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Solution structure of a DNA double helix with consecutive metal-mediated base pairs

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Abstract

Metal-mediated base pairs represent a powerful tool for the site-specific functionalization of nucleic acids with metal ions. The development of applications of the metal-modified nucleic acids will depend on the availability of structural information on these double helices. We present here the NMR solution structure of a self-complementary DNA oligonucleotide with three consecutive imidazole nucleotides in its centre: In the absence of transition metal ions, a hairpin structure is adopted with the artificial nucleotides forming the loop. In the presence of silver(I) ions, a duplex comprising three imidazole–Ag^{+}–imidazole base pairs is formed. Direct proof for the formation of metal-mediated base pairs was obtained from $^{1}J(^{15}N,^{107/109}Ag)$ couplings upon incorporation of ^{15}N-labelled imidazole. The duplex adopts a B-type conformation with only minor deviations in the region of the artificial base pairs. This structure represents the first example of a metal-modified nucleic acid with a continuous stretch of metal-mediated base pairs.

Nucleic acids such as DNA are becoming increasingly popular as versatile building blocks in nanobiotechnology as a result of their superb, predictable self-assembling properties and the high rigidity of their double helices on the nanoscale.[1] Their applicability can be extended even further by the introduction of functional groups such as metal ions. One recently established method for the site-specific functionalization of nucleic acids with metal ions is based on the use of metal-mediated base pairs.[2-5] Such base pairs comprise natural or artificial nucleobases and rely on coordinative bonds to a central metal ion instead of (or in addition to) hydrogen bonds. Depending on the choice of nucleosides, metal ions, and oligonucleotide sequence, a plethora of metal-modified double helices can be generated. Even if focussing only on the use of *artificial* nucleosides, examples exist for DNA duplexes containing one or two metal-mediated base pairs interspersed between natural ones,[6-8] DNA double helices with continuous stretches of metalated base pairs,[9-11] and DNA duplexes with different metal-mediated base pairs at pre-defined positions.[12] When including also metal-mediated base pairs from natural nucleosides,[13-15] even more combinations can be envisaged.

In addition to DNA, other nucleic acids such as RNA,[16] GNA[17] or PNA[18, 19] have been modified with metal-mediated base pairs, too. Experimentally derived structural information on such metal-mediated nucleic acids is very scarce: only one DNA double helix comprising two non-neighbouring metal-mediated base pair has been structurally characterized as yet.[20] In addition, the structure of one metal-containing GNA duplex is known.[21] Neither of these contains a continuous stretch of metalated base pairs despite the enormous interest in this type of modification. Interestingly, neither of the helices adopts the canonical B-type structure either: The DNA duplex containing two pyridine-2,6-dicarboxylate–Cu^{2+}–pyridine base pairs crystallizes in the Z-conformation, probably as a result of the propensity of Cu^{2+} ions to bind additional axial ligands (here: furanose O4' of a neighbouring thymidine and O6 of a neighbouring guanine).[20] The structural distortion that is necessary to place these ligands above and below the square planar Cu^{2+} coordination environment provided by the artificial

nucleosides leads to a destabilization of the B-conformation and a concomitant stabilization of the Z-conformation. The GNA duplex on the other hand adopts a conformation that is entirely different from the canonical A-, B- or Z-forms and is largely a result of the unnatural backbone.[21]

To fill the gap and gain structural insight into a nucleic acid double helix that actually contains metal ions along its helical axis (as shown in most cartoons representing metal-mediated base pairs), we here determined the first structure of an oligonucleotide double helix that contains three consecutive silver(I)-mediated base pairs. In this paper, we describe the solution structure of the resulting DNA double helix as determined by NMR spectroscopy.

Results

The modified DNA adopts a hairpin in solution

For our structural investigations we used a self-complementary sequence with three consecutive artificial imidazole nucleosides in its centre (Fig. 1). We could previously show that such a type of sequence adopts a hairpin structure with the artificial nucleosides being placed in the loop in the absence of transition metal ions. A rearrangement to form a regular double helix with neighbouring metal-mediated base pairs takes place in the presence of silver(I) ions.[22] Based on this observation, which is also known from other nucleic acids, other nucleobases and other metal ions,[16] we substituted the 1,2,4-triazole nucleosides used in those previous experiments by imidazole nucleosides due to their superior silver(I)-binding properties.[23] Moreover, the presence of three hydrogen atoms in imidazole as compared with two hydrogen atoms in triazole yields more ^{1}H NMR resonances, thereby increasing the number of constraints as necessary for the structure refinement.

