophilized to dryness and redissolved in D_2O as already described.

The stock solution used for the Hg(II) titration was prepared as follows: 6.7 g of $Hg(ClO_4)_2$ (99+% from Alpha Products) was dissolved in 50 mL of H_2O. A small amount of white precipitate which became yellow and finally red on standing was filtered off by means of a fine porosity (G4) filter. The clear solution was fairly stable when kept cool, and only a minute amount of precipitate appeared after several weeks. The concentration of Hg(II) was determined by atomic absorption spectroscopy: [Hg(II)] = (0.25 ± 0.01) M. Aliquots of metal salt solution (10–45 μL) were added directly into the NMR tubes with a micropipette.

After finishing the final NMR experiment on the Hg(II)-containing sample A, 20-80 μL of 0.9 M KCN was added to the DNA solution, and ^{1}H NMR spectra were recorded after each aliquot added.

^{1}H NMR Spectroscopy. The ^{1}H NMR experiments were performed at 400.13 MHz on a Bruker AM-400 WB spectrometer. In order to obtain a reasonable resolution all experiments involving only the nonexchangeable proton signals were carried out at 310 K. The temperature was lowered to 292 K while observing the exchangeable protons to counteract the effects from exchange with water protons. Two different one-dimensional proton spectra were recorded for the samples

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A and B dissolved in H_2O, at a probehead temperature of ca. 292 K. (i) The water resonance was suppressed by a weak irradiation field (ca. 70 Hz) during the recycling delay to obtain acceptable spectra in the chemical shift regions close to the water resonance. The spectral width was 8333 Hz, the pulse width 9.5 μs, the recycling delay 2 s, 8192 data points were acquired in quadrature detection mode, and 32 transients were averaged. (ii) The water resonance was suppressed by selective excitation of the exchangeable and aromatic protons by a time-shared Redfield "2-1-4" -pulse centered at 2580 Hz upfield from the water resonance.[25] The 15° pulse was 11.0 μs, and optimal values for the asymmetric delays "a" and "b" were 26.7 and 27.3 μs, respectively. The spectral width was 8621 Hz, the recycling delay 1.5 s, 4096 data points were acquired in quadrature detection mode, and 72 transients were averaged.

One-dimensional "truncated driven noe" (TOE) difference spectra were recorded for the H_2O solution prior to the Hg(II) titration of DNA sample A and after the final amount of Hg(II) had been added.[26] The imino proton resonances were saturated by a weak irradiation field, ca. 15 Hz, using the decoupler. The spectrum was excited by means of a time-shared Redfield "2-1-4" pulse as already described. A total of 256 transients were averaged for each spectrum, the recycling delay was 2.0 s, and irradiation times in the range 35–350 ms were used.

The ^{1}H NOESY[27,28] spectra were recorded in the pure-absorption mode with quadrature detection using the hypercomplex acquisition scheme.[29] A total of 1024 complex points in t_2 were collected for each of 1024 t_1 increments, 32 transients were averaged for each increment, the spectral width was 4000 Hz, and a relaxation delay of 2 s was used. The probehead temperature was kept at 310 K. The following mixing times were employed for both the Hg(II)-free DNA sample A and after the addition of Hg(II): 0, 50, 100, 150, 200, and 300 ms. The total experimental times were 23–26 h. In all cases the mixing time was randomly varied (±10–30 ms) in order to suppress (actually smear out along F_1!) zero-quantum J cross peaks.[28] We have modified the standard NOESY experiment to completely eliminate all baseline artifacts except for offsets along F_2. These offsets are easily removed by baseline correcting software. The modified NOESY scheme is based on spin-echo detection in both t_1 and t_2 accompanied with careful timing of the acquisition to avoid undesired effects from the RF filters.[30] As an extra advantage, the only phase correction needed is the zeroth order correction in F_2. This modification has proven invaluable in the effort to obtain reliable cross peak volumes from NOESY spectra with crowded diagonals of broad resonance lines.

^{1}H DQF-COSY spectra were collected for both the Hg(II)-free and Hg(II)-containing DNA sample A. They were acquired in the pure-absorption mode[31] with quadrature detection using the TPPI method.[32,33] The spectral width was 3125 Hz, 1024 complex points were acquired for each of the 1024 t_1 increments, 32 transients were averaged for each increment, and a relaxation delay of 1.5 s was used. The temperature was 310 K. The total experimental time was 19 h. To avoid "rapid-pulsing" artifacts and the necessity of using "dummy" transients, a phase cycling scheme due to Derome and Williamson was used.[34] We have modified this scheme by finding a permutation of the transients which include full four-step CYCLOPS,[35] axial peak suppression, and "rapid-pulsing" artifact cancellation within 32 transients.[36]

NOESY spectra were taken in 90% H_2O/10% D_2O solution of the Hg(II)-free and the Hg(II)-containing DNA sample A. The preparation and mixing pulses were relatively strong nonselective pulses, 63 μs 90° pulses, providing nearly uniform excitation along F_1. In order to suppress the water resonance, the detection pulse was substituted for a time shared Redfield "2-1-4" pulse as described above. Owing to the nonlinear phase distortions of "2-1-4" spectra, magnitude mode NOESY spectra were used. They were recorded with quadrature detection, 2048 complex points were acquired for each of 400 t_1 increments, and 64 transients were averaged for each increment. The spectral width was 8333 Hz, the recycling delay 2 s and the mixing time 400 ms. The sample temperature was 292 K, and the experimental time was 22 h.

^{15}N NMR Spectroscopy. Two-dimensional ^{1}H-detected ^{1}H–^{15}N HMQC shift correlation experiments were performed on a Bruker AM-400 WB spectrometer at a ^{15}N frequency of 40.562 MHz and at natural abundance of ^{15}N: $90_x(^1H)$–$1/2J_{NH}$–$90_\phi(^{15}N)$–$t_1/2$ – $180_x(^1H)$–$t_1/2$ – $90_x(^{15}N)$-acquire(^{1}H).[37] Spectra were acquired for both the Hg(II)-free and Hg(II)-containing DNA sample A in D_2O at 310 K. The experiment was optimized for two-bond ^{1}H–^{15}N connectivities involving nonexchangeable protons, where $\Delta = 1/2J_{NH} = 50$ ms. Coupling constants $^2J_{NH}$ for guanosine and adenosine are found in the range 10–17 Hz,[38,39] except for $^2J_{N9\text{-}H8}$ which is found to be 3.7–5 Hz.[38] The corresponding coupling constants for the pyrrole-like nitrogen N1 of the pyrmidines are likely to be found in the same range.[40] The data were acquired in quadrature detection mode and yielded absolute value spectra. The ^{1}H and ^{15}N spectrals widths were 4000 and 6203 Hz, respectively. The data were acquired into 1024 complex points along t_2, 1024 (2048 with Hg(II) added) transients were averaged for 128 t_1 values (80 with Hg(II) added), the recycling delay was 1.5 s, and the experimental time was 71 h (89 h with Hg(II) added).

NMR Data Processing. The NMR data were processed on a Micro VAX 3100-80 and a Silicon Graphics IRIS 4D/35 using the programs FTNMR and FELIX.[41] The one-dimensional ^{1}H FIDs obtained from H_2O solutions were multiplied by an exponential window prior to Fourier transformation. Typically, 2 Hz was added to the line widths. The spectral baselines were corrected by third-order polynomial fits or cubic spline interpolations. The spectra were referenced to the water resonance set to 4.82 ppm at 292 K. DQF-COSY and NOESY data were zero-filled to 2048 complex points along t_2, multiplied by a 90° phase-shifted and squared sine function, and subjected to a complex Fourier transform. The spectra along F_2 were phase corrected (only a zeroth-order correction for the NOESY spectra) and baseline corrected by subtraction of an offset fitted to the edges of the NOESY spectra; the DQF-COSY spectra were corrected by a third-order polynomial fit. Along t_1, the data were zero-filled and apodized in an identical manner and finally subjected to a real (for TPPI data) or complex (hypercomplex data) Fourier transform. Prior to Fourier transformation along t_1, the first t_1 increment (F_2 spectrum) was multiplied by 0.5 to avoid the t_1 noise originating from improper scaling in the discrete Fourier transform algorithm.[42] This correction is incorporated into the Fourier transform routines of FELIX. The chemical shift of the HDO resonance was set to 4.68 ppm, while the sample temperature was kept at 310 K (*vide supra*). The NOESY data obtained from DNA in 90% H_2O/10% D_2O solution were apodized with a squared and skewed (0.4) sine bell and subjected to a complex Fourier transform. Along t_1, the data were zero-filled to 2048 complex points, apodized with a plain sine bell window, Fourier transformed (complex), and the magnitude spectra were calculated. The ^{1}H–^{15}N data were multiplied by a squared and phase-shifted (90°) sine bell along t_2 and subjected to a complex Fourier transform. The data were zero-filled to 256 complex points along t_1, multiplied by a phase-shifted (45°) sine bell function, Fourier transformed (complex), and the magnitude spectra were calculated. The chemical shifts of the ^{15}N resonances were referenced to G2 N9, its chemical shift set arbitrarily to –207.2 ppm, to obtain shift values close to those of monomeric nucleotides,[38] but with the signs reversed.

