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Concerted Intercalation and Minor Groove Recognition of DNA by a Homodimeric Thiazole Orange Dye

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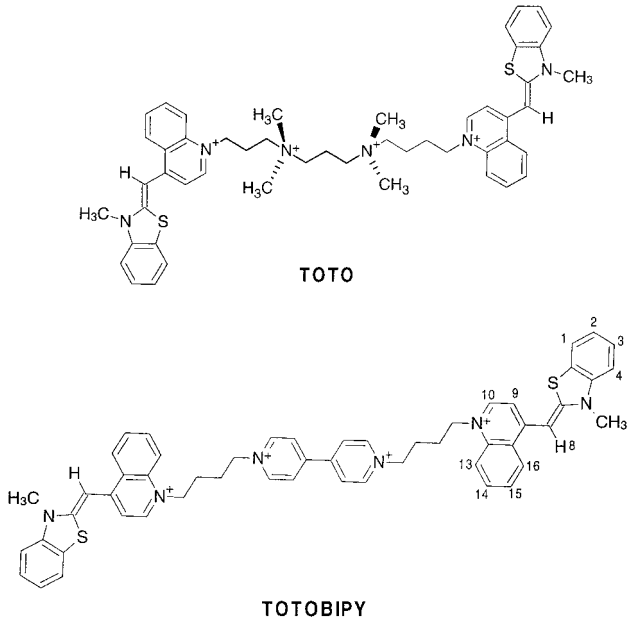
The thiazole orange dye TOTO binds to double-stranded DNA (dsDNA) by a sequence selective bis-intercalation. Each chromophore is sandwiched between two base pairs in a (5′-CpT-3′):(5′-ApG-3′) site, and the linker spans two base pairs in the minor groove. We have used one- and two-dimensional NMR spectroscopy to examine the dsDNA binding of an analogue of TOTO in which the linker has been modified to contain a bipyridyl group (viologen) that has minor groove binding properties. We have investigated the binding of this analogue, called TOTOBIPY, to three different dsDNA sequences containing a 5′-CTAG-3′, a 5′-CTTAG-3′, and a 5′-CTATAG-3′ sites, respectively, demonstrating that TOTOBIPY prefers to span three base pairs. The many intermolecular NOE connectivities between TOTOBIPY and the d(CGCTTAGCG):d(CGCTAAGCG) oligonucleotide in the complex shows that the bipyridyl-containing linker is positioned in the minor groove and spans three base pairs. Consequently, we have succeeded in designing and synthesizing a ligand that recognizes an extended recognition sequence of dsDNA as the result of a concerted intercalation and minor groove binding mode.

INTRODUCTION

The study of molecular recognition and in particular the chemical requisites for the formation of specific drug−DNA[1] complexes has been an active research area (Johnson and Boger, 1996). The goal is to rationally design novel molecules, capable of sequence-specific DNA-recognition, by exploiting a combination of both steric and hydrogen bond donor−acceptor complementarity. The very high affinity for DNA exhibited by bis-intercalating agents make them interesting candidates for the design of bimodal DNA-binding molecules by combining this high affinity with the sequence selectivity exhibited by minor groove binding agents.

Recent work in our laboratory has focused on ^{1}H NMR studies of the binding-mode and sequence selectivity of *N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis-{3-[4-[(3-methyl-2(*3H*)-benzothiazolilyden)methyl]quinolinium-1-yl]propyl}-1,3-propandiammonium tetraiodide (TOTO, Scheme 1) with various dsDNA oligonucleotides (Bondensgaard and Jacobsen, 1999; Petersen et al., 1999; Bunkenborg et al., 1999; Jensen et al., 1998; Johansen and Jacobsen, 1998; Petersen and Jacobsen, 1998; Faridi et al., 1997; Stærk et al., 1997; Hansen et al., 1996; Spielmann et al., 1995; Jacobsen et al., 1995). We have shown that TOTO binds sequence selectively to double-stranded DNA (dsDNA) by bis-intercalation. Each chromophore is sandwiched between two base pairs in a d(5′-py-*p*-py-3′):d(5′-pu-*p*-pu-3′) site, and the linker spans two base pairs in the minor groove. We have examined the binding of TOTO to various dsDNA oligonucleotides and found that the 5′-CTAG-3′ sequence (or analogues of that) is the most favorable binding motif (Bunkenborg et al., 1999).