The proton resonances of the oligonucleotide in the absence of silver(I) ions were

assigned by [^{1}H,^{1}H]-NOESY spectra on the basis of the sequential walks along the H1' and aromatic protons (Fig. 2a). Three additional regions (H2'/aromatic, H2''/aromatic and aromatic/aromatic) served to cross-validate the assignments. Typical resonances for a B-type helical conformation were observed for nucleotides T1 – T7 and A11 – A17. The [^{1}H,^{1}H]-NOESY spectrum acquired in H_2O shows a large overlap of the imino proton resonances. Nevertheless, 13 interstrand correlations (mainly between adenine-H2 and H1' on the opposite strand) prove the formation of seven distinct A-T Watson-Crick base pairs. Only very weak and broad aromatic resonances could be observed for the three imidazole moieties, and consequently the sequential walk could not be followed throughout the loop. Nevertheless, based on the sugar-sugar region in the NOESY spectrum as well as TOCSY and ^{13}C,^{1}H-HSQC spectra, the three artificial nucleotides could be reliably identified and assigned. The [^{31}P]-NMR spectrum shows a set of signals between 0 and –0.5 ppm and a small broad peak at 0.45 ppm being consistent with a general hairpin structure.

However, it is difficult to differentiate unambiguously between a hairpin structure and a regular double helix by simple [^{1}H]-NMR experiments. We thus determined the hydrodynamic radius r_H of the oligonucleotide by DOSY (diffusion-ordered spectroscopy) spectra as well as DLS (dynamic light scattering) measurements. These two independent methods gave identical values within the error limits of 1.46±0.09 and 1.4±0.2 nm, respectively. A theoretical calculation of r_H using well-established equations[16] and assuming a hairpin structure is in excellent agreement with the experimental values observed by DOSY and DLS (Supplementary Table S1), thereby providing evidence that indeed a hairpin structure was formed in the absence of silver(I) ions.

The solution structure of this modified DNA hairpin is shown in Fig. 3a. The structure determination of the oligonucleotide in the absence of silver(I) ions is based on 354 conformationally restrictive NOE distance restraints (Table 1). The DNA adopts a stable hairpin structure with a well-defined helical region closed by an unstructured loop composed

of the three imidazole moieties. The overall r.m.s.d. of all heavy atoms from the 20 lowest energy structures is 1.6±0.4 Å (Table 1) and the independent superposition of the helical region results in an r.m.s.d. of 0.9±0.3 Å. The poor definition of the loop region is well in line with the small number of resonances observed and probably due to the semi-protonated artificial nucleotides: The pK_a of the imidazole base is estimated to be around 6.4 (imidazole β-nucleoside pK_a = 6.01±0.05,[23] plus the influence of the phosphate group of 0.39±0.04 log units[24]) and consequently close to the pH of 7.2 as used in the measurements. Obviously, no stable hydrogen bonding network is established between the imidazole moieties under these conditions.

In the presence of silver(I), a duplex structure is adopted

After the addition of one equivalent of silver(I) ions (for details see Methods), i.e. the amount needed to form a duplex with three metal-mediated base pairs, the NMR spectra change considerably. Generally, the quality of the spectra improves significantly, and especially in the sequential walk region many new resonances appear (Fig. 2b). The intense and sharp resonances belonging to the imidazole moieties indicate a rigid structure around the imidazole nucleotides as is expected for the formation of imidazole–Ag^+–imidazole base pairs with the N3 nitrogen atoms coordinating the silver ions (Fig. 1). The sequential walk can now be followed throughout the entire sequence, including the central artificial imidazole nucleotides. As a result of the palindromic sequence and the C_2 symmetry of the double helix, resonances of 17 nucleotides, i.e. exactly half the total number, can be observed and assigned. Interestingly, all H2 and H4 proton resonances of the imidazole moieties display a remarkable upfield shift of 0.40 – 1.08 ppm (Supplementary Table S2) being indicative of increased stacking interactions. The resonances of the nucleotides neighbouring the imidazole moieties show slight changes in chemical shift compared to the hairpin, whereas the helical nucleotides display a very similar pattern. All ^{31}P NMR resonances were found to be in the region typical

for a regular B-helical structure.[25] The hydrodynamic radius r_H as determined by DOSY and DLS increases by about 30% to 1.9±0.3 nm and 1.8±0.2 nm, respectively. Again, the theoretically calculated radius – this time assuming an extended double helical structure – perfectly matches the experimental values (Supplementary Table S1). The increase in r_H by about 30% is typical for a conformational change from hairpin to regular duplex and correlates well with previous observations.[16, 22]