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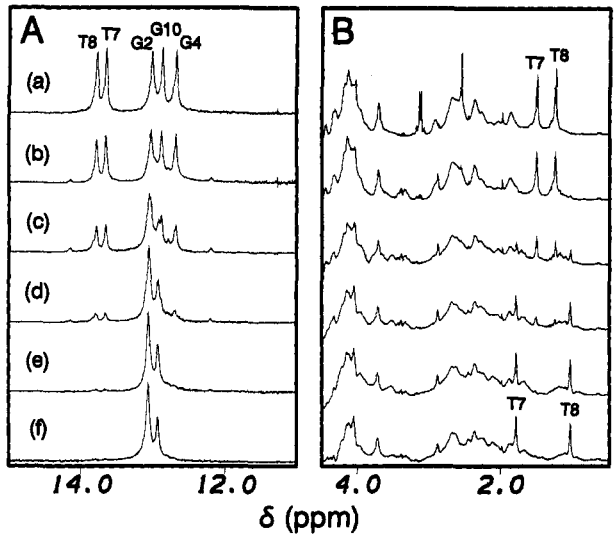

Figure 1. 400-MHz ^{1}H NMR spectra obtained from a 4.8 mM solution of the duplex form of the dodecamer [d(CGCGAATTCGCG)]$_2$ in a buffer containing 170 mM sodium phosphate (pH 7.0) and 200 mM $NaClO_4$ (sample A) in 90% H_2O/10% D_2O and with successive amounts of $Hg(ClO_4)_2$ added. The Hg(II) concentrations were as follows: (a) 0 (b) 6, (c) 13, (d) 19, (e) 15, and (f) 28 mM. The spectra in the left column (A) show the imino proton region. These spectra are obtained by means of a time-shared Redfield "2-1-4" pulse to avoid the excitation of the H_2O resonance. The spectra in the right column (B) show part of the sugar proton region and the thymine methyl resonances. For these spectra the H_2O resonance has been suppressed by means of presaturation. The probehead temperature was kept at 292 K.

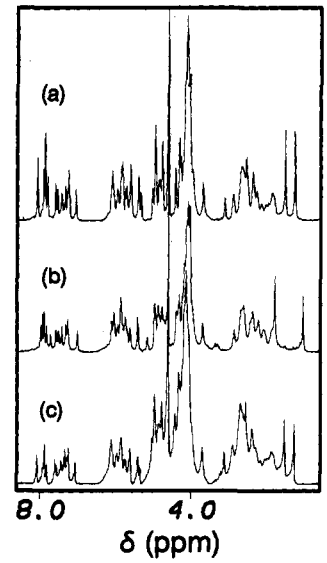

Figure 2. 400-MHz ^{1}H NMR spectra of the *same* dodecamer [d(CGCGAATTCGCG)]$_2$ sample (sample A, see caption of Figure 1) in D_2O: (a) no $Hg(ClO_4)_2$, (b) 28 mM Hg(II), and (c) 28 mM Hg(II) + 180 mM KCN. Spectra (a) and (b) are taken from the first t_1 increments of the 300 ms NOESY spectra shown in Figure 3 and 4, respectively, recorded under *identical* experimental conditions, acquisition parameters, etc. Spectrum (c) is recorded in a separate 1D experiment under the same conditions. An absolute intensity scale is used (FTNMR/FELIX: AI = 1), and by assuming similar relaxation behavior in the cases (a)–(c), corresponding resonance integrals are directly comparable. However, signal intensities are only approximately representing proton concentrations due to slight differences in signal widths and shapes for the cases (a)–(c) (see text). The low intensity of the proton signals in the region 7.7–8.1 ppm of spectrum (c) is due to enhanced proton/deuteron exchange at position H8 of the purines in the presence of Hg(II) (see text). The probehead temperature was always kept at 310 K.

Results and Discussion

The dodecamer [d(CGCGAATTCGCG)]$_2$ is self-complementary, and we will primarily refer to the numbering scheme for one strand: 5′-d($C_1G_2C_3G_4A_5A_6T_7T_8C_9G_{10}C_{11}G_{12}$)-3′. When considering cross-strand interactions, we will occasionally use a prime (′) to denote the second strand.

Hg(II) Titration and 1D ^{1}H NMR Spectra. The titration of sample A, 4.8 mM [d(CGCGAATTCGCG)]$_2$ in 0.2 M $NaClO_4$ and 0.17 M sodium phosphate, with $Hg(ClO_4)_2$ was monitored by ^{1}H NMR in H_2O solution. Solvent suppression was achieved by means of selective excitation and presaturation in an alternating manner to emphasize the imino/amino and nonexchangeable proton regions, respectively. By increasing the concentration of Hg(II), the signals of the thymine imino hydrogens, T7 NH3 and T8 NH3, disappear as well as those of the guanine imino protons G4 NH1 and G10 NH1 (Figure 1A). Simultaneously, there is a buildup of intensity close to the original shift (13.02 ppm) of G2 NH1 at 13.06 ppm, and a new resonance gradually appears at 12.93 ppm as well. Moreover, at lower Hg(II) concentrations weaker resonances emerge at 12.20, 12.79, and 14.14 ppm, while disappearing again at higher Hg(II) concentrations. The most prominant change in the nonexchangeable part of the proton spectrum is the reduction in the intensities of the thymine proton resonances and the concomitant buildup of intensity at 1.05 and 1.80 pm (Figure 1B). This implies that there is slow exchange between two forms on the NMR time scale. The original methyl proton signals from the dodecamer have nearly vanished at an Hg(II) concentration of about 24 mM corresponding to about five Hg(II) ions added per oligomer duplex molecule. The observations indicate a transition from the normal B-form of the duplex [d(CGCGAATTCGCG)]$_2$ to an apparently new well-defined form interacting with Hg(II). The transient signals at 12.20, 12.79, and 14.14 ppm (Figure 1A) indicate the emergence of a third *intermediate* conformation during the Hg(II) titration.

By carrying out the same titration on sample B (5.4 mM of DNA duplex in the more dilute buffer: 10 mM sodium phosphate, pH 7.0) the spectral changes at low Hg(II) concentrations ($< \approx 8$ mM) are the same. However, at high concentrations all nonexchangeable proton resonances broaden severely, and all imino proton resonances completely vanish except for very weak and broadened T7 NH3 and T8 NH3 resonances (results not shown). Increasing the buffer concentration of the solution of sample B to 0.2 mM $NaClO_4$ and 0.17 mM sodium phosphate restores the spectral pattern shown in Figure 1. In an analogous Hg(II) titration experiment with only 0.34 mM of DNA duplex (sample C in dilute buffer (10 mM sodium phosphate, pH 7.0), the ^{1}H spectral changes were nearly identical to those of sample A (concentrated DNA/concentrated buffer, results not shown). These observations may be explained by Hg(II)-induced deprotonation (*vide infra*). On further addition of $Hg(ClO_4)_2$ to sample C all proton resonances gradually become weaker and broader. No further experiments are performed on samples B and C.

