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Microscopic Rearrangement of Bound Minor Groove Binders Detected by NMR

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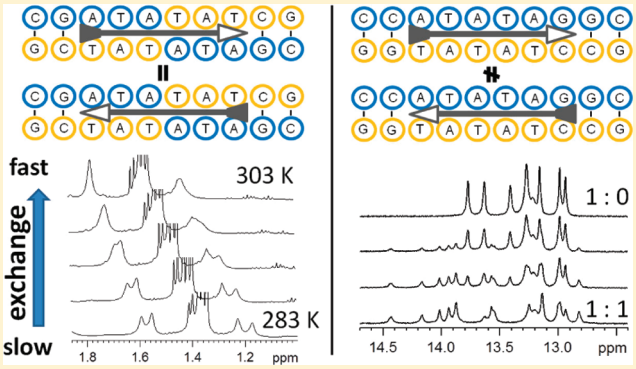
Supporting Information

ABSTRACT: Thermodynamic and structural studies are commonly utilized to optimize small molecules for specific DNA interactions, and, thus, a significant amount of binding data is available. However, the dynamic processes that are involved in minor groove complex formation and maintenance are not fully understood. To help define the processes involved, we have conducted 1D and 2D NMR in conjunction with biosensor-SPR experiments with a variety of compounds and symmetric, as well as asymmetric, AT tract DNA sequences. Surprisingly, the NMR data clearly show exchange between equivalent binding sites for strongly binding compounds like netropsin and DB921 (K_a > 10^8 M^{-1}) that does not involve dissociation off the DNA. A quantitative analysis of the data revealed that these bound exchange rates are indeed much faster than the macroscopic dissociation rates which were independently determined by biosensor-SPR. Additionally, we could show the existence of at least two 1:1 compound DNA complexes at the same site for the interaction of these compounds with an asymmetric DNA sequence. To explain this behavior we introduced a model in which the ligand is rapidly flipping between two orientations while in close association with the DNA. The ligand reorientation will contribute favorably to the binding entropy. As the potential of minor groove binders to form more than a single complex with asymmetric, as well as symmetric, duplexes is widely unknown, the consequences for binding thermodynamics and compound design are discussed.



INTRODUCTION

The specific external control of cellular gene expression by designed agents is a key goal of chemical biology and offers many advantages in understanding gene function.[1−7] An attractive method to establish gene control is by directly and specifically targeting genes with synthetic small molecules.[3−11] Sequences for most of the 20−25000 human genes, as well as the sequences of the genomes of a large number of disease causing microorganisms, have been determined, and all of them are potentially susceptible to external control.[12,13] Designed compounds that have biological activities by targeting duplex,[6−9] triplex and triplet-repeat,[10,14,15] or quadruplex DNA structures[5,11,16] have been identified and are under development. Duplex DNA minor groove binding compounds have been found to be particularly effective in therapeutic areas that include antiparasitic, antimicrobial, anticancer, and other applications and are a very promising area for development.[6−9,17−19] In order to optimize binding of small molecules for specific DNA interactions thermodynamic, kinetic, and structural studies of complex formation with a wide range of compounds were carried out. In addition, the dynamics of water molecules at the DNA backbone were thoroughly investigated.[20,21] It has long been recognized that the binding kinetics is important and may be dominant in determining biological activity.[22] However, even though a significant amount of binding data are available, the dynamic processes that are involved in minor groove complex formation and maintenance are not fully understood. As part of a recent study that identified more than one compound binding mode at a single DNA minor groove binding site, NMR results were obtained that suggested much faster exchange kinetics between bound compounds than would generally be expected for strong binding groove interactions.[23]

In order to address the questions of how common the multiple bound species are at a single site and to better quantify possible exchange among the bound agents, we have carried out 1D, 2D NMR, and biosensor-surface plasmon resonance (SPR) experiments on minor groove binding compounds which cover a range of chemical and conformational space and which have a range of macroscopic binding affinities. The binding exchange behavior of minor groove binding dications, such as those in Figure 1, was investigated with several alternating AT tract

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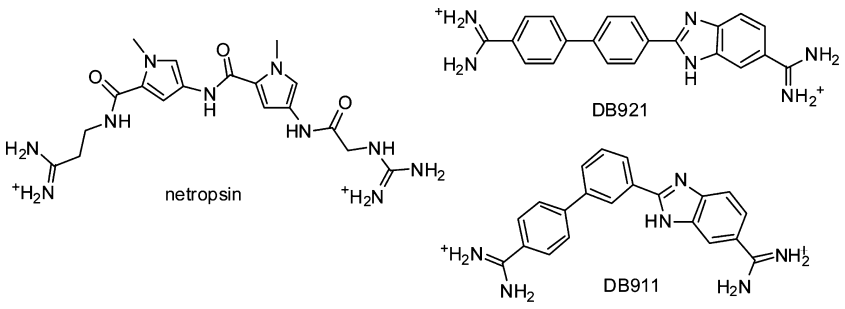

Figure 1. Compounds used in this work.