Silver(I)-mediated base pairs are formed in the DNA double helix

To provide final proof that imidazole–Ag^+–imidazole base pairs are formed, we synthesized ^{15}N-labelled imidazole nucleosides and incorporated these into the oligonucleotide. The [^{15}N] HSQC spectra of the oligonucleotide in the absence and presence of silver(I) are shown in panels c) and d) of Fig. 2. Prior to the addition of the transition metal ions, only a few not very well-resolved cross-peaks are visible, indicating the presence of a rather unstructured hairpin loop. The ^{15}N chemical shifts cluster around 188 ppm for the N1 atoms (involved in the glycosidic bonds) and 221 ppm for the N3 atoms (Supplementary Table S2). In the presence of one equivalent of silver(I), the resolution of the HSQC spectrum improves dramatically indicating a more rigid structure and thus being well in line with the formation of a double helix. All nitrogen resonances experience an upfield shift: The N1 resonances shift to about 185 ppm ($\Delta\delta \approx -3$ ppm) while the N3 resonances move to about 206 ppm ($\Delta\delta \approx -15$ ppm). This significant upfield shift clearly indicates a metal coordination to the N3 positions[26] and is also indicative of increased stacking interactions as expected in a helix. Most importantly, the ^{15}N resonances of the N3 atoms show a splitting of about 75 Hz in the presence of silver(I) ions (Fig. 4a). This splitting is attributed to a direct coupling between the nitrogen and the silver atoms, i.e. a $^1J(^{15}N, ^{107/109}Ag)$ coupling, thereby providing the first direct proof for the formation of an azole–Ag^+–azole base pair. To the best of our knowledge this is also the first time that such a coupling has been measured in solution at ambient temperature.

The solution structure of the resulting double helix is shown in Fig. 3b. The structure was determined based on 848 conformationally restrictive NOE distance restraints (Table 1). During structure refinement a non-crystallographic symmetry (NCS) term was applied to accomplish perfect C_2 symmetry as based on the NMR spectra (see above). The overall r.m.s.d. of all heavy atoms from the 20 lowest energy structures is 1.3±0.4 Å. The individual superposition of the artificial nucleotides results in an r.m.s.d. of 0.5±0.2 Å. A total of 198 NOE-derived distance constraints were obtained just for the artificial nucleotides, including 6 interstrand and 36 direct stacking NOEs of the imidazole rings. The large number of distance restraints obtained for these residues helps tremendously to define precisely the orientation of the artificial bases. Overall, the metalated DNA retains its B-form helix upon formation of Ag^+ mediated base pairs in its centre.

Discussion

The solution structure shown in Fig. 3b provides the first structural insight into a nucleic acid with consecutive metal-mediated base pairs. It also proves that a DNA double helix can adopt the regular B-type conformation while at the same time arranging metal ions along its helical axis (Fig. 4b), as is evident from the base pair parameters shown in Fig. 5 and Supplementary Fig. S1, calculated with the programme 3DNA.[27] In both Figures, the absolute values of the respective parameters are shown on the right whereas the values relative to those of average B-DNA[28] are given on the left. Tilt τ, roll ρ, inclination η and global displacement of all nucleobases adopt values close to those of B-DNA (Supplementary Fig. S1). Interestingly, small deviations from the B-type structure are observed for helical twist Ω and helical rise h (Fig. 5): The helical twist Ω adopts values of about 36°, with the exception of the two central base pair steps between the three metal-mediated base pairs. The helical twist of these three metal-mediated base pairs amounts to only ~28°, indicating a slight unwinding of the double helix. This unwinding appears to be caused by an opening of the minor groove, while the

major groove remains largely unchanged (Supplementary Fig. S2). The small but significant structural change could be used to design small molecules than specifically target this type of metal-mediated base pairs. Along the same line, the helical rise *h* is typically in the range of 3.4 Å, again with significant deviations from this value in the central region of the double helix (Fig. 5): *h* increases to 4.3(1) Å between adjacent A–T and Im–Ag^{+}–Im base pairs and to 4.1(3) Å between consecutive metal-mediated base pairs.

Going along with the latter value of *h*, the metal-metal distances between neighbouring silver(I) ions along the helical axis amount to 3.92 Å and 3.97 Å for the lowest-energy structure, and vary between 3.79 and 4.51 Å for the 20 best structures. The total value of almost 4 Å for the lowest-energy structure appears to rule out direct metal-metal interactions. This distance is unexpectedly large, especially considering that attractive argentophilic interactions are frequently observed between closely spaced silver(I) ions.[29] On the other hand, the high variation – also manifested in the relatively large standard deviation of the helical rise *h* for the central two base pair steps – shows that the energy differences between conformations with differing metal-metal distances are small. As a result, only minor energetic changes can lead to structures with a significant metal-metal interaction. For example, a recent theoretical inspection of DNA containing copper(II)-mediated base pairs shows that metal-metal distances down to 3.22 Å are possible within the structural context of a DNA double helix.[30] At this point it remains unclear whether the observed metal-metal distances are real or whether they are merely the result of an overestimation of the electrostatic repulsion by the force field applied during the structure calculations with X-PLOR-NIH.