Figure 2 shows that added mercury salt induces reduction in the intensities of the nonexchangeable protons of the DNA dodecamer sample A. Hg(II) addition is also accompanied by a slight overall spectral line broadening. Moreover, some resonances appear to have "tails" or humps at low intensities, reminiscent of bad field homogeneity. This feature may indicate the presence of some minor species. The line broadening may be due to intermediate rate exchange between these minor species and the postulated major one. There is also a possibility for differences in the molecular sizes and molecular shapes of the normal duplex DNA dodecamer and a DNA/Hg(II) complex, which in turn give rise to different molecular motions and therefore differences in relaxation behavior. In this study, we will focus our attention on the major component which is responsible for the well-defined spectrum of sample A in Figure 2b but briefly discuss the influence from possible minor species in this sample when appropriate.

It is well-known that proton–deuteron exchange occurs at nucleobase positions G H8 and A H8 of DNA dissolved in D_2O. There will be a notable reduction ($\approx$10%) in the intensities of the

Table 1. ^{1}H Resonance Assignment for the Nonexchangeable Protons of the DNA Dodecamer d[CGCGAATTCGCG]$_2$, Sample A in D_2O:[a] (i) without Hg(II) and (ii) in the Presence of Hg(II)

	^{1}H chemical shifts[b,c] (ppm)						
residue	H6/H8	H2/H5/CH$_3$	H1′	H2′	H2″	H3′	H4′
C1	7.56	5.83	5.73	1.88[d]	2.36	4.66	4.04
	0.04	0.06	0.03	0.03	0.01	0.02	0.01
G2	7.92		5.86	2.63	2.70	4.94	4.32
	0.03		0.00	0.02	−0.04	0.02	0.01
C3	7.24	5.35	5.60	1.81	2.25	4.79	4.18
	0.03	0.08	0.11	−0.04	−0.02	0.01	−0.07
G4	7.82		5.41	2.63	2.73	4.97	4.29
	−0.09		−0.23	−0.40	−0.36	−0.08	−0.14
A5	8.08	7.26	5.97	2.67	2.90	5.04	4.42
	−0.09	0.18	0.08	−0.05	−0.02	−0.05	−0.01
A6	8.07	7.61	6.13	2.54	2.88	4.98	4.44
	−0.23	0.33	−0.24	−0.32	−0.21	−0.24	−0.05
T7	7.07	1.26	5.87	1.95	2.53	4.78	4.11
	−0.05	−0.21	0.09	0.16	0.08	−0.07	0.14
T8	7.35	1.52	6.07	2.14	2.51	4.87	4.18
	−0.01	0.28	−0.01	−0.10	−0.07	−0.03	−0.14
C9	7.44	5.62	5.64	2.03	2.38	4.84	4.13
	0.09	0.01	0.17	0.06	0.05	0.04	0.07
G10	7.87		5.83	2.61	2.68	4.97	4.34
	0.03		0.04	0.02	0.04	0.01	0.02
C11	7.29	5.41	5.76	1.85	2.29	4.78	4.13
	0.00	0.01	0.01	0.01	0.01	0.01	0.00
G12	7.88		6.11	2.58	2.37	4.65	4.14
	0.01		0.01	0.00	0.00	−0.01	0.00

[a] 4.8 mM solution of the duplex form of dodecamer in a buffer containing 170 mM sodium phosphate (pH 7.0) and 200 mM $NaClO_4$ in 99.996% D_2O at 310 K. [b] The assignment is based on a DQF-COSY spectrum and NOESY spectra with 50–300 ms mixing times. For each residue the first line lists the chemical shifts prior to Hg(II) addition, whereas the second line represents the changes in chemical shifts induced by 28 mM of $Hg(ClO_4)_2$, $\delta_{DNA+Hg(II)} - \delta_{DNA}$. The values are referenced to the residual HDO peak, for which the chemical shift has been set to 4.63 ppm. [c] The H5′/H5″ resonances are not included, of which only a few were possible to assign utilizing standard DQF-COSY and NOESY techniques. [d] The H2′ and H2″ resonances were distinguished by observing the differences in the intensities of the intraresidue H6/H8-H2′ and H6/H8-H2″ cross peaks in the 50 ms NOESY spectrum, the latter type of cross peaks having the lowest intensity.[46] A B-type DNA conformation is assumed, where the corresponding internuclear distances are ca. 2.2 and 3.6 Å, respectively. The assignment is corroborated by the observed difference between the intraresidue H1′–H2′ and H1′–H2″ cross peaks, the latter showing the largest intensity; the B-type DNA distances are ca. 3.0 and 2.3 Å, respectively. Moreover, these observations are completely consistent with the fact that the interresidue (n)H2″–(n + 1)H8/H6 cross peaks are more intense than the (n)H2′–(n + 1)H8/H6 cross peaks; the B-type DNA distances are ca. 2.4 and 3.8 Å.

G H8 resonances over a period in the order of 100 h at 310 K in 0.1 M NaCl/D_2O, while the exchange of A H8 is slower under these conditions.[43] We have observed this effect on the G H8 resonances of [d(CGCGAATTCGCG)]$_2$ at about the same order of exchange rates. By introducing Hg(II), the proton–deuteron exchange of the A/G H8 protons accelerate to such rates that the intensities of all A/G H8 resonances become significantly reduced. Over the time span needed for the completion of a series of NOESY experiments with six mixing times some of these intensities are reduced by about 30–40%, making quantitative use of certain cross peak volumes difficult.

After finishing all NMR experiments on the Hg(II)-treated DNA sample, we added KCN to the sample in about 2–6-fold excess to the amount of Hg(II) added. Figure 2 shows that the original spectrum of Hg(II)-free DNA is more or less regenerated, thus confirming the observations made by Gruenwedel and Cruikshank about the reversibility of the interaction of Hg(II) with DNA.[12] The aromatic H8 resonances have reduced intensities caused by exchange with deuterium upon standing at 277 K for a couple of months. On standing the solution became slightly miscolored, and a minute amount of precipitate appeared as well. A slight general broadening of the resonances in comparison with the original sample is therefore not surprising.

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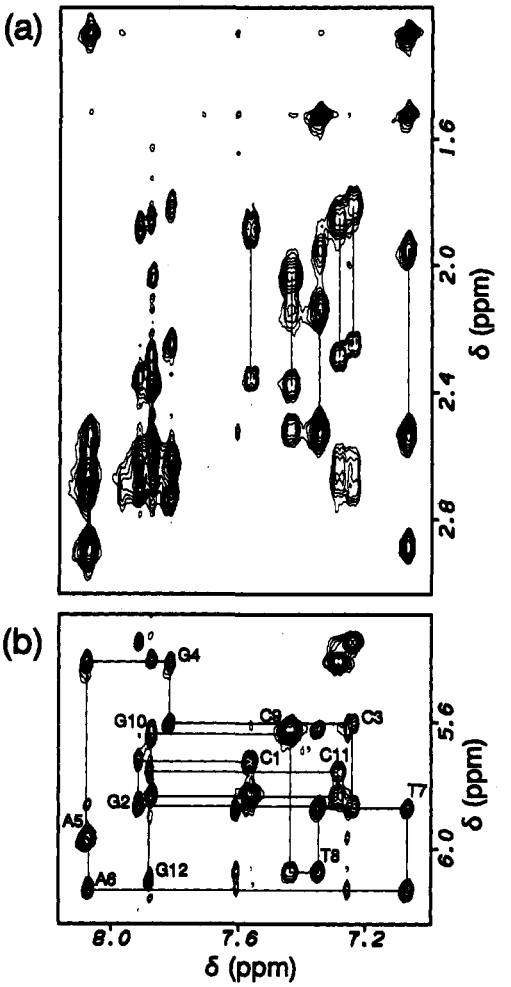

Figure 3. Contour plots taken from a 300 ms NOESY spectrum obtained at 310 K of the dodecamer [d(CGCGAATTCGCG)]$_2$ (sample (A), see caption of Figure 1) in D_2O without Hg(II) added: (a) The H6/H8-H2′/H2″/CH$_3$ cross peak region is aligned with (b) the H6/H8-H1′/H5 region. In (a) the lines are connecting the chemical shifts of geminal pairs of H2′ and H2″ protons. In (b) the sequential connectivities are indicated by a continuous line. The intraresidue H6/H8-H1′ cross peaks are labeled with their residue number. The remaining cross peaks (no line) in region (b) involve the C H5 and A H2 protons.