DNA sequences. NMR provides information on the microscopic exchange of bound molecules, while SPR provides the macroscopic dissociation rate constants. Several symmetric and nonsymmetric DNA sequences with different base pairs flanking the AT binding site were used in the experiments. The alternating AT sequence is of particular interest since such sequences are frequently bent by minor groove binding compounds.[24,25] A-tract sequences are intrinsically bent, and complex formation with minor groove binders produces only little change in bending. Each of the compounds in Figure 1, for example, causes very extensive bending when bound to alternating AT sequences but only small changes with A-tracts. The results show that the macroscopic dissociation for all compounds is much slower than the rate of exchange of the compounds between binding modes at the alternating AT site. These results clearly illustrate that the compounds move between binding sites while still bound to DNA. Because these events are not captured by methods that are only sensitive to a net affinity, these findings are not only relevant for our understanding of minor groove binding but they have potentially important ramifications in compound design as well.

MATERIALS AND METHODS

Sample Preparation. Netropsin was purchased from Sigma Aldrich, St. Louis, and was found to be pure by NMR and mass spectrometry analysis. The compounds DB921 and DB911[26] were synthesized according to published protocols. The DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT, Coraville, IA) with HPLC purification. The DNA sample was dialyzed three times against DI water using a Spectra/Por (Spectrum Laboratories, Inc., Rancho Dominguez) dialysis membrane with a cutoff of 1,000 Da, then lyophilized, and finally redissolved in BPES phosphate buffer with varying NaCl content (20 mM phosphate, 0.1 mM EDTA, pH 7.0). For NMR experiments the final duplex concentration was about 0.4 mM. For experiments involving exchangeable protons, a 90% H_2O−10% D_2O (Sigma Aldrich, St. Louis) sample was used. The DNA-compound complex was formed by titrating ligand into the DNA solution until saturation of the oligonucleotide as judged by the imino proton spectral region. For experiments with nonexchangeable protons the DNA sample was lyophilized twice and redissolved in 99.994% D_2O (Isotec, Miamisburg).

NMR Experiments. The NMR data were collected on a Bruker Avance 500 or 600 MHz NMR spectrometer equipped with a TXI cryoprobe or QXI probe, respectively. The water resonance was used as a reference for experiments at constant temperature (4.94 and 5 ppm at 283 and 278 K, respectively). Data were not corrected for chemical shift changes in temperature dependent studies. The spectra used for the determination of exchange rates were acquired with 16 K data points and processed using a Gaussian window function (LB = −0.3, GB = 0.3). Water suppression for 1D titrations was achieved by applying a W5 pulse sequence.

Phase sensitive 2D NOE experiments in D_2O were typically collected with 2048 × 600 data points in the two dimensions and 16 scans per t_1 increment. A series of mixing time from 50 to 250 ms was used, and the spectral width was set to 5 kHz. Suppression of the residual water signal was achieved by applying a weak presaturation pulse during mixing time and the 3 s recycle delay. Linear prediction with 25 coefficients in t_1 and zero filling gave a symmetrical 4 K × 4 K matrix. Both dimensions were apodized with shifted squared sine bell functions (SSB = 3).

The phase sensitive 2D ROESY experiments in D_2O were collected with 2048 × 600 and 2048 × 800 data points in the two dimensions, 32 and 64 scans per t_1 increment and a spectral width of 5 and 4.3 kHz at 600 and 500 MHz, respectively. A 150 ms cw spin lock pulse was used with a spin lock power of 27 and 14.94 dB at a B1 field strength of 5.5 and 11.2 kHz at 600 and 500 MHz, respectively. Suppression of the residual water signal was achieved by applying a weak presaturation pulse during the 2 s recycle delay. Linear prediction with 20 coefficients in t_1 and zero filling gave a symmetrical 4 K × 4 K matrix. Both dimensions were apodized with shifted squared sine bell functions (SSB = 3).

In addition to the 1D line-shape analysis,[27] as described in the Results, 2D exchange spectroscopy (EXSY) was used to estimate the chemical exchange rate. A two site exchange model with equal population of the sites and equal spin−lattice relaxation times implemented gives the relationships[28]

$$k = \frac{1}{t_m}\ln\frac{r+1}{r-1} \quad (1)$$

$$r = \frac{I_{AA} + I_{BB}}{I_{AB} + I_{BA}} \quad (2)$$

where k is the exchange rate constant, t_m is the mixing time, and I_{AA}, I_{BB} are diagonal and I_{AB}, I_{BA} are cross peak intensities.