In summary, we have presented the first structural insight into a B-type DNA double helix containing consecutive metal-mediated base pairs. The solution structure of a self-complementary oligonucleotide comprising three central imidazole–Ag^{+}–imidazole base pairs shows that the metal ions are located along the helical axis. In the sole previous structure of a

DNA duplex with metal-mediated base pairs, the metal ions were located at the periphery of the helix due to the Z-type conformation that was adopted in that structure.[20] The unprecedented observation of $^{1}J(^{15}N, ^{107/109}Ag)$ couplings in solution at room temperature provides a direct proof for the formation of the metal-mediated base pairs. An analysis of the base pair parameters shows that the incorporation of the artificial metal-containing base pairs proceeds without major conformational distortions. A slight unwinding of the helix in the region of the imidazole–Ag^{+}–imidazole base pairs provides the opportunity for the development of small molecules that selectively recognize this structural motif. Silver-silver distances between consecutive metal-mediated base pairs amount to 3.92 Å and 3.97 Å for the lowest-energy structure. Nevertheless, direct silver–silver interactions cannot be ruled out completely because the duplex structure seems to be rather dynamic, thereby allowing shorter metal-metal distances without large energy barriers.

Methods

Preparations

3,5-Di-*p*-toluoyl-1,2-dideoxy-*β*-1-(^{15}N-imidazol-1-yl)-D-ribofuranose **1**

^{15}N imidazole was synthesized according to a modified literature procedure:[31] To a solution of glyoxal (40 wt.% solution in water, 11.3 mmol, 1.29 mL) and formaldehyde (36.5 wt.% solution in water, 11.3 mmol, 851 µL), $^{15}NH_4Cl$ (1.02 g, 18.7 mmol, 98% ^{15}N) was added, and the pH adjusted to zero by addition of concentrated HCl. After stirring at 95 °C for 2 h, the reaction mixture was cooled to ambient temperature, treated with solid KOH until pH 10, and the solvent was removed in vacuo. A Kugelrohr distillation (100 °C, 10^{-3} mbar) was performed to separate ^{15}N imidazole from side product KCl. This crude ^{15}N imidazole (375 mg, 5.35 mmol assuming 100% purity) was dissolved in MeCN (40 ml), and NaH (60% in oil, 257 mg, 6.42 mmol) was added at 0 °C. After stirring for 30 min at 0 °C, 3,5-di-O-*p*-

toluoyl-α-D-*erythro*-pentofuranosyl chloride[32] (2.5 g, 6.4 mmol) was added in four portions every 20 minutes. The solvent was removed in vacuo after stirring for additional 2 h, and the remaining solid was dissolved in CH_2Cl_2 (50 ml), washed with H_2O (30 ml) and dried (Na_2SO_4). The solvent was removed, and the crude product purified by column chromatography (SiO_2, CyHex:CH_2Cl_2:Et_3N 50:30:8) yielding 1.2 g (2.9 mmol, 54 %) of **1**.
For the characterization of **1** and further synthetic steps towards the DMT- and phosphoramidite protected imidazole nucleoside, see Supplementary Information.

Oligonucleotide preparation

Phosphoramidites of the natural nucleosides as well as CPGs were purchased from Glen Research. The oligonucleotides were prepared using an Expedite 8909 synthesizer in the DMT-off mode and purified by HPLC with a Nucleogen 60-7 DEAE column following standard procedures. For details see Supplementary Information.

NMR spectroscopy

All oligonucleotide NMR spectra were recorded on a Bruker AV700 MHz spectrometer equipped with a CP-TXI z-axis pulsed-field gradient CryoProbe®, except the ^{31}P spectra which were obtained on a Bruker AV2-400 MHz spectrometer equipped with a QNP probe. Non-exchangeable resonances were assigned from [^1H,^1H] NOESY (250 ms mixing time, 293, 298 and 303 K), [^1H,^1H] TOCSY (50 ms mixing time, 298 K), [^1H,^{13}C] HSQC (298 K) and long-range [^1H,^{15}N] HSQC ($^1J_{NH}$ = 20 Hz, 298 K) spectra in D_2O. Only very few exchangeable proton resonances could be assigned due to large overlap of the thymine N3H resonances. DOSY spectra (298 K) were acquired and processed as previously described.[16] All ^1H,^1H and ^1H,^{13}C spectra were recorded at natural isotope abundance, whereas ^1H,^{15}N spectra were recorded with DNA containing 98% enriched ^{15}N imidazole moieties. NMR data were processed with TOPSPIN 1.3 and 2.0 (Bruker) and analyzed by using Sparky

(http://www.cgl.uscf.edu/home/sparky/). NOE peak volumes were integrated with the Gaussian peak fitting function in Sparky.