2D ^{1}H NMR Spectra of Nonexchangeable Protons. The assignment of the nonexchangeable proton resonances of the dodecamer [d(CGCGAATTCGCG)]$_2$ has been published, based on NOESY/COSY spectra[21] and TOCSY spectra.[44] The buffers used were different from ours (10 mM phosphate, pH 7.0, versus 170 mM sodium phosphate, pH 7.0, and 200 mM sodium perchlorate). Through a reassignment of the nonexchangeable protons of the Hg(II)-free sample using standard techniques,[21,45,46] we find new values for comparison with Hg(II)-induced chemical shifts. The results agree very closely with those already published, except for an interchange of the chemical shifts of G12 H2′ and G12 H2″ (Table 1).[21] Thorough ^{1}H NMR investigations have shown that the dodecamer under study adopt a B-DNA conformation,[22,23] and in the outset of the resonance assignment we will assume that this is still the case after Hg(II) has been added to the solution.

The cross peak regions H8/H6-H5/H1′ and H8/H6-H2′/H2″/CH$_3$ of 300 ms NOESY spectra of the dodecamer (sample A) without and with Hg(II) added are shown in Figure 3 and 4,

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(46) Scheek, R. M.; Boelens, R.; Russo, N.; van Boom, J. H.; Kaptein, R. *Biochemistry* **1984**, *23*, 1371–1376.

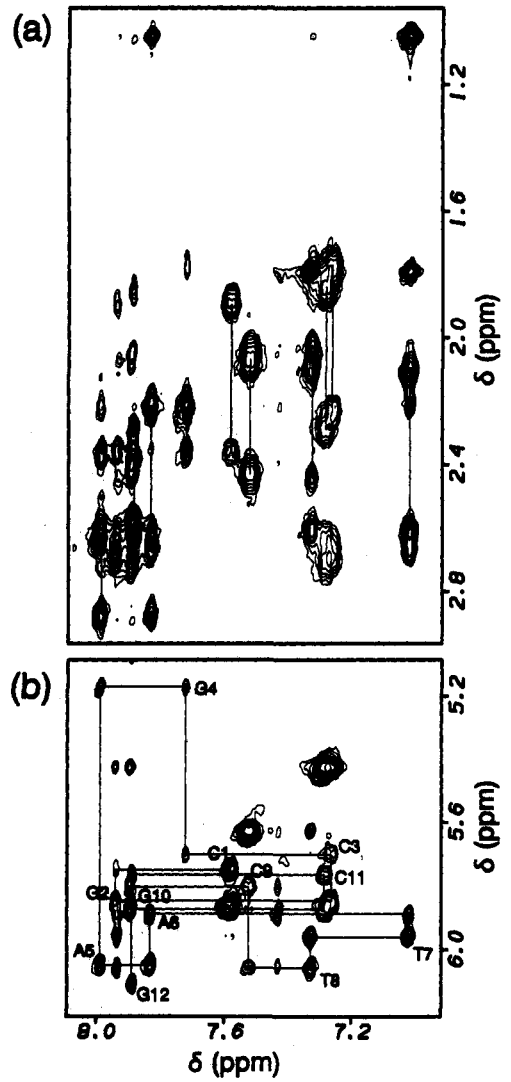

Figure 4. Contour plots taken from a 300 ms NOESY spectrum obtained at 310 K of the dodecamer [d(CGCGAATTCGCG)]$_2$ (sample A, see caption of Figure 1) in D_2O with 28 mM $Hg(ClO_4)_2$ added: (a) The H6/H8-H2′/H2″/CH_3 cross peak region is aligned with (b) the H6/H8-H1′/H5 region. In (a) the lines are connecting the chemical shifts of geminal pairs of H2′ and H2″ protons. In (b) the sequential connectivities are indicated by a continuous line. The intraresidue H6/H8-H1′ cross peaks are labeled with their residue number. The remaining cross peaks (no line) in region (b) involve the C H5 and A H2 protons.

respectively. A complete "walk" of sequential NOE connectivities can be completed using both these regions,[21,45,46] indicating that the dodecamer remains in a right-handed double-helical conformation after the addition of Hg(II). Moreover, cross peaks are found between cytosine H5 or thymine CH_3 and H6/H8 on the residue on their 5′-side, i.e., $(n+1)$C H5/T CH_3–(n)H6/H8, where n is the residue number counted in the 5′ → 3′ direction. These interactions are additional characteristics of right-handed DNA. All characteristic cross peaks involving A5/A6 H2 are observed as well. Especially, even the interstrand interactions are observed with Hg(II) added: The cross peaks between A5 H2 and C9′ H1′ and between A6 H2 and T8′ H1′ of opposite strands. These interactions represent further evidence for an intact right-handed DNA helix. Furthermore, in the 300-ms NOESY spectrum without Hg(II), cross peaks are found for all the expected aromatic interbase (n)H6/H8–$(n+1)$H6/H8 interactions. With Hg(II) added all these cross peaks are still observed, but the cross peak G4 H8–A5 H8 and those between terminal bases, C1 H6′–G2 H8 and C11 H6–G12 H8, appear with reduced intensity. In this region of the spectrum, a new cross peak appears (above the diagonal only): A6 H2–T7 H6. Within the frames of normal B-DNA geometry, the distance between these protons is too long (>6.0 Å) to give rise to an observable cross peak, either intrastrand, A6 H2–T7 H6, or interstrand, A6 H2–T7′ H6, where T7 and T7′ are found on opposite strands related through symmetry. This cross peak is not observed in the 300-ms NOESY spectrum of [d(CGCGAATTCGCG)]$_2$ prior to the introduction of Hg(II), and it is thus likely that the appearance of the cross peak is caused by minor structural changes. This is discussed in a later paragraph.

The proton chemical shift changes of [d(CGCGAATTCGCG)]$_2$ induced by Hg(II) are summarized in Table 1. Most of the chemical shifts with Hg(II) added differ by less than ±0.10 ppm from those of teh Hg(II)-free dodecamer. The three terminal G·C base pairs of the palindromic dodecamer are barely displaying any shift changes at all, while G4 exhibits large upfield shifts of its ribose protons (–0.14 to –0.40 ppm). The anomeric proton of C9 undergo a significant downfield shift (0.20 ppm). The adenosines A5 and A6 show downfield shifts for H2 (0.18 and 0.33 ppm) and upfield shifts for H8 (–0.23 and –0.09 ppm). The thymine methyl protons are shifted in opposite directions, –0.21 ppm and 0.28 ppm for T7 and T8, respectively. We also find large upfield shifts for the ribose protons of A6 (–0.21 to –0.32 ppm) but not for A5.

In a study of the interaction of Hg(II) with thymine and guanine monomers, no significant shift changes on proton resonances were observed.[16] The significant proton shift changes of our "mercury complex" strongly suggest that these are due to conformational changes induced by interaction with Hg(II) rather than intrinsic shift effects at the monomer level due to direct binding of Hg(II) itself, in agreement with Young *et al.*[15] The chemical shifts of the protons of individual nucleotides in DNA oligomers are largely determined by aromatic ring currents of the nucleobases. If a Hg(II) ion replaces a proton in one of the A···T hydrogen bonds of the A·T base pairs, the stacking geometry and the relative orientation of sugar moieties and nucleobases of the central part of DNA helix change. Consequently the ring current shifts of the involved proton resonances may become altered. It is difficult to relate the extent of chemical shift changes to specific conformational changes. However, the observed shift effects in the AT tract and at the boundary between the AT and GC parts are likely to be determined by such changes.