Biosensor-Surface Plasmon Resonance (SPR). Biosensor-SPR experiments were conducted with a Biacore 2000 (Biacore, Uppsala, Sweden) instrument as previously described.[29,30] 5′-Biotin-labeled ATATA DNA hairpin (5′-GCG ATA TAC GTC TCC GTA TAT CGC-3′) was immobilized on four-channel Biacore SA sensor chips via noncovalent streptavidin capture. The compounds at different diluted concentrations in degassed and filtered cacodylic acid buffer with 0.01% surfactant P-20 were injected over the DNA surface for selected times. A glycine solution (10 mM, pH 2.0) was used for surface regeneration. Kinetic analysis was performed

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Table 1. Overview of DNA Sequences and Numbering Scheme of Residues for the CG/CG Sequence

abbreviation	sequence	self-complementary
CG/CG	5′-CGA TAT ATC G-3′	yes
CC/GG	5′-CCA TAT ATG G-3′	yes
GG/CC	5′-GGA TAT ATC C-3′	yes
CCA	5′-CCA TAT AGG C-3′	no
	3′-GGT ATA TCC G-5′	

5′-	C1	G2	A3	T4	A5	T6	A7	T8	C9	G10	-3′	strand A
3′-	G20	C19	T18	A17	T16	A15	T14	A13	G12	C11	-5′	strand B

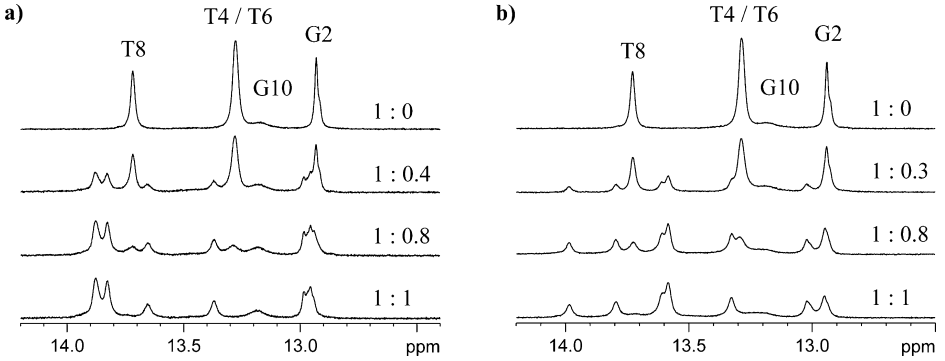

Figure 2. Changes in the imino proton spectral region of CG/CG during titration with netropsin a) and DB921 b) at 283 K in BPES buffer containing 80 mM NaCl. Duplex to compound ratios are indicated on the right.

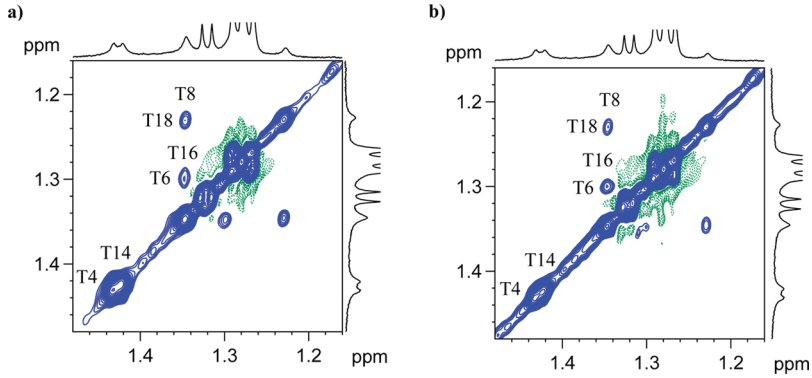

Figure 3. Methyl proton region of the a) 250 ms NOESY and b) 150 ms ROESY spectrum of the netropsin-CG/CG complex at 283 K at 600 MHz. Crosspeaks between methyl groups are labeled. Due to the similar chemical shifts of the T4 and T14 methyl resonances the crosspeaks are partially obscured by the diagonal peaks.

by global fitting of the binding data with mass-transport-limited kinetic 1:1 binding models (eqs 5 and 6)[29,31,32]

$$A + B \rightleftarrows AB, \quad K_a = [AB]/[A][B] = k_a/k_d \tag{3}$$

$$[A]_{t=0} = 0, \quad [B]_{t=0} = RU_{max}, \quad [AB]_{t=0} = 0 \tag{4}$$

$$d[AB]/dt = k_a[A][B] - k_d[AB] \tag{5}$$

$$d[A]/dt = k_t([A_{bulk}] - [A]) - (k_a[A][B] - k_d[AB]) \tag{6}$$

where [A] and $[A_{bulk}]$ are the concentrations of the compound at the sensor surface and in the bulk solution, respectively, [B] is the concentration of the immobilized DNA without bound compound, and [AB] is the concentration of the complex; k_a is the association, and k_d is the dissociation rate constant.