Scheme 1. Chemical Structure of TOTO and TOTOBIPY with Numbering Schemes



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[1] Abbreviations: BIPY, 4,4′-bipyridyl; DNA, deoxyribose nucleic acid; DMSO, dimethyl sulfoxide; dsDNA, double-stranded DNA; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DQF-COSY, double quantum filtered correlation spectroscopy; EDTA, ethylenediaminetetraacetic acid; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RMD, restrained molecular dynamics; TOCSY, total correlation spectroscopy; TOTO, *N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis-{3-[4-[(3-methyl-2(*3H*)benzothiazolilyden)methyl]quinolinium-1-yl]propyl}-1,3-propandiammonium tetraiodide; TOTOBIPY, *N*,*N*′-bis-{3-[4-[(3-methyl-2(*3H*)benzothiazolilyden)methyl]-quinolinium-1-yl]butyl}-4,4′-bipyridinium tetraiodide.

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The thiazole orange chromophore of TOTO has been shown to prefer to intercalate at a d(5′-C*p*T-3′):d(5′-A*p*G-3′) base pair step. The bis-intercalation of TOTO obeys the nearest neighbor exclusion principle with the linker spanning two bases in the minor groove. The short positively charged polyamine linker of TOTO contributes to the d(5′-T*p*A-3′):d(5′-A*p*T-3′) minor groove preference due to the favorable negative electrostatic potential in the AT-step minor groove (Bunkenborg et al., 1999). Earlier we have synthesized derivatives of TOTO with extended methylene linkers between the quinolinium ring and the quaternary ammonium nitrogen. One of these derivatives was found to predominantly bis-intercalate in the 5′-CTGAG-3′ site (Stærk et al., 1997), but a longer polyamine linker than the one in TOTO reduces the sequence selectivity (Bunkenborg et al., 1999).

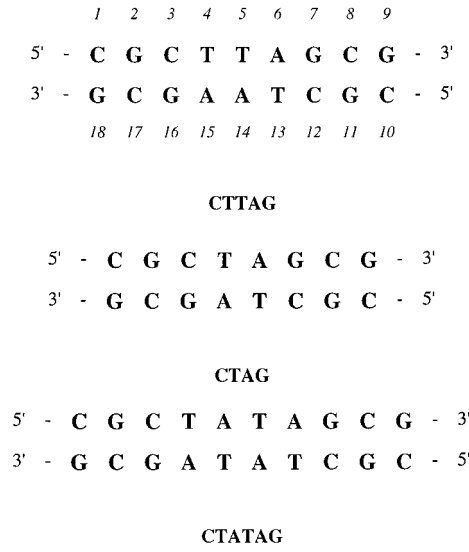
How do we avoid the reduction in sequence selectivity when trying to extend the recognition sequence for new TOTO analogues? A rather obvious suggestion is to replace the flexible linker with a more rigid element endowed with some of the typical minor groove binding characteristics. The 4,4′-bipyridyl (viologen) element is well suited for this purpose because it forms a rigid positively charged aromatic linker. Viologen has been shown to endow bis-intercalators with a preference for AT base pairs by serving as a minor groove binder (Takenaka et al., 1997). Consequently, we have synthesized (Deligeorgiev et al., 2000) and investigated the DNA-binding properties of a TOTO analogue with the polyamine linker substituted by a 4,4′-bipyridyl containing linker: *N*,*N*′-bis-{3-[4-[(3-methyl-2(*3H*)benzothiazolilyden)methyl]quinolinium-1-yl]butyl}-4,4′-bipyridinium tetraiodide (TOTOBIPY, Scheme 1). In this paper, we present the results of one- and two-dimensional ^{1}H NMR studies of complexes between dsDNA and TOTOBIPY. We have investigated the binding of TOTOBIPY to three different dsDNA sequences containing the 5′-CTAG-3′, the 5′-CTTAG-3′ and the 5′-CTATAG-3′ sites, respectively. All these sequences contain two d(5′-C*p*T-3′):d(5′-A*p*G-3′) base pair steps as preferred by the thiazole orange chromophore of TOTO, but separated with a different number of base pairs. Thus, we were able to show that TOTOBIPY preferentially spans three base pair steps as the result of a concerted intercalation and minor groove binding mode. The many intermolecular NOEs of the TOTOBIPY−CTTAG complex demonstrates that the BIPY-element is situated in the minor groove as expected.