^{1}H NMR Spectra of Exchangeable Protons. The exchangeable proton resonances of the dodecamer without Hg(II) added have been reassigned by using a 400-ms NOESY spectrum and one-dimensional TOE difference spectra of sample A in 90% H_2O/10% D_2O to obtain new values for comparison (spectra not shown). The results agree with those already published.[47-49] One-dimensional TOE difference spectra are used to assign the imino protons of the Hg(II)-treated dodecamer (Figure 5). The assignment is also aided by a 400 ms NOESY spectrum (not shown). After the addition of 25 mM Hg(II) to the 4.8 mM dodecamer solution (sample A) two resonances, at 13.06 and 12.93 ppm, remain in the imino region of the proton spectrum (Figure 1), the former about twice as intense as the latter. Irradiation of the resonance of 13.06 ppm gives NOE enhancements at 8.37, 8.48, 7.44, 6.59, and 6.46 ppm. In analogy with the results for the Hg(II)-free dodecamer the relatively narrow resonances at 8.37 and 8.48 ppm are due to hydrogen-bonded cytosine amino protons, while the broader resonances at 6.59 and 6.46 ppm are due to cytosine amino protons which are not hydrogen-bonded. Therefore, two G NH1 resonances overlap at 13.06 ppm. The NOE enhancement at 7.44 ppm can be explained by the short distance, ca. 3.7 Å, between A5 H2 and G4 NH1, if we assume that the B-like conformation of the DNA duplex is retained. Thus, one of the overlapping imino proton resonances at 13.06 ppm can be assigned to G4 NH1. Irradiation of the

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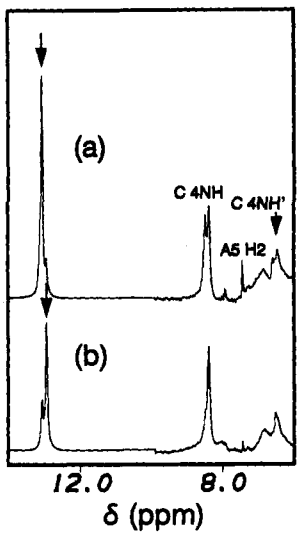

Figure 5. 400-MHz TOE difference spectra obtained at 292 K of the dodecamer [d(CGCGAATTCGCG)]$_2$ (sample A, see caption of Figure 1) in 90% H_2O/10% D_2O with 28 mM $Hg(ClO_4)_2$ added. The excitation of the H_2O resonance was avoided by using a time-shared Redfield "2-1-4" detection pulse. For the two resolved imino proton frequencies which were saturated (arrows), data from ten different irradiation times (35–350 ms) were averaged: (a) irradiation at 13.06 and (b) at 12.88 ppm. The right halves of the spectra are magnified (×5) for the convenience of presentation. C 4NH' denotes the amino proton which is *not* participating in the Watson–Crick hydrogen bonding.

resonance at 12.93 ppm give NOE enhancements at 8.39 and 6.59 ppm, indicating the presence of one G NH1 cross-relaxing with the amino protons of its cytosine base pair partner. The C NH4 (C NH_2) resonances at 8.39 and 6.51 ppm are unambiguously assigned to the same base pair as the G NH1 resonance at 12.93 ppm, but it is difficult from the TOE spectra to determine unequivocally to which of the base pairs, G2·C11 or C3·G10, these resonances belong. However, the NOESY spectrum shows a very strong diagonal peak for this resonance, while the *two* overlapping ones at 13.06 ppm are weak in comparison (not shown). This effect may be explained by greater proton exchange with H_2O, which itself experiences the relatively strong and nonselective preparation and mixing pulses. Therefore, the G NH1 resonance at 12.93 and its C NH4 cross-relaxing partners must belong to the G·C base pair which is least exposed to the solvent, most probably the base pair C3·G10. This conclusion is corroborated by the presence of the expected intraresidue NOESY cross peak between C3 H5 at 5.43 ppm and the C3 amino protons 6.51 and 8.39 ppm. Owing to the imino proton overlap at 13.06 ppm, it is impossible to determine which of the cytosine NH_2 protons belong to the base pair G4·C9 and G2·C11. These ambiguities may be resolved by resorting to the NOESY spectrum, where we find the intraresidue cross peaks between C9 H5 and the amino proton signals at 6.46 and 8.37 ppm and between C11 H5 and the amino proton signals at 6.59 and 8.48 ppm. Thus, all G NH1 and C NH4 resonances of the G·C base pairs are assigned, except for the terminal ones, which exchange too rapidly at the chosen temperature (Table 2). There are no significant differences between the nonexchangeable parts of the ^1H spectrum obtained at this temperature (292 K) and those obtained at 310 K.

The transient signals at 12.20, 12.79, and 14.14 ppm (Figure 1A) have not been assigned, but they may represent imino proton signals belonging to an intermediate conformation adopted by the DNA dodecamer during the Hg(II) titration (Figure 1A). The chemical shift values may indicate unusual hydrogen bond interactions. Work is in progress in our laboratory to investigate the origin of these transient signals.

The assignment of the exchangeable protons shows that the G N1–H···N3 C hydrogen bonds are intact in the presence of Hg(II), except for the terminal ones. In contrast, the T7 NH3 and

Table 2. ^1H Resonance Assignment for the Observed Exchangeable Protons of the DNA Dodecamer [d(CGCGAATTCGCG)]$_2$, Sample A[a] in H_2O: (i) without Hg(II) and (ii) in the Presence of Hg(II)

	^1H chemical shifts[b] (ppm)		
base pair	G NH1/T NH3	C NH4[c]	
G2·C11′	13.02	8.41	6.55
	13.06	8.48	6.59
C3·G10′	12.88	8.35	6.42
	12.93	8.39	6.51
G4·C9′	12.68	8.40	6.80
	13.06	8.37	6.46
A5·T8′	13.78		
	d		
A6·T7′	13.65		
	d		

[a] 4.8 mM of the duplex form of the dodecamer in a buffer containing 170 mM sodium phosphate (pH 7.0) and 200 mM $NaCl_4$ in 90% H_2O/10% D_2O at 292 K. [b] The assignment is based on magnitude mode NOESY spectra with a 400 ms mixing time and one-dimensional TOE difference spectra. For each base pair, the first line lists the chemical shifts prior to Hg(II) addition, whereas the second line shows the chemical shifts after the introduction of 28 mM $Hg(ClO_4)_2$. The numbering of the residues are the same on both strands; the two symmetrically related strands are distinguished by attaching a prime (′) to the residue number of the second nucleotide of each base pair. The chemical shifts are references to the H_2O peak, for which the chemical shift has been set to 4.82 ppm. [c] The cytosine amino proton which participate in the Watson–Crick hydrogen bonds resonate at lowest field. [d] Denotes the resonances which were not observed after the addition of Hg(II).

T8 NH3 ^1H signals are absent from the spectra. This observation suggests that Hg–N3 bonds are established at these sites,[15] or that Hg(II)-binding occur at other sites on adenine and thymine and thereby allowing the thymine NH3 protons to exchange more rapidly with solvent H_2O.

If Hg(II) binding at N3 indeed takes place through proton loss, it may provide an explanation for the less ordered "Hg(II) complex" in the weak buffer (sample B, 10 mM sodium phosphate, pH 7.0) as manifested through an ill-defined ^1H spectrum (not shown). The capacity of the low concentration buffer will be exceeded by the released protons from T7 N3 and T8 N3, and the pH will decrease. In this picture, sample A (200 mM $NaClO_4$/170 mM sodium phosphate, pH 7.0) is not affected by such a pH effect. pH dependent melting studies of the dodecamer [d(CGCGAATTCGCG)]$_2$ have demonstrated that the oligomer is destabilized below pH ≈ 5.5 due to protonation of cytosine N3 (and adenine N1), thus disrupting the Watson–Crick base pairs.[50] Tentatively, we may sketch some possibilities for a disordered state at low pH in the presence of Hg(II) (sample B): The DNA dodecamer may adopt a completely random coil state with Hg(II) attached to T7 N3, or the A·T base pairs may be kept in register through Hg-assisted cross-links, with dangling CG ends. There is also a possibility that a bond can be established between Hg(II) and A NH6 (A NH_2), again accompanied with the release of a proton. In this case there may in addition exist a bond between Hg(II) and T O4. In contrast to sample A (*vide infra*), we have no experimental evidence regarding the stoichiometry of the "Hg(II) complex" and Hg(II)-binding sites of sample B (low salt/weak pH buffer). A random coil state of the DNA dodecamer may open the possibility for other binding sites than those of the duplex form. Moreover, in the low salt environment enhanced aggregation due to nonspecific Hg(II) binding may become important. Aggregation may explain the severely broadened ^1H spectrum of this sample. A simple test of whether proton release really occur could be done by monitoring the pH by means of a reference compound during the Hg(II) titration.