RESULTS

Strong Binding Compounds and Symmetric DNA. *1D NMR Titrations.* The binding of several diamidines as well as netropsin to alternating AT tract DNA was initially investigated by 1D NMR titrations. The different compounds as well as DNA sequences and a DNA base numbering scheme are given in Figure 1 and Table 1. Proton NMR assignments for the CG/CG oligonucleotide were done by using standard protocols for B-DNA.[33] Figure 2 shows imino proton spectra of this self-complementary 10mer in the presence of netropsin and DB921. Due to symmetry only five imino protons are observed for the free DNA. With the addition of an asymmetric ligand the complex is also asymmetric, and a more complex spectrum is obtained. At ligand to DNA ratios below 1:1 both free and complexed DNA coexist in solution and give rise to sharp peaks. As expected for strong binding ($K_a > 10^8$ M^{-1}, see the biosensor-SPR section for details) of small ligands like netropsin and DB921, the intensities of the free DNA proton peaks decrease, and no significant signal broadening or chemical shift changes are observed for the DNA imino protons with increasing compound concentrations. At the final titration step the 1:1 compound-DNA complex is observed

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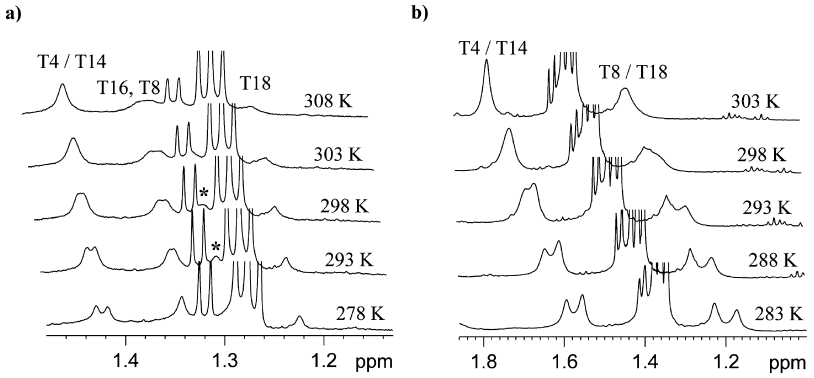

Figure 4. Temperature dependence of the methyl resonances of the a) 1:1 CG/CG-netropsin complex at 600 MHz and b) the 1:1 CG/CG-DB921 complex (BPES buffer containing 20 mM NaCl) at 500 MHz. At 278 K T6 is not visible in a) due to signal overlap but can be identified at 293 and 298 K as indicated by the asterisk.

exclusively. We do not observe any exchange processes between complexed and free DNA as expected for a very slow exchange rate between these states, and this is consistent with the SPR data. In addition to these 1D spectra, 2D NOESY experiments were recorded for assigning the resonances of the ligand-DNA complexes. Surprisingly, strong crosspeaks between symmetry related protons like e.g. T4 H6/T14 H6 or A7 H8/A17 H8 are observed for both DB921- and netropsin-DNA complexes. Similarly for the methyl groups, equivalent crosspeaks between the T6/T16 and T8/T18 (netropsin-DNA complex, Figure 3a) or T4/T14 and T8/T18 (DB921-DNA complex, data not shown) methyl protons are seen. The distance between these protons >9−16 Å (standard B-DNA) is far too large to give rise to possible NOE crosspeaks as NOEs can usually be detected between protons that are less than 6 Å apart from each other.[34] In addition, the evaluation of a related netropsin-ATAT DNA structure[35] gives carbon−carbon distances of equivalent methyl groups >7.5−14 Å. Therefore, these crosspeaks cannot be due to nuclear overhauser effects but must be due to chemical exchange. This was further confirmed by 2D ROESY spectra, which allow crosspeaks caused by direct NOEs to be distinguished from chemical exchange crosspeaks simply by their signs. Crosspeaks having the same sign as diagonal peaks are due to chemical exchange processes; those having an opposite sign are caused by direct NOE effects.[36] The peaks between the equivalent methyl groups (Figure 3b and Figure S1 in the Supporting Information for the DB921-CG/CG complex) can thus be clearly attributed to chemical exchange processes rather than NOEs. The behavior observed for the interaction of netropsin and DB921 with CG/CG is also observed with the closely related CC/GG (Figure S2, Supporting Information) and GG/CC (Figure S3, Supporting Information) sequences, and it is clear that flanking sequences have only a modest effect on exchange in the AT complexes. In summary, while macroscopic exchange between bound and free DNA cannot be seen and therefore has to be slow, significantly faster microscopic exchange processes of bound ligands that do not involve escape from the DNA are readily observable by NMR.

Exchange Rates of Bound Compounds. In order to characterize the microscopic exchange further and determine the exchange rates the behavior was studied at different temperatures. These studies revealed the fast exchange processes to be a characteristic of netropsin- and DB921-CG/CG complexes. For the T4/T14 methyl groups of the netropsin-CG/CG complex (Figure 4a) two individual peaks with equal integral can be observed at 278 K. Upon gradually increasing the temperature the originally separated peaks broaden and merge into one peak as coalescence is reached at around 300 K. As temperatures are further increased a sharpening of the merged peaks can be observed. Chemical shift changes and signal broadening are also observed for the T6/T16 and T8/T18 pairs of methyl protons, but due to signal overlap the coalescence temperatures could not be determined for these resonances.