Structure calculations

NOE distances were estimated from the integrated peak volumes obtained from the [^{1}H,^{1}H] NOESY spectra acquired at 298 K with a mixing time of 250 ms. Distances were calibrated by using the CALIBA macro in DYANA.[33] The NOEs were grouped into four categories, corresponding to strong (1.8-3.0 Å), medium (1.8-4.5 Å), weak (3.0-6.0 Å) and very weak (4.0-7.0 Å). Structure calculation of the hairpin was then performed following standard procedures using DYANA 1.5[33] and XPLOR-NIH 2.15.0.[34] The imidazole (IMI for XPLOR-NIH) residue was inserted into the parameter and topology files by using the structural parameters from previous DFT calculations.[23] The IMI residue was then patched into the oligonucleotide like a standard nucleotide by the DYANA and XPLOR program, respectively. For details see Supplementary Information.

Structure calculation of the duplex was performed with X-PLOR-NIH.[34] Torsion angles and sugar pucker restraints were set based on the NMR data as described for the hairpin. The backbone torsion angles were set to exclude the *trans* range and cover the B-DNA range. The sugar pucker was restrained to *S*-type and the glycosidic torsion angle χ was set to –120±20° (*anti*) for all residues, apart from the imidazole moieties, which were left unrestrained. First, an extended structure was generated including the geometrical parameters of the Im-Ag$^+$-Im base pair obtained from DFT calculations.[23] Starting from the extended strand a set of 2000 structures was calculated using NOE distances and dihedral restraints. Besides the planarity and hydrogen bond distance restraints of the natural base pairs, planarity and distance restraints for the Im-Ag$^+$-Im base pairs were also included to maintain the coordinative bond properties. Based on the perfect C_2 symmetry observed in the NMR spectra showing only one half of the duplex, a non-crystallographic symmetry (NCS) term was introduced. The 200

structures with lowest energies were used for further refinement using additional RAMA and ORIE database terms. The twenty lowest-energy structures out of 200 calculated of the hairpin and the duplex were visualized and analyzed by using MOLMOL.[35]

Structure coordinates are deposited at the Protein Data Bank (hairpin: 2K68, duplex: 2KE8), and the NMR chemical shift assignments at the BioMagResBank (hairpin: 15860, duplex: 16138).

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Author contributions

JM and RKOS designed research, NM and DB performed syntheses, SJ performed the NMR experiments and structure calculations, SJ and RKOS analyzed data, and JM, RKOS, SJ and NM wrote the manuscript.

Competing Financial Interests statement

The authors declare no competing financial interests.

Figure Legends

Figure 1: Schematic depiction of the hairpin to duplex transition of the oligonucleotide under investigation. The nucleotide numbering scheme is also given with the helical regions in green, the imidazole nucleotides in gold and the Ag^+ ions in blue.

Figure 2: Comparison of sections of the [^{1}H,^{1}H] NOESY and [^{1}H,^{15}N] HSQC spectra of the hairpin (a,c) and the metalated helix (b,d). The sequential walk is indicated by solid and dotted lines. It can be seen well that more and sharper resonances are visible for the metalated duplex (b,d) compared to the hairpin (a,c).

Figure 3: Lowest energy structures of the hairpin (a) and duplex containing the Ag^+-mediated imidazole base pairs in its centre (b). The B-form helical regions are coloured in green, the imidazole nucleobases in gold and the Ag^+ ions are shown as blue spheres. This Figure has been prepared with MOLMOL.[35]

Figure 4: Direct evidence for the formation of imidazole-Ag^+-imidazole base pairs. a) Section of the [^{1}H,^{15}N] HSQC (see also Fig. 2) with the magnification of the Im8 N3-H2 and N3-H5 cross peaks as an example to illustrate the $^1J(^{15}N, ^{107/109}Ag)$ coupling of 75 Hz. b) Top view on the three consecutive metal-mediated base pairs along the helix axis. The backbone ribbon is displayed for the whole duplex. This panel has been prepared with MOLMOL based on the lowest energy structure.[35]

Figure 5: Helical twist Ω and helical rise h for the silver(I)-containing double helix. Both parameters are global parameters based on C1'-C1' vectors. Values relative to those of average B-DNA are given on the left scale, absolute values on the right scale.

Table 1: NMR restraints and structural statistics for the hairpin and metal-modified duplex structures. All statistics are given for the respective 20 lowest energy structures out of 200 calculated structures.