Upon addition of Hg(II) to DNA in the stronger buffer (sample A, 200 mM $NaClO_4$/170 mM sodium phosphate), a slight overall broadening of the DNA ^1H resonances as well as resonance "tails" were observed (Figures 1 and 2). The tails or humps suggest that a small fraction of the DNA dodecamer is present as Hg(II)-induced aggregates in this sample as well. The overall line

broadening may be explained by exchange processes where both DNA/Hg(II) complexes in duplex form and aggregates take part.

Structural Information from the NOESY Spectra. Results from ^{1}H NMR in H_2O solution indicate that Hg(II) ions bind to thymine N3/O4 participating in Watson–Crick hydrogen bonding. However, there are no signs of dramatic changes in the secondary structure as judged from NOESY spectra, indicating that a right-handed helical DNA conformation is retained. Gruenwedel and Cruikshank have suggested that Hg(II) may promote a transition from right-handed B-DNA to left-handed Z-DNA conformation.[12] Our ^{1}H NMR spectra of the duplex–Hg(II) complex show no evidence of a left-handed Z-DNA conformation. The interresidue distances G H8–C H1′/C H2′/C H2″ in 5′-CG-3′-segments of typical B-DNA are ca. 2.8, 3.6, and 2.5 Å, respectively,[51] and the corresponding NOESY cross peaks are observed. In Z-DNA the analogous distances are too long (>5.0 Å) to give rise to observable cross peaks. The interresidue distances 5′-H8/H6–H2′-3′ and 5′-H8/H6–H2″-3′ in B-DNA are ca. 3.8 and 2.8 Å, respectively, being consistent with the observed cross peaks and the proton resonance assignment (Table 1). The corresponding distances for A-DNA are ca. 1.6 and 3.2 Å. In an A-DNA-like conformation the intraresidue distances H6/H8′–H2′ and H6/H8′–H2″ are 3.8 and >5.0 Å, respectively. In B-DNA, they are 2.2 and 3.6 Å, and both corresponding cross peaks are observed, the former being notably stronger.

There is quite convincing evidence for an intact right-handed helical DNA conformation from the NOESY spectra, and the disappearance of the thymine imino proton resonances during the Hg(II) titration can be explained by the insertion of Hg(II) ions between the complementary adenines and thymines on opposite strands of the DNA duplex. Alternatively, the loss of the thymine imino proton signals may be explained by a duplex to hairpin transition induced by Hg(II). The charactertistic shifts of the thymine methyl resonances observed in our study resemble those observed in a duplex-hairpin transition experiment.[52] We may distinguish between the hairpin and "bulged" duplex models by considering the interresidue 5′-H1′/H2′H2/′–H6/H8-3′ NOESY cross peaks used for the sequential assignment procedure. For the Hg(II) complex of our dodecamer all these cross peaks were observed. For hairpins, however, the sequential assignment of the nonexchangeable protons could not be completed across the postulated stem-loop boundary,[52,53] Extra evidence for the existence of a duplex, in contrast to a hairpin molecule, can be found from the measurement of the rotational correlation time of the whole molecule.[54,55] For the dodecamer with and without Hg(II) added, the sum of the volumes of the diagonal peaks of C3 H5 and C11 H5 was calculated for zero mixing time, and the sums of the volumes of the C3 and C11 H5–H6 cross peaks were calculated for all mixing times, 0–300 ms. The volumes of cross peaks symmetry related in pairs about the diagonal were averaged. The ratios between the cross peak intensity at each mixing time and diagonal peak intensity at zero mixing time was plotted against the mixing time for both samples (Figure 6).[56] The correlation times were calculated from the initial cross-relaxation rates[54] and gave 3.3 and 3.4 ns for the dodecamer with and without Hg(II), respectively, indicating approximately equal molecular sizes in the two cases. Thus, a Hg(II)-induced transition from duplex to monomeric hairpin structures can be ruled out. In comparison, the rate of recovery after selective inversion of the C1 H6 resonance has been found to be 4.2 *vs* 2.0 s^{-1} for the duplex and hairpin forms of [d(CGCGTATACGCG)]$_2$, respectively.[52]

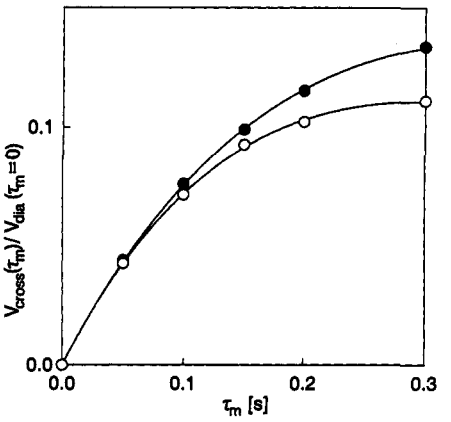

Figure 6. Buildup of cytosine H5–H6 cross peak volumes from NOESY spectra obtained at 310 K of the dodecamer [d(CGCGAATTCGCG)]$_2$ (sample A, see caption of Figure 1) in D_2O. The sum of the intrabase C3 H5–H6 and C11 H5–H6 cross peak volumes at each mixing time (0–300 ms) are divided by the sum of the corresponding diagonal peak volumes at zero mixing time. The volumes of symmetry related cross peaks were averaged. The open circles represent cross peak volumes from NOESY spectra of the DNA sample without Hg(II), while the filled circles represent those with 28 mM $Hg(ClO_4)_2$ added.

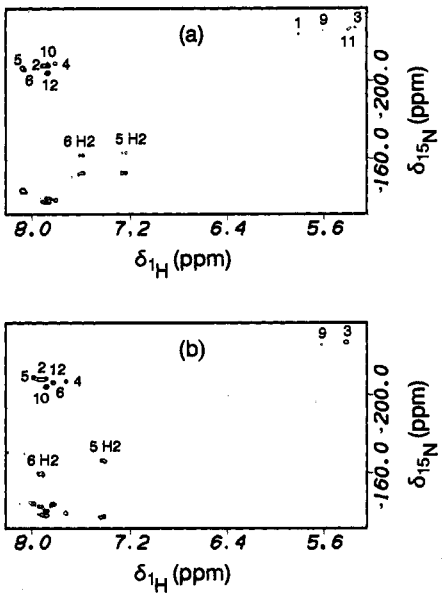

Figure 7. ^{1}H-detected ^{1}H–^{15}N HMQC spectra obtained at 310 K of the dodecamer [d(CGCGAATTCGCG)]$_2$ (sample A, see caption of Figure 1) in D_2O: (a) no Hg(II) added and (b) 28 mM $Hg(ClO_4)_2$ added. Only one cross peak is labeled at each ^{1}H chemical shift involved: The H8–N7, H6–N1, and H2–N3 cross peaks of the purines, cytosines, and adenines, respectively, are labeled with their residue numbers. The H8–N9 and H2–N1 cross peaks are therefore not labeled.

^{15}N NMR Spectroscopy. ^{15}N NMR is a sensitive probe for detecting specific nitrogen binding sites on the nucleobases. For instance, addition of equimolar amounts of $HgCl_2$ to adenosine, guanosine, and cytidine is shown to cause upfield shifts of the nitrogen resonances N1, N7, and N3, respectively, indicating binding of Hg(II) at those sites.[18] It is not feasible to directly observe ^{15}N resonances at natural abundance at the low concentrations of DNA used in this study; being only ca. 10 mM in equivalent sites. We have used ^{1}H-detected ^{1}H–^{15}N HMQC spectroscopy which greatly enhances the sensitivity of natural abundance ^{15}N spectroscopy.[37] Prior to Hg(II) addition most ^{15}N signals of [d(CGCGAATTCGCG)]$_2$ from nitrogen nuclei which couples through two chemical bonds to nonexchangeable protons are observed (Figure 7). The thymine N1 resonances are absent. It may be explained by a very small $^2J_{N1-H6}$ coupling constant, the analogous value for uracil has been found to be 3.5 Hz.[40] After the addition of Hg(II) there is a pronounced decrease

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Table 3. ^{15}N Resonance Assignment for the DNA Dodecamer d[CGCGAATTCGCG]$_2$, Sample A in D_2O:[a] (i) without Hg(II) and (ii) in the Presence of Hg(II)