EXPERIMENTAL PROCEDURES

Materials. Purified DNA oligonucleotides were purchased from DNA Technology (Aarhus, Denmark) and used without further purification. The non-self-complementary single-stranded DNA oligomers were added to an equivalent amount of the complementary strand and duplexes formed by annealing from 80 °C to room temperature over 2 h. In this work, three different oligonucleotides have been used (Scheme 2).

TOTOBIPY was synthesized as described earlier (Deligeorgiev et al., 2000). A stock solution of TOTOBIPY in DMSO-d_6 was used for complex formation with the dsDNA oligonucleotides by the procedure described earlier (Jacobsen et al., 1995). A phosphate buffer containing ethylenediaminetetraacetic acid (EDTA) to complex paramagnetic ions, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as a chemical shift reference and NaN_3 as antibacterial agent was added, giving a final concentration in the NMR sample of 10 mM P_i (pD = 7.0), 0.025 mM EDTA, 0.1 mM DSS, and 0.01 mM NaN_3. The complexes were redissolved in 500 μL of 99.96% D_2O (from Cambridge Isotope Laboratories) to yield a concentration of 2 mM. For experiments in H_2O, a mixture of 90% H_2O/10% D_2O (500 μL) was used. The samples remained stable for months.

Scheme 2. Numbering Schemes for the Three dsDNA Duplexes Used



NMR Experiments. All NMR experiments on the DNA−dye complexes were performed on a Varian Unity 500 MHz NMR spectrometer and a Varian INOVA 750 MHz NMR spectrometer. NOESY spectra in D_2O were acquired with a mixing time of 200 ms using 2048 complex points in t_2 and a spectral width of 5000 Hz for a total of 512 t_1-experiments with 64 scans each at 298 K. For the CTTAG−TOTOBIPY complex, an additional NOESY spectrum in D_2O was acquired at 750 MHz with a mixing time of 200 ms using 4096 complex points in t_2 and a spectral width of 7500 Hz for a total of 600 t_1-experiments with 48 scans each at 298 K. NOESY spectra in H_2O were acquired using 2048 complex points and a spectral width of 10 000 Hz using a NOESY pulse sequence where the last 90° pulse was replaced by a pulse containing a notch to suppress the water signal (Stein et al., 1995). This ensured suppression of the water signal together with a linear excitation profile over the whole spectral width.

TOCSY experiments in D_2O were acquired with mixing times of 90 ms using 2048 complex points in t_2 and a spectral width of 5000 Hz with total of 512 t_1 experiments with 64 scans each. DQF-COSY experiments in D_2O were acquired using 2048 complex points in t_2 and a spectral width of 5000 Hz with total of 1024 t_1 experiments with 64 scans each.

The acquired data were processed using FELIX (version 97.2, Biosym/MSI, San Diego, CA). The spectra were assigned in the conventional way as described in the literature (Wijmenga and van Buuren, 1998).

Molecular Modeling. A model structure of the CTTAG−TOTOBIPY complex was obtained by restrained molecular dynamics (RMD) calculations using the *Discover* program (MSI, San Diego, ver. 2.9.7) with a modified AMBER force field. Modifications were made to take the thiazole sulfur atoms and the bipyridyl group into account. Graphical displays were obtained by the *Insight*II program (MSI, SanDiego, ver. 97.2).

