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Simultaneous Binding of a Polyamide Dimer and an Oligonucleotide in the Minor and Major Grooves of DNA

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Abstract—The effect of the polyamide ImPyPy-Dp (Im = *N*-methylimidazole-2-carboxamide, Py = *N*-methylpyrrole-2-carboxamide, and Dp = dimethylaminopropylamide), which binds as an antiparallel dimer in the Watson–Crick minor groove, on pyrimidine•purine•pyrimidine triple helix stability was investigated. A DNA restriction fragment was designed which contained two triple helix sites, one which overlapped a minor groove ligand site (proximal), and a control site 13 base pairs away (distal). Using quantitative DNase I footprint titration experiments the equilibrium association constant of oligonucleotide 5′-TTTTTm^{5}CTTTm^{5}CTTTm^{5}CT-3′ (**1**) to each site was measured in the absence and presence of the polyamide dimer. Our data indicate that triple helix formation is compatible with a polyamide dimer binding in the minor groove of DNA at an overlapping site. No cooperative effect of the polyamide dimer on the equilibrium association constant of oligonucleotide **1** was observed. Copyright © 1996 Elsevier Science Ltd

Introduction

High-resolution X-ray crystal structures of protein-DNA complexes have revealed a diverse repertoire of structures for DNA recognition.[1–3] Proteins such as BZIP bind sequence-specifically in the major groove,[1] while others, such as TBP, bind exclusively in the minor groove.[2] Still others, such as Hin, make specific contacts *simultaneously* in the major and minor grooves of double helical DNA.[3] In the area of nonnatural ligand design for DNA recognition, it has been demonstrated that oligonucleotides bind sequence specifically in the major groove of DNA by triple helix formation.[4] Antiparallel polyamide dimers bind sequence-specifically in the minor groove.[5–7] The compatibility of oligonucleotide-directed triple helix formation with simultaneous binding of a polyamide dimer in the minor groove has not yet been explored.

Pyrimidine motif triple helix formation

Oligonucleotide-directed triple helix formation is a valuable technique for sequence-specific recognition of double helical DNA.[4] One recognition motif involves a short pyrimidine oligonucleotide binding parallel to a purine-rich strand in the major groove of Watson–Crick duplex DNA through the formation of a local triple helix.[4a,8,9] Sequence specificity is derived from Hoogsteen base pairing between the pyrimidine bases of the oligonucleotide and the purine strand of duplex DNA to form T•(AT) and C$^+$(GC) triplets.[8,9]

Minor groove binding polyamides

Recently, polyamides containing *N*-methylpyrrole and *N*-methylimidazole amino acids have been shown to bind as antiparallel, side-by-side dimers in the minor groove of DNA.[5–7] Within the 2:1 polyamide:DNA model, an imidazole on one ligand opposite a pyrrole carboxamide on the second ligand recognizes a G•C base pair, while a pyrrole carboxamide/imidazole combination targets a C•G base pair.[5,7] A pyrrole carboxamide/pyrrole carboxamide pair is partially degenerate for A•T and T•A base pairs.[5–7] The polyamide ImPyPy-Dp has been shown to bind to the mixed sequence 5′-(A,T)G(A,T)C(A,T)-3′ with a first-order association constant of $\sim 2 \times 10^5$ M^{-1} (Fig. 1).[5]

Simultaneous binding of ligands to DNA

Several studies have reported that binding of the natural product distamycin or netropsin in the minor groove of DNA is compatible with triple helix formation, although the small molecule thermally destabilizes the triple helix.[10] However, these studies were generally performed with short A,T rich duplexes. The minor groove width of DNA is approximately 3.4 Å for netropsin binding as a 1:1 complex to A,T rich sequences[11] compared with 6.8 Å for models of 2:1 polyamide:DNA complexes.[5,6] Therefore, 2:1 binding polyamides may have a very different effect on triple helix stability than the 1:1 binding natural products.