An estimate of the rate of exchange of netropsin between two binding sites was obtained by using the standard relationship for two-site exchange with equal population of the sites[27]

$$k_{coal} = (\pi/2^{1/2})\Delta\nu \quad (7)$$

where $\Delta\nu$ = 7 Hz is the chemical shift difference between the exchanging spins T4/T14. Thus, an approximate exchange rate constant of 16 s^{-1} and an apparent lifetime $1/k_{coal}$ = 63 ms at the coalescence temperature of 300 K could be determined. An independent estimation of the exchange rate constant of 2D EXSY spectra with mixing times of 250, 150, and 80 ms could additionally be obtained for the T8/T18 pair of protons. The derived rates (1.8−2.15 s^{-1}) at 283 K support the data obtained by the 1D line-shape method at higher temperature. The temperature dependent studies of the DB921-CG/CG complex (Figure 4b) give results similar to those for the netropsin-DNA interaction. For both the T4/T14 as well as the T8/T18 methyl groups two individual peaks can be observed at 283 K, and coalescence is reached at around 298 K for T8/T18 and roughly at 295 K for T4/T14. The chemical shift differences between the exchanging spins T4/T14 and T8/T18 of $\Delta\nu$ = 20 and 28 Hz, respectively, translate to approximate exchange rate constants at the coalescence temperatures of 44 s^{-1} ($1/k_{coal}$ = 23 ms) at 295 K and 62 s^{-1} ($1/k_{coal}$ = 16 ms) at 298 K. The coalescence points were reached at slightly different temperatures for these different exchanging protons within the same complex. However, for the same process the chemical shift difference between the exchanging protons is determined by the precise chemical environment in the different forms. In general, this will be different for different pairs of exchanging groups and may thus lead to different coalescence temperatures.

Strong Binding Compounds and Nonsymmetric DNA. *1D NMR Titrations.* In analogy to the exchange observed for binding of an asymmetric ligand to a symmetric oligonucleotide exchange is also expected for closely related asymmetric

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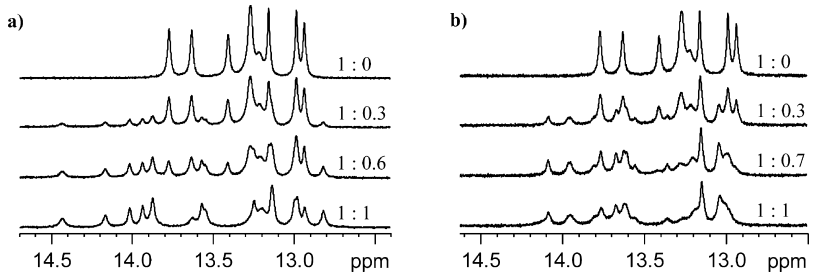

Figure 5. Changes in the imino proton spectral region of CCA during titration with a) netropsin and b) DB921 at 278 K in BPES buffer containing 20 mM NaCl. Duplex to compound ratios are indicated on the right.

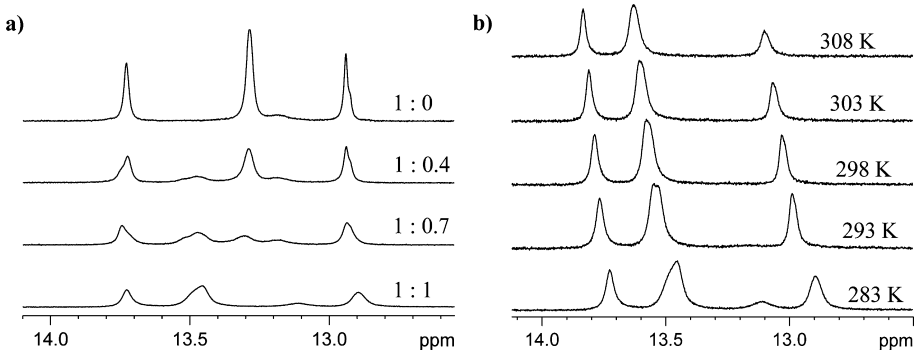

Figure 6. a) Titration of the self-complementary 10mer duplex CG/CG with DB911 at 283 K (BPES buffer containing 80 mM NaCl). Duplex to compound ratios are indicated on the right. b) Influence of elevated temperatures on the 1:1 DNA-compound complex.

sequences like CCA. This sequence contains the same AT core sequence as the CG/CG duplex but features different flanking sequences resulting in an asymmetric duplex. Consequently binding of an asymmetric ligand inevitably leads to the formation of 2 different complexes (see Figure 8 and the Discussion for details). This is readily apparent from the NMR spectra recorded for the CCA duplex. The interactions of netropsin and DB921 with this sequence are shown in Figure 5. Eight peaks can be easily distinguished for the free CCA oligonucleotide in the top parts of these figures, and, by taking into account solvent exchange and the apparent signal overlapping between 13.1 and 13.3 ppm, the expected number of 10 imino protons is obtained. The tight binding for both compounds to this sequence, as described for the related CG/CG oligonucleotide, is again revealed by the disappearance of free DNA peaks at the final titration step (e.g., peak at 13.4 ppm). Also, a drastic increase in the number of imino protons is seen, which clearly shows the coexistence of two nonequivalent 1:1 compound-DNA complexes.