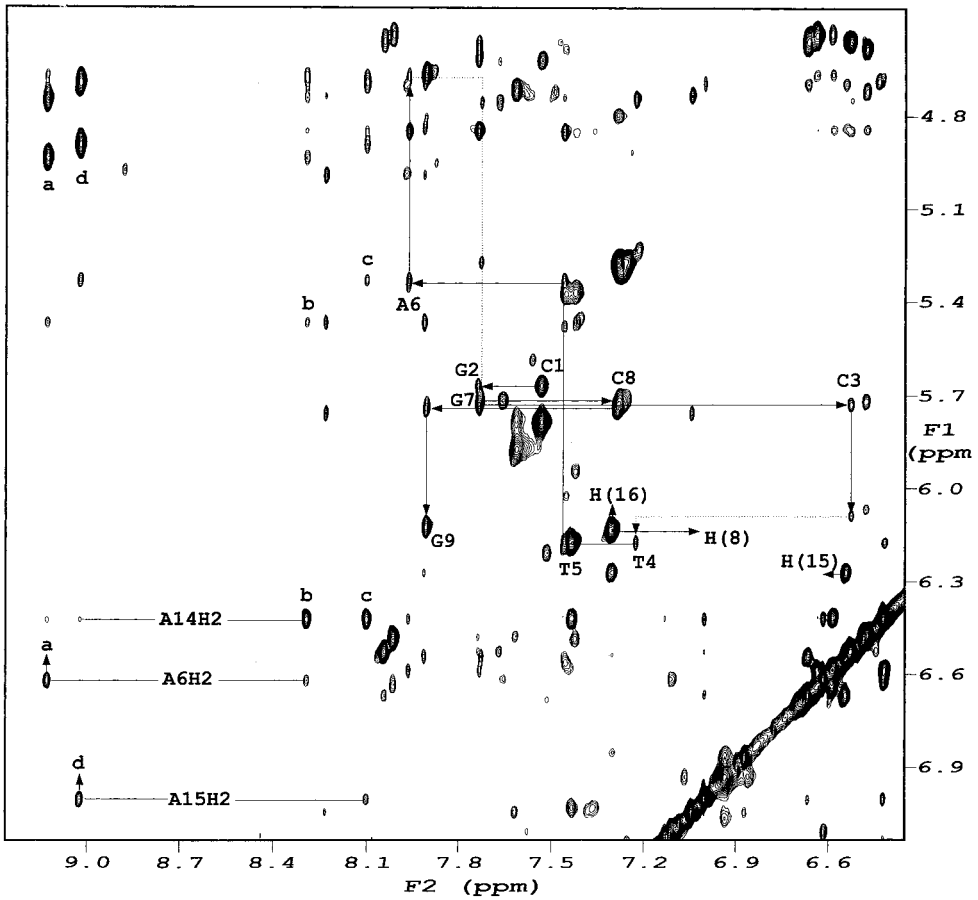


Figure 1. The aromatic to H1′ region of a 750 MHz NOESY spectrum (mixing time of 200 ms) of the CTTAG-TOTOBIPY complex at 25 °C. The sequential $H1'_{(n-1)}$-$H6/H8_{(n)}$-$H1'_{(n)}$ connectivity pattern is shown with solid lines for the 5′-CGCTTAGCG-3′-strand. Interruptions at the 5′-C3pT4-3′ and the 5′-A5pG6-3′ binding sites are indicated with broken lines. A few crucial cross-peaks between TOTOBIPY and the DNA are indicated and in the asymmetric complex there are four distinct bipyridyl proton resonances (Figure 2). Intramolecular cross-peak of the chromophore in TOTOBIPY are also indicated.

The dsDNA strands were built in *InsightII*, and the TOTOBIPY ligand was docked manually into the binding site. Restraints between hydrogens on the DNA and the ligand were obtained from the 200 ms NOESY spectrum using the isolated spin pair approximation. The restraints were divided into classes of weak, medium, and strong connectivities. The three base pairs at each end of the dsDNA were fixed in the B-DNA form by generic restraints. Twenty structures were calculated using the following RMD protocol: initially 100 and 1000 cycles of steepest descent and conjugate gradient energy minimizations, respectively. This was followed by 28 ps of RMD (steps of 1 fs) with the following temperature profile: 600 K for 4 ps and then cooled to 200 K in 50 K steps of 3 ps each. Force constants of 50 kJ/mol were used. Finally, the structures were energy minimized using conjugate gradient until the derivative was less than 0.01 Å.