	^{15}N chemical shifts[b] (ppm)			
residue	N1	N3	N7	N9
C1	-233.5			
G2			-137.9	-207.2
			-0.9	-0.6
C3	-226.8			
	0.1			
G4			-139.0	-208.4
			-0.5	0.8
A5	-152.6	-162.7	-143.7	-206.0
	15.3	-3.3	-0.5	-3.0
A6	-152.2	-161.5	-143.4	-205.3
	9.4	2.4	-0.7	-1.0
C9	-225.3			
	-0.2			
G10			-139.6	-203.7
			-0.9	-0.5
C11	-225.7			
G12			-137.6	-207.4
			-0.6	-0.9

[a] 4.8 mM solution of the duplex form of the dodecamer in a buffer containing 178 mM sodium phosphate (pH 7.0) and 200 mM $NaClO_4$ in 99.996% D_2O at natural abundance of ^{15}N. The temperature was 310 K. [b] The assignment is based on ^1H-detected ^1H-^{15}N HMQC correlation spectra and the ^1H resonance assignment from Table 1. For each residue, the first line lists the chemical shifts prior to Hg(II) addition, whereas the second line represents the changes in ^{15}N chemical shifts induced by 28 mM of $Hg(ClO_4)_2$, $\delta_{DNA+Hg(II)} - \delta_{DNA}$. The chemical shifts are referenced to the N9 resonance of G2, for which the shift value has been set to -207.2 ppm, corresponding very roughly to shifts relative to hypothetical internal NO_3^-. The ^{15}N chemical shifts of the Hg(II) free DNA dodecamer will thus be close to the ^{15}N shift values for monomer nucleotides dissolved in D_2O.[38]

in the signal-to-noise ratio, and the weak signals from C1 N1 and C11 N1 have vanished. Inspection of the spectra reveals that only the three-bond couplings of the adenine H2 protons to N1 or N3 are resolved.

The assignment of most of the ^{15}N resonances of [d(CGCGAATTCGCG)]$_2$ follows fairly straightforward from the HMQC spectrum (Figure 7) under the assumption that the orders of chemical shifts of the ^{15}N resonances within each residue are the same as those within monomer nucleosides or nucleotides (Table 3). The relative ^{15}N shifts agree within ±2.5 ppm with those reported for the corresponding monomer nucleotides.[38] The narrow shift dispersion among corresponding ^{15}N resonances in the Hg(II)-free dodecamer probably reflect the insensitivity of the base ^{15}N resonances to differences in base stacking or other conformational variations. The observed chemical shift changes accompanying Hg(II) addition must therefore be due to local effects from Hg(II) binding corresponding to those observed for Hg(II) monomer studies.[18] We find that most ^{15}N shifts are little affected by the presence of Hg(II), except for the A5/A6 N1 or A5/A6 N3 resonances which display significant shifts. We must consider two different possibilities for the assignment of these resonances, since there is a danger of signal crossing. The most plausible assignment for A5 N1/N3 and A6 N1/N3 is given in Table 3. A5/A6 N1 experience large (15.3 and 9.4 ppm) downfield shifts upon the addition of Hg(II), whereas A5/A6 N3 become less affected (-3.3 and 2.4 ppm). These results are contrary to what is observed for adenosine in DMSO, where equimolar addition of Hg(II) salt results in *upfield* shifts of N1 (-9.5 ppm) and N7 (-1.2 ppm) and *downfield* shifts (0.5–2.5 ppm) for the other nitrogens.[18] This was interpreted as genuine Hg(II) binding at N1, in analogy with protonation studies of adenine and pyridine.[38] Thus, in terms of these results our spectra are difficult to reconcile with a model where Hg(II) binds to A5/A6 N1 in the dodecamer. Interchanging the assignments of N1 and N3 for the adenines gives large upfield shifts for A5/A6 N1 (-13.4 and -6.9 ppm) and even larger downfield shifts for the N3 nitrogens (25.4 and 18.7 ppm). These shift effects on A5/A6 N1 may easily be reconciled with Hg(II) binding at N1, but the very large shift changes of A5/A6 N3 become difficult to explain.[18]

In Figure 7 the HMQC spectra of the dodecamer with and without Hg(II) are shown. The only observed spin–spin splittings are those of the A5/A6 H2–N1 and A5/A6 H2–N3 cross peaks ($^2J_{N1/3-H2} \approx 14$ Hz). No change in the splitting pattern of the HMQC cross peaks is observed upon addition of Hg(II). For neutral AMP in H_2O, geminal ^{15}N–^1H coupling constants have been determined: $^2J_{N1-H2} = 15.8$ Hz, $^2J_{N3-H2} = 17.1$ Hz, $^2J_{N7-H8} = 11.0$ Hz, and $^2J_{N9-H8} = 5$ Hz.[38] By protonation of N1 in 9-methylpurine $^2J_{N1-H2}$ is reduced appreciably ($\Delta J \approx 6$ Hz), whereas $^2J_{N3-H2}$ is less affected ($\Delta J < 2$ Hz).[57] These effects were explained by a change in the π-delocalization upon protonation. Analogous effects might be expected upon metal binding at N1 of adenine residues in oligomers.

Hg(II) Binding Sites. The ^{15}N chemical shift data and HMQC splitting pattern suggest that no chemical bond is estalished between Hg(II) and A5/A6 N1 in [d(CGCGAATTCGCG)]$_2$. Having eliminated A5/A6 N1 and N3, we are left with Hg(II) binding at the amino groups for explaining the *downfield* shift of the N1 resonances. We propose the formation of a complex where each of four Hg(II) ions are forming a bond to (i) a NH6 (A NH_2) through the release of a proton and (ii) to an enolic T O4 on the opposite strand, thus cross-linking all A·T base pairs. There is structural evidence of Hg(II) binding to both N3 and O4 in pyrimidines. In the Hg(II) complex of 1-methylthymine Hg(II) binds two thymine ligands at N3 in a linear arrangement, N3–Hg–N3′ (Hg–N3 = 2.04 Å).[58] In a corresponding Hg(II) complex of uracil a similar linear arrangement involves O4 (Hg–O4 = 2.71 Å).[59] There are also examples of Hg(II) binding to nucleobases involving a deprotonated amino group.[60] In an A·T basepair participating in Watson–Crick hydrogen bonding, the total distances O4···H–N6 and N–H···N1 are ca. 2.9 Å. Insertion of Hg(II) will increase the O4···H–N6 distance to about 5.0 Å. To accommodate the Hg(II) ions in the duplex one may envisage either a stretching or an opening of the A·T base pairs. The absence of the T NH3 resonances from the exchangeable ^1H spectrum may then be due to deprotonation or faster exchange with H_2O. If we rule out A N1 as a possible binding site for Hg(II), we do not have to simultaneously rule out T N3 as a binding site. Instead of the postulated T O4–Hg–N6 A arrangement, we may consider a T N3–Hg–N6 A arrangement. In this case the incorporation of the Hg(II) is accompanied by a stretching and a shear of the A·T base pairs.

Recently, the observation of $^2J_{Hg-H}$ and $^5J_{Hg-H}$ coupling constants in mixed Pt(II)–Hg(II) cytosine complexes was reported.[60] A thorough investigation of the properties of ^{199}Hg in the context of DNA-binding may provide useful information. We have not observed ^{199}Hg satellites in our ^1H spectra. Especially, no $^2J_{Hg-AH2}$ is observed for the A H2 ^1H resonances. The absence of these ^{199}Hg satellites seems to support our model where no A5/A6 N1–Hg exists. However, this is not conclusive: If there are such bonds, the ^{199}Hg satellites may be difficult to observe due to, e.g., efficient relaxation through chemical shift anisotropy at high resonance frequencies.

The rather large difference in ^{15}N chemical shift effects of A5 N1 (15.3 ppm) and A6 N1 (9.4 ppm) upon Hg(II) binding is puzzling. These chemical shifts may suggest that the binding modes of Hg(II) at the two central base pairs (A6′·A7 and A6·A7′) and the flanking ones (A5′·A8 and A5·A8′) are different. However, it is difficult to find conclusive evidence regarding the nature of this difference from our present data.