As a minimum first step toward the creation of nonnatural ligands which bind specifically and simultaneously in the major and minor grooves of DNA, we have carried out a study aimed at determining whether major groove binding oligonucleotides and minor groove binding polyamides are structurally compatible in overlapping sequence space on opposite sides of a double helix. We report here the energetics of triple

helix formation by a pyrimidine oligonucleotide bound in the major groove in the absence and presence of a polyamide dimer $(ImPyPy\text{-}Dp)_2$ bound in the minor groove of an overlapping binding site.

Results and Discussion

A plasmid (pMEP-2-ImN) has been constructed which contains two identical oligonucleotide binding sites, one overlapping a minor groove binding site (proximal **B**) and a control site thirteen bases away (distal **C**, Fig. 2). The target binding site for ImPyPy-Dp **A** overlaps site **B** by one base pair. Quantitative DNase I footprint titrations[12] were performed on the 255 base pair *Eco*RI/*Pvu*II restriction fragment from pMEP-2-ImN. The results of a typical experiment with oligonucleotide **1** in the absence of ImPyPy-Dp are shown in Figure 3(A). The DNase I cleavage patterns are the same for both triple helix binding sites. In addition, the first-order association constants of oligonucleotide **1** for both site **B** and site **C** in the absence of ImPyPy-Dp are $K_a = \sim 2.7 \times 10^7\ M^{-1}$ (Table 1). In the presence of 50 μM ImPyPy-Dp, the binding constant for oligonucleotide **1** remains the same within experimental error for both sites **B** and **C** (Table 1). Results of footprinting oligonucleotide **1** in the presence of 100 μM ImPyPy-Dp are shown in Figure 3(B). ImPyPy-Dp protects site **A** from cleavage, indicating that the dimeric polyamide complex forms in the presence of **1**. Also, the equilibrium association constant of oligonucleotide **1** for both **B** and **C** sites is unchanged in the presence of ImPyPy-Dp, consistent with simultaneous binding of the triple helix and ImPyPy-Dp (Fig. 3). The overlaid binding isotherms for oligonucleotide **1** for sites **B** and **C** in the absence and presence of ImPyPy-Dp are shown in Figure 4.

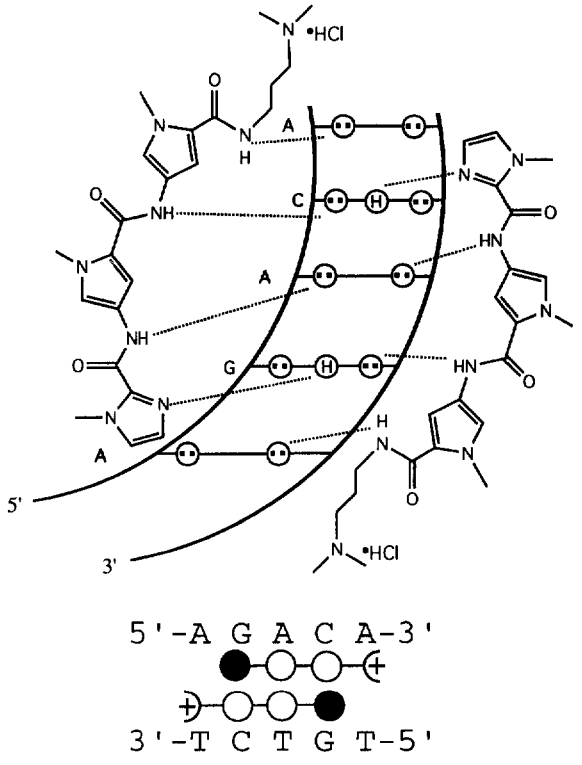

Figure 1. Binding model for the 2:1 complex of polyamide $(ImPyPy\text{-}Dp)_2$ with a 5′-AGACA-3′ sequence. Circles with dots represent lone pairs of N3 purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogen of guanine. Putative hydrogen bonds are illustrated by dashed lines. Ball and stick models are also shown. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively.