RESULTS

d(CGCTTAGCG):d(CGTAAGCG) (CTTAG) Oligonucleotide. The mixture of CTTAG + TOTOBIPY yields a spectrum with sharp lines from a single major form (~90%). The NOESY spectrum of this CTTAG−TOTOBIPY complex exhibits the characteristic features of dsDNA sequential connectivities from aromatic H6/H8 protons to both intra- and interresidue H1′ and H2′/H2″. However, the sequential connectivities are interrupted at the 5′-C3*p*T4-3′, 5′-C12*p*T13-3′, 5′-A6*p*G7-3′, and 5′-A15*p*G16-3′ base pair steps. This is clear evidence of bis-intercalation. Interruption of the sequential NOE connectivities is also observed in the cross-peak pattern of the methyl group of T4 and T13 for which the peak to C3 H6 and C12 H6, respectively, are missing. The NOESY spectrum of the complex in H_2O shows the normal Watson−Crick NOE connectivity pattern (Wüthrich, 1986).

The internal NOE connectivities in the chromophore of TOTOBIPY are a distinct feature of the NOESY spectra of the complex. Cross-peak patterns connect H16-H15-H14-H13, H8-$CH_3$6-H4-H3-H2-H1, and H9-H10, respectively. A very strong cross-peak between H8 and H16 and a cross-peak pattern connecting H9-H10-H13 establish the assignments of the individual protons and the conformation of the TOTOBIPY ligand. Resonances in the linker of TOTOBIPY were assigned by combined use of TOCSY and NOESY cross-peaks. On the basis of the cross-peak pattern in the NOESY spectrum, we conclude that the relative conformation of the two ring systems in each chromophore is that indicated in Scheme 1.

A large number of cross-peaks between TOTOBIPY protons and protons on the oligonucleotide was observed. A few of them are indicated in Figure 1 and further illustrated in Figure 2. These intermolecular cross-peaks clearly show that TOTOBIPY bis-intercalates in the (5′-CTTAG-3′):(5′-CTAAG-3′) site. In detail, sequential connectivities for the 5′-T4A5A6-3′ and 5′-T13T14A15-3′ sequences show that no intercalation takes place in this part of the oligonucleotide, proving that the linker spans

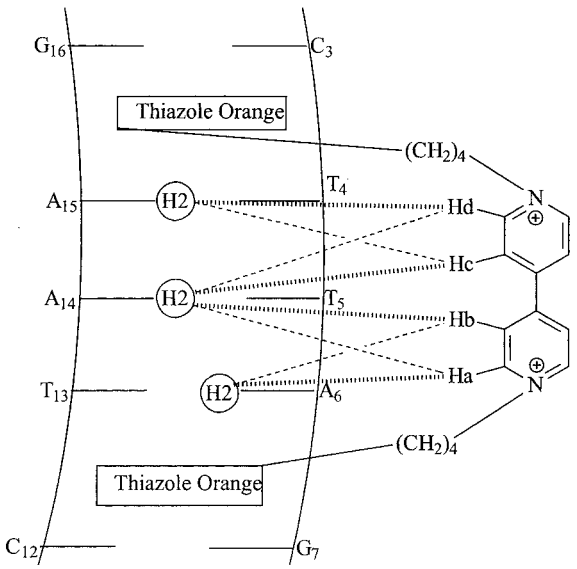


Figure 2. A few of the observed intermolecular NOE connectivities observed between the bipyridyl protons of the linker and the H2 on the adenines. Strong connectivities (| | |) and medium connectivities are both indicated (- - -).

three base pairs. Interruptions in the sequential $H1'_{(n-1)}$-$H6/H8_{(n)}$-$H1'_{(n)}$ pattern confirm the binding site. The aromatic protons on A6 and G7 have cross-peaks to H15 and/or H14 on one of the chromophores while the aromatic protons on A15 an G16 have cross-peaks to H15 and H14 on the other chromophore. Furthermore, an upfield shift of approximately 0.8 ppm of the methyl group proton resonances of T4 and T13 is observed while the methyl resonance of T5 is unchanged. All together, this establishes bis-intercalation in the 5′-C|*TTA*|G-3′ binding site with the linker spanning the three central bases. Cross-peaks in the NOESY spectrum unambiguously show that the linker is positioned in the minor groove. This is demonstrated by a large number of cross-peaks of which some are illustrated in Figure 2.