Due to signal overlap and signal broadening an assignment of the resonances to the individual 1:1 complexes is hampered and beyond the scope of this article. However we observe very strong crosspeaks between iminoproton resonances that are present at even very low mixing times (50 ms) in the NOESY spectra. Such unusually strong crosspeaks are not expected for NOE's between iminoprotons since their distance is generally >3.4 Å[33] but are consistent with exchange processes (Figure S4, Supporting Information).

More Weakly Binding Compounds. DB911 is an isomer of DB921 that has the more typical shape of curved minor groove binders (Figure 1), but which binds about a factor of 10 more weakly than DB921 and has a faster off rate (refs 25 and 37 and see below). Its interaction with the symmetric CG/CG sequence has been investigated with the same methods as used with DB921 for comparison (Figure 6a). Despite its weaker DNA binding ($K_a \sim 10^7$ M^{-1}) the coexistence of free and complexed DNA at ratios between 1:0 and 1:1 shows that exchange is slow on the NMR time scale as with the strong binding compounds. However, the peaks of the complex are significantly broadened. In analogy to DB921 a loss of symmetry would also be expected for the inherently asymmetric DB911-DNA complex. This is not seen; however, this behavior is in agreement with the observations made for the strong binding compounds above (Figure 4) if the coalescence temperature for the DB911-CG/CG complex is below 283 K. Low temperature studies in aqueous solutions are limited by sample freezing; however, increasing the temperature should result in an overall sharpening of the imino proton peaks. Indeed, apart from the broad peak at 13.1 ppm which quickly disappears, this is what can be observed in Figure 6b. Only above 298 K does the exchange with solvent start to outweigh the sharpening of the imino protons and a decrease in intensity is observed, especially for the most upfield resonance.

In agreement with a comparatively higher microscopic exchange rate without compound release is also the interaction of DB911 with the asymmetric CCA sequence as shown in Figure 7 at 278 K. In accordance to the results for netropsin and DB921 with this sequence free and complexed DNA coexist in solution at intermediate titration ratios (e.g., free DNA peaks at 13.3 and 13.6 ppm and bound DNA peak at 13.5 ppm) showing that exchange between both species is slow. However, in contrast to DB921 and netropsin there is no increase in the number of imino proton peaks at the final titration step and lines are significantly broadened. Temperatures that are impractical for aqueous solutions would be required for more extensive studies, but it is clear that additional exchange processes, that do not involve compound release, must be largely responsible for the line broadening.

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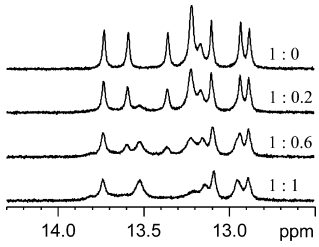

Figure 7. Changes in the imino proton spectral region of CCA during titration with DB911 at 278 K (BPES buffer containing 20 mM NaCl) at 600 MHz. Duplex to compound ratios are indicated on the right.

Biosensor-SPR: Macroscopic Dissociation Kinetics. To determine macroscopic dissociation kinetics constants for comparison to the exchange rate constants observed in NMR spectra, biosensor-SPR experiments were conducted with netropsin, DB921, and DB911 with an alternating DNA binding site (5′ GCG ATA TAC GTC TCC GTA TAT CGC-3′). Example sensorgrams for the three compounds are shown in the Supporting Information (Figure S5), and it is visually clear that both the association and dissociation rates for netropsin and DB921 are much slower than for DB911. Association and dissociation rate constants were determined for the three compounds by fitting the sensorgrams with a one-site binding model. As can be seen, the fits are excellent, and the association and dissociation rate and equilibrium constants are given in Table 2. Netropsin and DB921 have affinity constants $\geq 10^8$ M^{-1}, while the value for DB911 is approximately ten times lower ($K_a \sim 10^7$ M^{-1}).

DISCUSSION

Netropsin as well as the diamidines DB921 and DB911 are relatively strong minor groove binders with association constants in the range of ($K_a = 10^7$–10^9 M^{-1}) to AT binding sites (Table 2), and our work provides evidence for exchange processes between different bound states of these 1:1 complexes that are much faster than ligand dissociation. Similar observations were described earlier for the interaction of a very small number of compounds, generally with symmetric A-tract type-sequences and also involved different bound states of the complexes.[38−41] Clearly, the exchange behavior is an important feature of minor groove interactions that has not been widely recognized and to our knowledge no studies have been done with the alternating AT sequence or that combine NMR with macroscopic kinetics results such as those from SPR experiments.