	Hairpin	**Duplex**
NOE-derived distance restraints	354	848
natural base pairs	316	650
imidazole nucleotides Im8-Im10 / Im25-Im27	38	198
intra-nucleotide	147	366
inter-nucleotide ($\mid i-j \mid = 1$)	185	442
long-range ($\mid i-n \mid \geq 2$)	22	40
repulsive	0	0
NOE restraints per residue	20.8	24.9
NOE violation > 0.2 Å	0	0
dihedral restraints	148	218
dihedral violations > 5°	0	0
hydrogen-bond restraints	28	56
imidazole-imidazole restraints	-	18
r.m.s.d. (for all heavy atoms to the best structure [Å])		
overall	1.6±0.4	1.3±0.4
helix	0.9±0.3	0.6±0.1
Im8-Im10 / Im25-Im27	2.7±0.8	0.5±0.2

Graphical abstract

The solution structure of a B-type DNA duplex with consecutive silver(I)-mediated base pairs is presented.

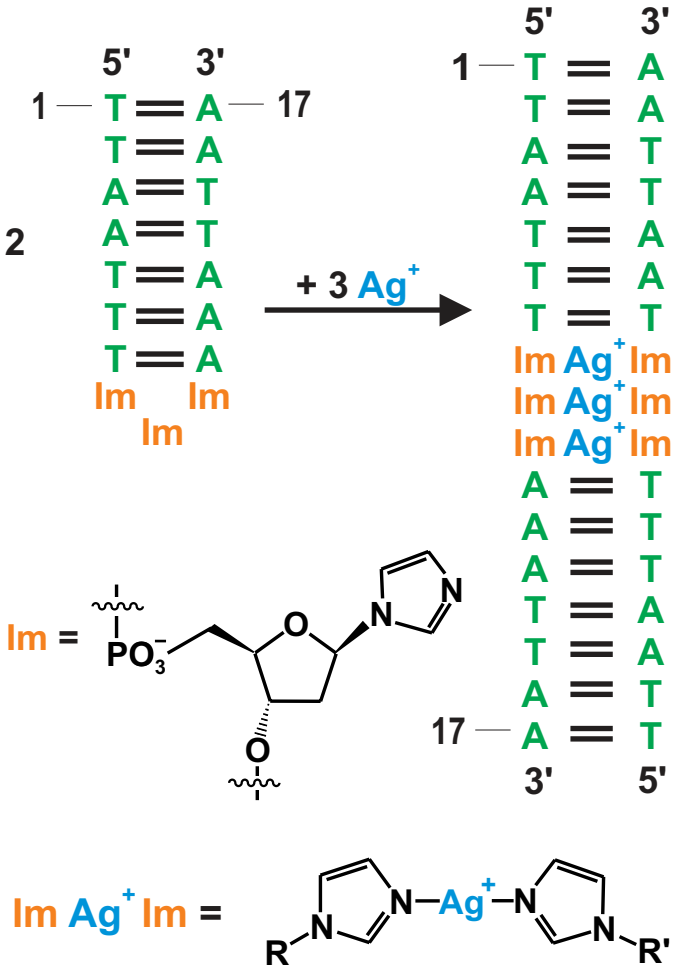


Figure 1

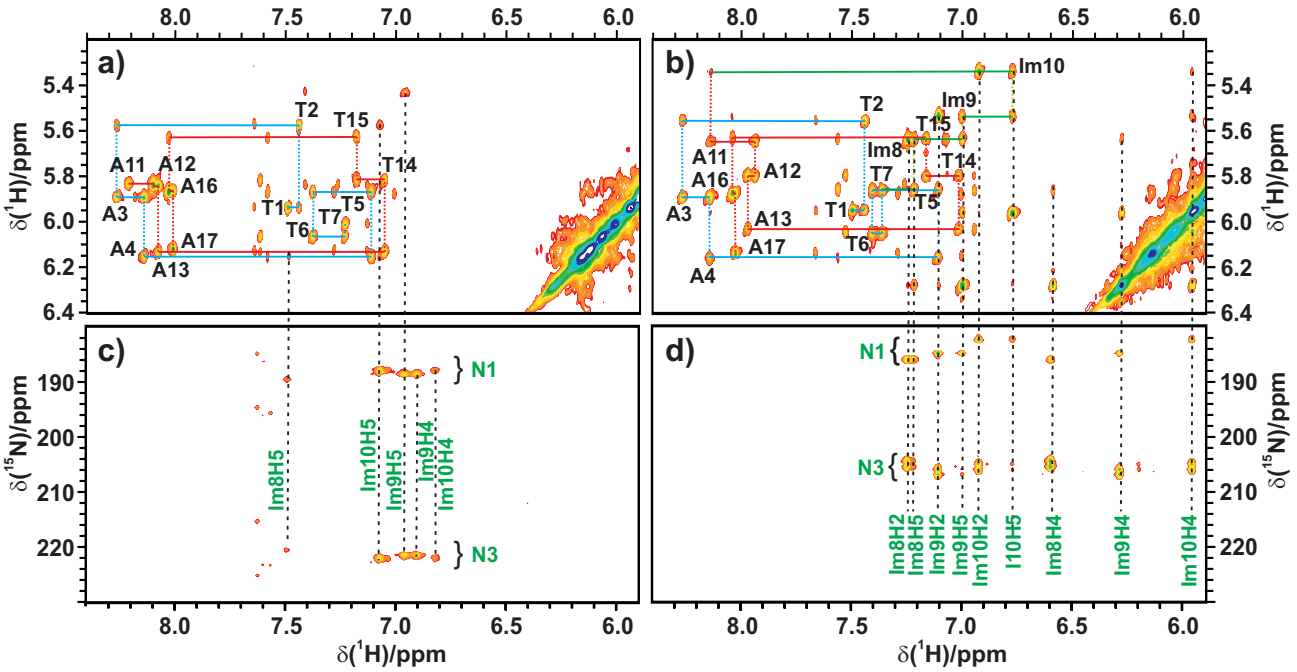

a)
b)
c)
d)
δ(¹H)/ppm
δ(¹⁵N)/ppm
8.0
7.5
7.0
6.5
6.0
5.4
5.6
5.8
6.0
6.2
6.4
190
200
210
220
A3
A4
A11
A12
A13
A16
A17
T1
T2
T5
T6
T7
T14
T15
Im8
Im9
Im10
N1
N3
Im8H5
Im10H5
Im9H5
Im9H4
Im10H4
Im8H2
Im9H2
Im10H2
I10H5
Im8H4