A remarkable feature of the complex of CTTAG−TOTOBIPY is the occurrence of only four resonances from the 4,4′-bipyridyl moiety. This suggests that the bipyridyl is undergoing rapid rotation within the NMR time scale.

On the basis of the observed NOE connectivities between TOTOBIPY protons and protons on the oligonucleotide, a model of the CTTAG−TOTOBIPY complex was calculated by restrained molecular dynamics. A picture of the model is shown in Figure 3. The model was obtained by using 22 intermolecular restraints to position the TOTO chromophores and 11 restraints to position the bipyridyl ring system in the minor groove. These constraints did not embrace the equivalence of the protons at the bipyridyl group due to the dynamic rotation. Consequently, the model obtained must not be mistaken for a representation of the structure of the complex. The model correctly positions the chromophores at the intercalations sites and the linker in the minor groove, but it does not claim to include detailed structural and dynamic properties of the complex.

d(CGCTAGCG)$_2$ (CTAG) Oligonucleotide. TOTOBIPY forms more than one complex with the CTAG oligonucleotide. Consequently, the 1D NMR spectrum had broad lines due to exchange between the complexes. Severe overlap in the 2D NMR spectra prevented conclusive evidence that would allow identification of the binding sites of TOTOBIPY, but the methyl group resonances suggest that there are two major forms present in almost equal amounts. One is a symmetric complex with single methyl resonance upfield shifted 0.8 ppm. Such an upfield shift is normally observed in binding sites containing a thymidine (Jacobsen et al., 1995; Hansen et al., 1996) and indicates normal bis-intercalation in the (5′-C|*TA*|G-3′)$_2$ binding site. The other major form has one downfield shifted methyl resonance and one ordinary methyl resonance suggesting an asymmetric complex with nonnearest neighbor bis-intercalation in the (5′-C|*TAG*|C-3′)$_2$ site.

d(CGCTATAGCG)$_2$ (CTATAG) Oligonucleotide. The mixture of CTATAG + TOTOBIPY yields two or more complexes with one major asymmetric form. This complex was established as containing a bis-intercalated TOTOBIPY in the 5′-T|*ATA*|G-3′ binding site with the linker spanning three central bases. Once again the linker suits a three base pair step. Basically, there are two identical such binding sites in the palindromic sequence, the other one being the 5′-C|*TAT*|A-3′ site. The line broadening is due to exchange between these two sites.

DISCUSSION

The sequence selectivity and the binding mode of the TOTOBIPY analogue to the three dsDNA oligonucleotides examined in this work is very similar to the one of TOTO. In the CTAG oligonucleotide, both TOTO and TOTOBIPY bind to the 5′-CTAG-3′ site, but in the case of TOTOBIPY, there also exists another major component in which the ligand is bis-intercalated in isolated intercalation sites: (5′-C|*TAG*|C-3′)$_2$. This demonstrates that the partiality of the TOTO chromophore for the (5′-C*p*T-3′):(5′-A*p*G-3′) site competes with the preference of the linker to span three base pairs. An equivalent situation is observed in the CTATAG duplex where more than one complex is formed with TOTOBIPY but still with a three base step spanning major form.

The model of the TOTOBIPY−CTTAG complex summarizes the major structural features. The thiazole orange chromophores are situated in their favorite positions in the two (5′-C*p*T-3′):(5′-A*p*G-3′) sites with the benzothiazole ring systems sandwiched between the purines. All these features are in good accordance with our earlier thiazole orange studies, and thus, the introduction of a new linker has not perturbed the positioning of the chromophores. As evidenced by a number of linker−DNA NOE connectivities, the linker is positioned in the minor groove, but the occurrence of only four resonances from the bipyridyl moiety suggest a dynamical averaging due to a fast rotation on the NMR time scale. This rapid rotation may be considered as an indication of the lifetime of the complex or as an indication of the wide space in the minor groove that allows rotation of the linker while bound to the dsDNA. However, there are no exchange cross-peaks between resonances of the two chromophores indication that the exchange is slow on the NMR time scale. Furthermore, the structure of dsDNA is dynamic and flexible. Consequently, the rotation of the linker might be coupled to the dynamic motions of the duplex without requiring a particular wide minor groove.