A direct comparison of our results with those of Katz[8] and Young *et al.*[15] and their specific cross-linking models requires

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NMR studies of oligonucleotides of different composition and probably greater lengths. However, our results support the general idea that Hg(II) cross-links DNA by forming bonds to certain sites on the nucleobases.[3,12] Furthermore, by our choice of DNA sequence a direct comparison of how single Hg(II) ions and Mn(II)/Zn(II) ions interacts with the same piece of duplex DNA is possible.[1,2] We find that while Hg(II) interrupts the Watson–Crick hydrogen bonds and establishes interstrand cross links, Mn(II) (and Zn(II)) interacts with N7 of certain guanine residues. These findings agree fairly well with those inferred from studies of the melting behavior of DNA in the presence of different divalent metal ions.[3]

Structure of DNA–Hg(II) Complex. The rotational correlations times show that the DNA dodecamer/Hg(II) complex is a dimer; no hairpin structure is formed. The Hg(II) titration (Figure 1) shows that about four Hg(II) ions are bound per dodecamer dimer. The exchangeable ^{1}H spectra (Figure 1 and Table 2) indicate that Hg(II) interacts with the AT tract of the duplex DNA dodecamer/4·Hg(II) complex. Furthermore, Hg(II) interfers with the A·T hydrogen bonds. This is confirmed by the ^{15}N chemical shifts. The presence of G N1–H···N3 C imino proton resonances and the insignificant changes of the chemical shifts of the nonexchangeable protons resonances belonging to G and C nucleotides indicate that a right-handed helical form of the duplex DNA/4·Hg(II) complex is retained for its GC parts. Moreover, it seems very unlikely that Hg(II) interacts with the GC parts at all.

The sequential assignment of the duplex DNA/4·Hg(II) complex and the semiquantitative interpretation of the NOESY spectra confirms that the GC parts of the duplex DNA/4·Hg(II) complex adopt a right-handed B-DNA like helical conformation. Oddly, the same conclusion seems to apply to the AT part of the complex. Even the characteristic interstrand cross peaks between A5 H2/A6 H2 and C9′ H1′/T8′ H1′ are observed. There are no indications of B to Z transition induced by Hg(II), nor are there any indications of right-helical parts of the complex reminiscent of A-DNA.

Based on these experimental observations and molecular modeling we suggest the following structural model: The duplex DNA dodecamer/4·Hg(II) complex adopts a right-handed double helical conformation. The structure of the GC parts (ends) which are not interacting with Hg(II) is very similar to normal B-DNA helices as present prior to Hg(II) addition. The AT (central) part of the duplex interacting with Hg(II) is also a right-handed helix, although a distorted one in order to accommodate the Hg(II) ions. The unmercurated GC ends and mercurated AT center of the complex are all linear, i.e., the right-handed duplex is not bent. One Hg(II) ion is inserted into each of the four A·T base pairs. This insertion into the Watson–Crick hydrogen bonds causes a stretching/opening of the A·T base pairs. The overall shape of the complex is thus that of a right-handed helical duplex with a slight "bulge" in its (central) mercurated AT part, which can be described as a tapering effect between the unmercurated GC and mercurated AT parts.

The part of the duplex which contains the four consecutive mercurated A·T base pairs cannot be an ordinary B-DNA helix. However, the NOESY spectra strongly indicate B-DNA characteristics even for this part of the duplex, like the successful sequential assignment of the ^{1}H resonances along the *complete* dodecamer strands. This may be explained by insertion of the Hg(II) ions between the A and T bases in such a way that the A·T base pairs are *opened* toward the major groove of the helix simultaneously with a slight stretching of these base pairs. This is most easily achieved by postulating the existence of T O4–Hg–N6 A bonds. The relative orientations of the sugar moieties and the nucleobases as well as the sugar conformations only need to be slightly altered, and the observation of certain B-DNA NOESY characteristics at a qualitative level is plausible. The opening of the A·T base pairs toward the major groove of the helix in concert with only slight base stretching, can explain why the cross strand A5 H2/C9′H1′ and A6 H2/T8′H1′ cross peaks are observed even if Hg(II) ions are inserted. These cross peaks are namely related to distances across the minor groove. The "new" cross peak between A6 H2 and T7 H6 does not fit directly into this picture. If it were to be interpreted as a direct interaction between these spins, a propeller twist of the central base pairs A6·T7′ and A6′·T7 may provide an explanation for its existence. However, a propeller twist in such a direction will inevitably cause the A6 H2 and T8′H1′ protons to move apart and their cross strand cross peak vanish, unless other slight rearrangements counteract this effect. It is even more difficult to envisage changes that may shorten the interstrand distance A6 H2–T7′ H6. Therefore, the "new" cross peak can probably best be explained by structural changes that would provide a more efficient spin-diffusion route than for the Hg(II) free dodecamer and where transient NOE enhancements between A6 H2 and T7 H6 (or T7′ H6) could pass via an intermediate spin, for instance T7 H1′ (or T7′ H1).

The way we think Hg(II) is inserted into the A·T base pairs may also explain why the chemical shifts of, e.g., T7 CH_3 and T8 CH_3 change differently upon incorporation of Hg(II). During the concerted opening and slight stretching of all A·T base pairs, the T8 nucleobase moves away from C9, and the orientation of the T8 CH_3 protons relative to C9's aromatic ring becomes altered. On the other hand, T7 CH_3 remain inside an intact consecutively stacked quartet of nucleobases A6/A6/T7/T8. The different effects from the orientations of the nucleobases T7 and T8 may cause different changes in the ring-current induced chemical shifts of the T7 CH_3 and T8 CH_3 protons, and therefore their chemical shifts move differently upon Hg(II) introduction as observed.

The G4 N1–H···N3 C9 base pair, adjacent to the mercurated AT tract, is intact as shown by the presence of its imino proton resonance at 292 K. Thus, the G4 N1–H···N3 C9 base pair seems to be more stable than the terminal G12 N1–H···N3 C1 base pair. This observation indicates a certain degree of discontinuity between the conformations of the central mercurated AT part and unmercurated GC ends of the complex.

Finally, we should stress although the overall picture of the DNA/Hg(II) complex should be reasonable, in order to work out the exact details of the structure, e.g., distance geometry calculations based upon high quality time dependent NOESY spectra are required.[22,23] Moreover, to test the proposed Hg(II)-bonding scheme, we plan to carry out gradient-enhanced ^{1}H detected ^{1}H–^{15}N HSQC spectroscopy at low temperatures in H_2O.[61,62] This will enable us to detect the ^{15}N signals of A5/A6 NH6 and T7/T8 N3. These ^{15}N nuclei couples strongly to the amino/imino protons through single bonds,[39,63,64] but no observable couplings to nonexchangeable protons exist. Another possible approach would be to use specifically ^{15}N-labeled nucleotides. Furthermore, by means of ^{1}H–^{15}N correlation spectra with better sensitivity and timing it should be possible to detect T N1 signals just as well as those of the cytosines.

Conclusion

In this study we have demonstrated that Hg(II) interacts selectively with the AT-tract of the DNA dodecamer duplex $[d(CGCGAATTCGCG)]_2$.

The ^{1}H NMR spectral patterns show that there is a Hg(II)-induced conformational transition from the original B-DNA form to a new conformer. The transition is completed at an approximate 5:1 ratio between [Hg(II)] and the concentration of the duplex form of the dodecamer. At intermediate stages of the titration ^{1}H NMR signals representing the two conformers coexists,

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indicating that there is slow exchange between the forms on the NMR time scale. The disappearance of the imino resonances of the thymines only, upon Hg(II) addition, show that the metal ion interferes with the Watson–Crick hydrogen bonds of the A·T base pairs. The changes induced by Hg(II) in the ^{1}H and ^{15}N chemical shifts and certain NOESY cross peaks confirm that Hg(II) only interacts with the AT tract. Nearly equal rotational correlation times of the dodecamer and the Hg coplex rule out the possibility of a Hg(II)-induced duplex to hairpin transition. The conformational changes induced by Hg(II) can be considered as perturbations of the normal B-DNA geometry, like stretching/opening of the A·T base pairs, producing a "bulge" in the AT tract of the dodecamer.

We cannot assess from the present data the exact binding site(s) for Hg(II) in the dodecamer, but the metal ions are almost certainly inserted into the A·T base pairs. In one model consistent with the data four Hg(II) ions form covalent bonds with the four A·T base pairs, involving A5/A6 NH_2 and T7/T8 O4 on opposite strands.

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