A schematic representation for the binding of an asymmetric compound to a self-complementary sequence is given on the left-hand site of Figure 8. The symmetric DNA offers two identical binding sites for the ligand and an asymmetric ligand may bind with its head oriented toward the "upper" or toward the "lower" end of the DNA. Upon ligand binding, as can be seen, the original symmetry of the self-complementary oligonucleotide is lost which results in a doubling of the DNA resonances. We suggest that the observed exchange processes between the bound states of the oligonucleotides as observed in the NOESY and ROESY experiments are due to a reorientation of the compound between the equivalent binding sites. Irrespective of the mechanism involved, an increase in the rate of the ligand reorientation on the DNA from slow to fast on the NMR time scale, e.g. by elevating the sample temperature, leads to deceptively simple spectra. A consequence of this is the temperature dependent effect of the CG/CG- netropsin and CG/CG- DB921 complexes. These are inherently nonsymmetric because of the bound ligand, but above their coalescence temperatures they exhibit fewer resonances than expected due to the fast ligand reorientation on the DNA (Figure 4).

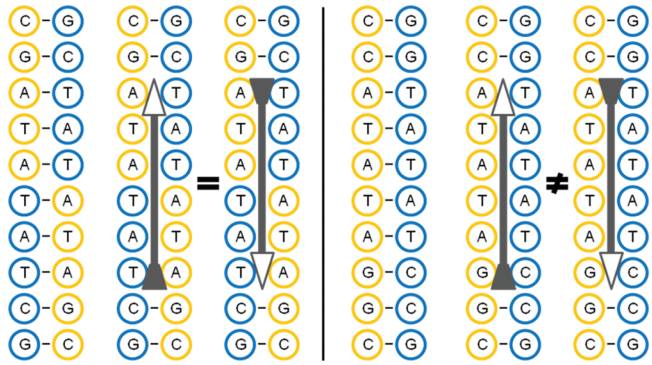

Figure 8. Schematic representation of binding of an asymmetric compound to a self-complementary (left) and a nonself-complementary (right) DNA sequence.

A schematic representation for the interaction of asymmetric ligands with nonself-complementary duplexes is given on the right-hand site of Figure 8. Depending on the conditions 10 imino protons can be expected for a free DNA decamer. In cases where the flanking sequences enforce a single ligand orientation on the DNA the number of imino proton resonances would remain the same in the complex. If on the other hand the flanking sequences do not restrict the ligand orientation, then two distinct complexes are obtained which result in a correspondingly more complex imino proton spectrum as can be seen for the interaction of netropsin and DB921 with the CCA sequence (Figure 5). Moreover, we can also observe imino peaks that are exchange related (Figure S4, Supporting Information). The potential of some minor groove binders to form more than a single 1:1 complex to nonself-complementary sequences is further confirmed in a recent study, showing that netropsin forms two individual 1:1 binding modes to an asymmetric DNA hairpin.[23]

It is interesting to note that in contrast to the closely related DB921, the interaction of the asymmetric DB911 with the CG/

Table 2. Kinetic and Thermodynamic Values Determined by SPR and NMR

compounds	K_a (1/M)	K_d (M)	k_a (1/Ms)	k_d (1/s)	$1/k_d$ (s)	k_{coal} (1/s)[a]	$1/k_{coal}$ (ms)[a]
netropsin	4.1×10^8	2.4×10^{-9}	2.6×10^7	0.06	17	16[b]	63[b]
DB921	1.0×10^8	1.0×10^{-8}	1.0×10^6	0.01	100	44[c]/62[d]	23[c]/16[d]
DB911	1.2×10^7	8.3×10^{-8}	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]

[a]As determined by NMR. See text for details. [b]At 300 K. [c]At 295 K. [d]At 298 K. [e]The kinetics of DB911 with DNA are too fast for accurate determination by SPR and NMR.

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CG sequence (Figure 6a) does not lead to an increase in the number of imino proton peaks. However, significant line broadening is present, particularly for the interaction with the asymmetric CCA sequence (Figure 7). Also, upon increasing the temperature the peaks sharpen (Figure 6b) suggesting a faster exchange. We note that in consistence with the macroscopic dissociation constant indicating slow transfer to solution (Table 2) no exchange between ligand-bound and ligand-free DNA can be observed. These observations are in agreement with fast microscopic rearrangements for bound ligands as described above. As observed at elevated temperatures for the netropsin- and DB921-CG/CG complexes, the equivalent DB911-CG/CG complexes are in fast exchange between binding modes under the given, low temperature conditions and thus seem to retain the original symmetry of the DNA. Accordingly, a high exchange rate between different 1:1 DNA compound complexes might be responsible for the significant signal broadening observed for the binding to the nonself-complementary CCA sequence.