Earlier, we have shown (Stærk et al., 1997) that approximately three methylene groups from the chromophore are required to position the middle part of the linker correctly in the minor groove. A model of the TOTOBIPY ligand shows that this middle part of the linker in this dye has a length of approximately 10.5 Å. This length is suited for spanning about three base steps

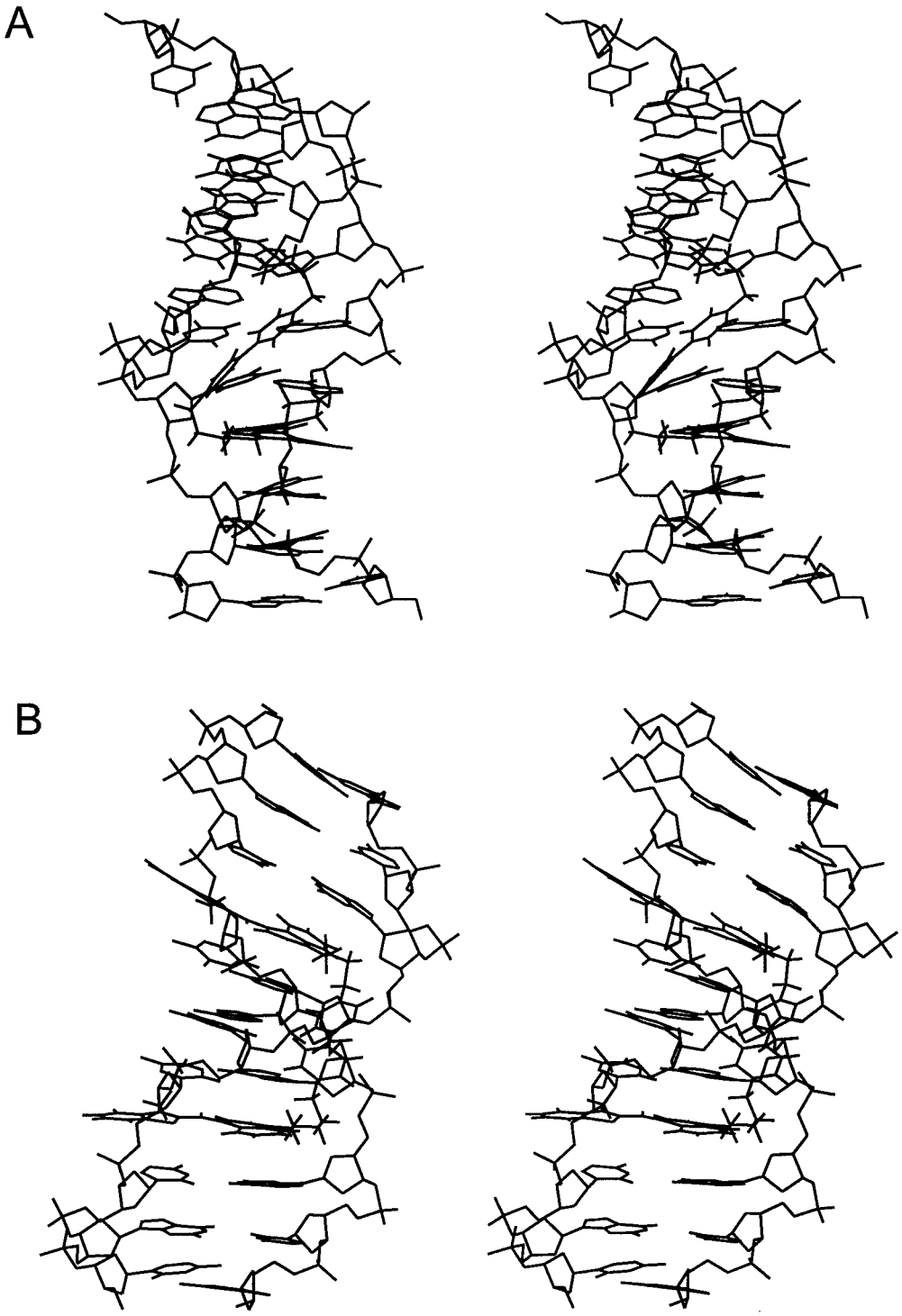


Figure 3. Stereoview of the model of the CTTAG-TOTOBIPY complex obtained by restrained molecular dynamics calculations. The model at the bottom is viewed into the minor groove. The model at the top is turned 90°.