Figure 2

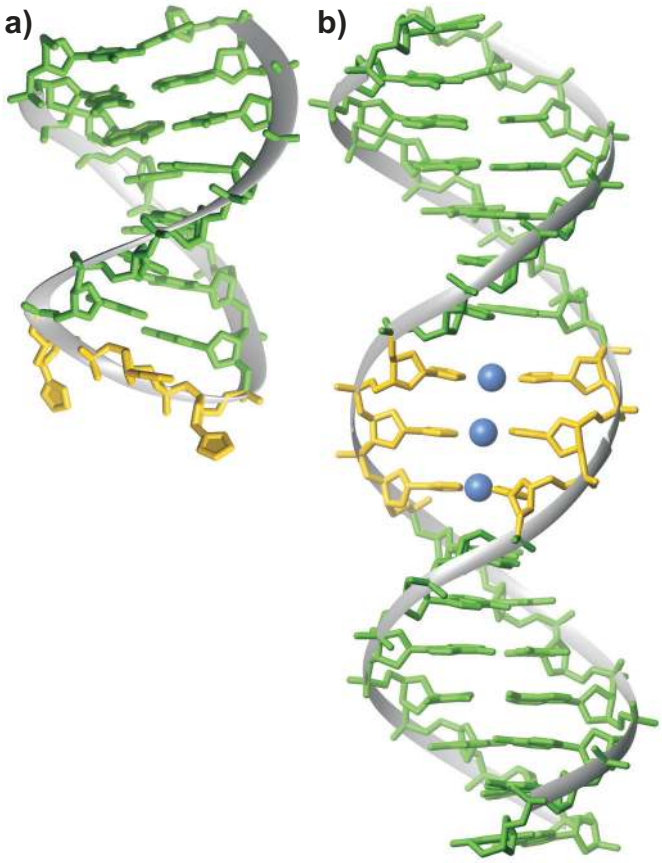


Figure 3

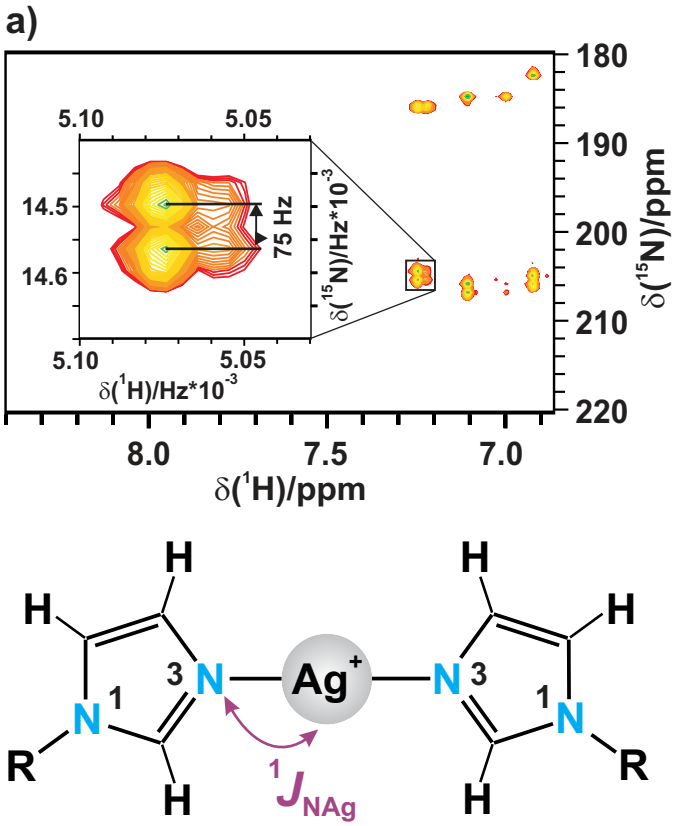

a)
180
190
200
210
220
δ(15N)/ppm
8.0
7.5
7.0
δ(1H)/ppm
5.10
5.05
14.5
14.6
75 Hz
δ(15N)/Hz*10-3
δ(1H)/Hz*10-3
H
N
Ag+
R
1JNAg
b)

Figure 4

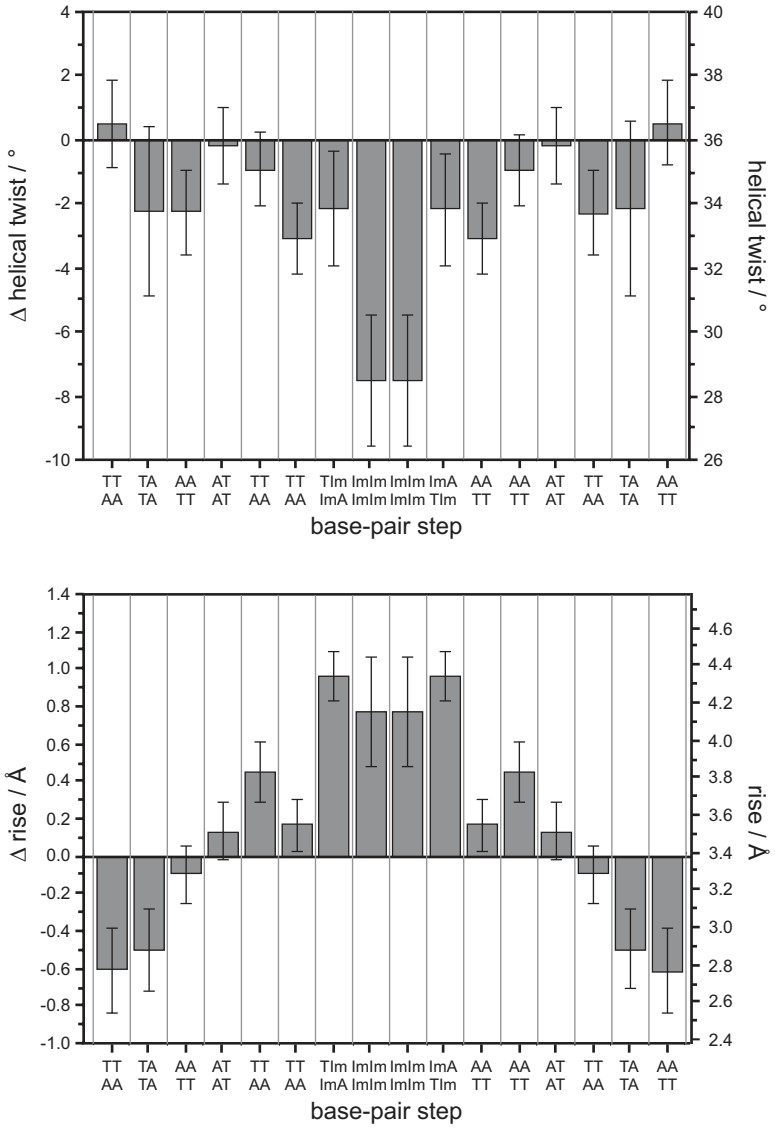
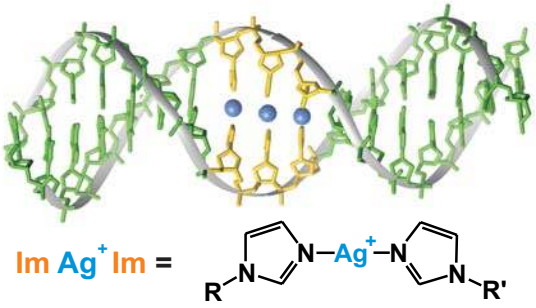

Δ helical twist / °
helical twist / °
base-pair step
Δ rise / Å
rise / Å
base-pair step
TT AA
TA TA
AA TT
AT AT
TT AA
TT AA
TIm ImA
ImIm ImIm
ImIm ImIm
ImA TIm
AA TT
AA TT
AT AT
TT AA
TA TA
AA TT

Figure 5



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