The experimental data presented here for netropsin and DB921 with an alternating (AT) sequence as well as those for Hoechst 33258,[40] SN 6999,[41] and netropsin[38] with A-tract-type sequences in earlier studies clearly show chemical exchange between equivalent binding sites of self-complementary DNA sequences. Two models are readily envisioned to account for the ligand reorientation:

In the first model the ligand must be completely released from the DNA prior to binding in the alternate orientation on the DNA. Thus, the temporary presence of both free DNA and free compound is required during the reorientation. The kinetics in this model are captured by the dissociation rates off the DNA that are measured via SPR.

In the second model the ligand does not dissociate off the DNA and maintains associated with the DNA prior to rebinding in the alternate orientation on the DNA. The positive charge of the compound aids in keeping it in contact with the anionic potential of the DNA.

The NMR data provide evidence for exchange between two forms of complexed DNA exclusively, and we do not observe any exchange involving free DNA. Also, the NMR bound exchange rates are fast and translate to apparent lifetimes in the range of tens of ms around room temperature. In comparison to that the SPR kinetics results (Table 2) show off rates at 298 K that are in the range of seconds. Thus, the lifetime of the DNA-compound complex is much longer than that of a single orientation of the drug toward the DNA. Taken together, all experimental data largely support a model with microscopic flipping motions without compound release.

Finally, it is interesting that the microscopic bound exchange rate constant is larger for DB921 than for netropsin. The macroscopic values are reversed, however, with DB921 having a longer bound state lifetime. This might be explained by the different binding mechanisms of the compounds. Whereas netropsin has the classical crescent shape for a tight fit in the minor groove, DB921 is rather straight and needs an additional water molecule to mimic the shape of classical minor groove binders.[26,37,42] Clearly, the flexibility of the latter binding mechanism with its induced fit would allow for faster microscopic rearrangements. In principal, it might also be easier for this system to stabilize intermediate binding states by bridging water molecules explaining the longer macroscopic bound state lifetime.

The exchange between binding sites has interesting consequences especially for binding thermodynamics. At temperatures around or below 300 K the flipping rates of netropsin and DB921 were estimated to ∼16 Hz and ∼50 Hz, respectively, and with an individual ITC injection step having a typical duration in the range of seconds, it is clear that the flipping motion is inherently contributing to the thermodynamic values determined by this and other methods with similar measurement time scales. The population of two instead of just one microscopic binding states ("up" and "down" orientations versus just one of those, cp. Figure 8) contributes favorably to the binding entropy.

The potential of the investigated minor groove binders to bind to the same asymmetric oligonucleotide forming different 1:1 complexes is another factor that deserves attention. As these complexes all share the same molecular weight and assumingly similar spectral properties, they might stay undetected or lead to surprising observations. A recent example is the observation of a "dip" in ITC binding isotherms upon binding of netropsin to a DNA hairpin. Several suggestions including a hairpin to duplex transition were made to explain this unusual behavior, and it took a combined approach of mass spectrometry, PAGE gels, and NMR to finally reveal that two distinct 1:1 compound-DNA complexes caused the unusual shape of the binding isotherm.[23]

CONCLUSIONS

In summary, the experimental data presented here as well as those from earlier studies clearly show that classical minor groove binders can exchange between binding sites of DNA sequences. Our results support a model in which the ligand stays associated with the DNA during the reorientation and the reorientation contributes favorably to the binding entropy. Additionally, our results directly show the existence of at least two 1:1 compound DNA complexes for the interaction of netropsin and DB921 with the asymmetric CCA sequence revealing the potential of minor groove binders to form more than a single complex with asymmetric duplexes. The key conclusion from these studies is that although the macroscopic dissociation lifetime for strong binding minor groove-DNA complexes is on the order of many seconds, the bound state lifetime for exchange between binding sites is much shorter. It should be emphasized that these bound state dynamic transitions are in addition to any local thermal fluctuations that occur within a specific binding site. The complexes in this work are for alternating sequence DNA, but the literature suggests that different binding site sequences show similar dynamic transitions.[23,38−41] In addition, DB921 and netropsin have very different complexes with the DNA minor groove,[26,35,37,39,42−44] and it is also clear that the exchange behavior occurs for many minor groove complex types. The DB911 results clearly illustrate that even a small decrease in affinity can lead to a drop in the coalescence temperature that obscures the exchange processes. This observation suggests that microscopic exchange is a quite common phenomenon that is frequently missed due to a fast exchange rate.

ASSOCIATED CONTENT

Supporting Information

A ROESY spectrum of the DB921-CG/CG complex, 1D titration spectra of netropsin with the CC/GG and GG/CC sequences, a 50 ms NOESY spectrum of the CCA-netropsin complex, a 150 ms NOESY spectrum of the free CCA

oligonucleotide, and figures of the SPR kinetic analysis of netropsin, DB921, and DB911 binding to the ATATA sequence. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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