but too short for spanning four. In the CTTAG duplex, TOTOBIPY was observed to give nonnearest neighbor bis-intercalation in oligonucleotides with isolated (5′-CpT-3′):(5′-ApG-3′) base pairs separated by three base pairs. This sequence contains the preferred intercalation sites separated by three base pairs and does thus fulfill both binding requirements of TOTOBIPY. Consequently, we have succeeded in designing and synthesizing a ligand that recognizes DNA in a concerted intercalation and minor groove binding mode that extends the recognition sequence.

In our earlier attempts to elongate the binding sequence, we have tried adding additional methylene groups to the flexible polyamine linker (Bondensgaard and Jacobsen, 1999; Stærk et al., 1997). Although the TOTO13 with the longest linker was capable of spanning three base steps there was a loss of selectivity, e.g., TOTO13 formed more than one complex with the 5′-CTTAG-3′ sequence (Stærk et al., 1997) with a major form in the 5′-C|*TTA*|G-3′ site. The rigid TOTOBIPY only forms one complex and thus the rigid linker seems to hold the chromophores in place. Shedding flexibility and enhancing affinity by introducing rigid elements seems to be a general trend. The importance of the linker has been extensively investigated (Assa-Munt et al., 1985a,b) for acridine bis-intercalators. It was found that while

uncharged polymethylene linkers lead to rapid exchange of the bis-intercalator between different intercalation sites in a $d(AT)_5$:$d(AT)_5$ duplex, the introduction of positively charged spermine linker stabilized the complex due to a retarding of the migration of the intercalator between different binding sites. A further loss of flexibility with a concomitant enhancement in binding affinity was gained by introducing a rigid pyrazole linker. Likewise, a significant improvement in DNA affinity was noted on exchanging a polymethylene linker with a diphenyl-ether (Cory et al., 1985). The affinity gain seems mainly to stem from van der Waals contacts with the walls of minor groove and loss of motional freedom—both of these necessitating steric complementarity between the linker and the target sequence. The bipyridyl linker is probably more sequence selective than the flexible polyamine linker, because the rigid linker will sterically clash with the guanine amino-group protruding into the minor groove while the polyamine linker can adapt more easily. The linear geometry of bipyridyl is probably not optimal for minor groove binding as can be surmised from the family of crescent-shaped minor groove binders (e.g., berenil, distamycin etc). However, the unwinding caused by the intercalating chromophores seems to make this isoconcavity demand less severe.

Thiazole orange (TOTO) and oxazole yellow (YOYO) homodimer polyamine dyes bind avidly to dsDNA by bis-intercalation with a high fluorescence enhancement upon binding (Rye et al., 1992; Glazer and Rye, 1992). The enhancement is attributed to the loss of rotational freedom between the thiazole and quinolinium moieties of the chromophores when inserted between the DNA nucleobases (Larsson et al., 1994). The unaltered chromophore positioning indicates that the new linker does not interfere with the fluorescence properties. Both TOTO and YOYO have been found to cause photocleavage of DNA, though the mechanism depends of the dye:DNA ratio (Åkerman and Tuite, 1996). At high dye:DNA ratios, externally bound dye cleaves in an oxygen-dependent manner, and this mechanism is more efficient than the oxygen-independent cleavage by bis-intercalated dye occurring at lower dye:DNA ratios. Earlier studies have shown that an electron transfer from intercalated ethidium to externally bound methyl-viologen can be photoinduced (Fromherz and Rieger, 1986) and that the one-electron reduced viologen reacts with oxygen leading to cleavage of DNA. A viologen linker containing acridine bis-intercalator has been shown to photocleavage dsDNA (Takenaka et al., 1992). The sequence-selective binding of TOTO-TOTOBIPY combined with its potential photocleaving properties makes it an interesting molecule for further studies.

ACKNOWLEDGMENT

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