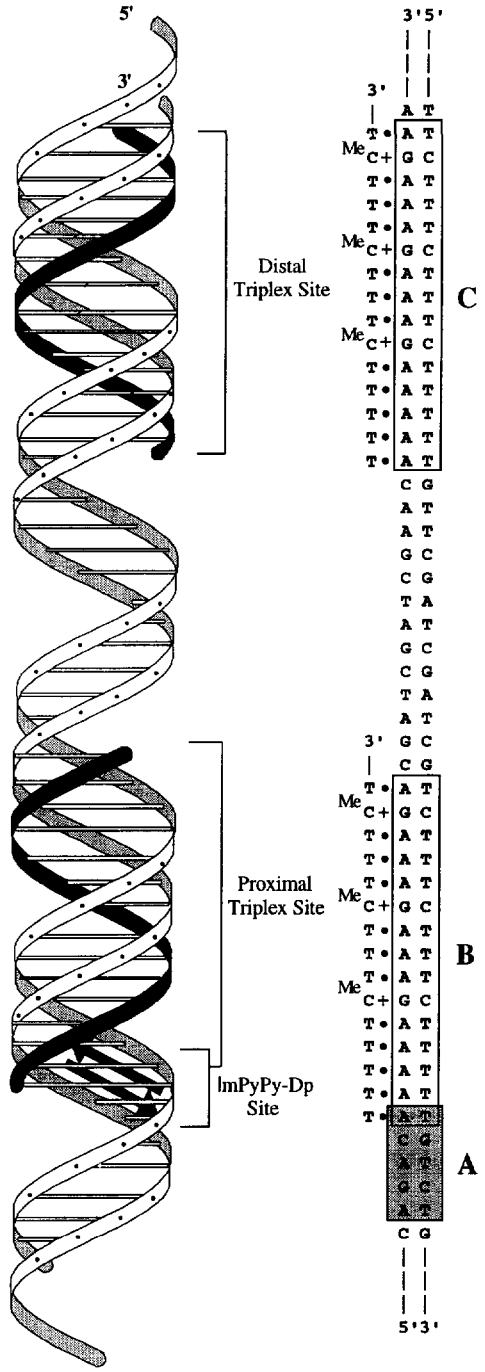

Figure 2. Ribbon model of fragment showing binding sites for ImPyPy-Dp **A**, oligonucleotide **1** (proximal **B**), and oligonucleotide **1** (distal **C**). ImPyPy-Dp is represented in the ribbon model by antiparallel black arrows.

The results described above show that binding of ImPyPy-Dp in the minor groove is compatible with triple helix formation in the major groove at an overlapping site, although no cooperativity was observed in this system. These findings are consistent with previous results, which showed that ImPyPy-Dp is compatible with the major groove binding protein GCN4, but no cooperative interactions are observed.[13] Simultaneous binding of ImPyPy-Dp with a triple helix is also consistent with current literature studies demonstrating the compatibility of the minor groove binders, distamycin A and netropsin, with triple helix formation.[10]

ImPyPy-Dp differs from the natural products distamycin and netropsin in that it binds in the minor groove as a dimer, even at low concentrations.[5] In addition, the minor groove width for 1:1 distamycin or netropsin binding to A,T rich sites is approximately 3.4 Å[11] compared with 6.8 Å for models of the 2:1 complexes.[5,6] Since the oligonucleotide **1** site **B** overlaps the ImPyPy-Dp binding site **A**, positive or negative cooperativity between **1** and ImPyPy-Dp might be expected. Based on the equilibrium association constants for oligonucleotide **1** in the absence and presence of ImPyPy-Dp, no cooperativity of binding is observed (Table 1). In a similar experiment, we also found the binding of distamycin A to be compatible with triple helix formation, but not cooperative (data not shown). The fact that distamycin A and ImPyPy-Dp give similar results is surprising since the minor groove widths for these two ligand binding sites are expected to be different. This suggests that the triple helix can accommodate changes in the width of the minor groove of overlapping duplex DNA.

Early fiber diffraction studies on triple helical poly(dT)•poly(dA)•poly(dT) complexes indicated that all three strands in this complex are in the A DNA conformation and therefore have C3′-*endo* sugar

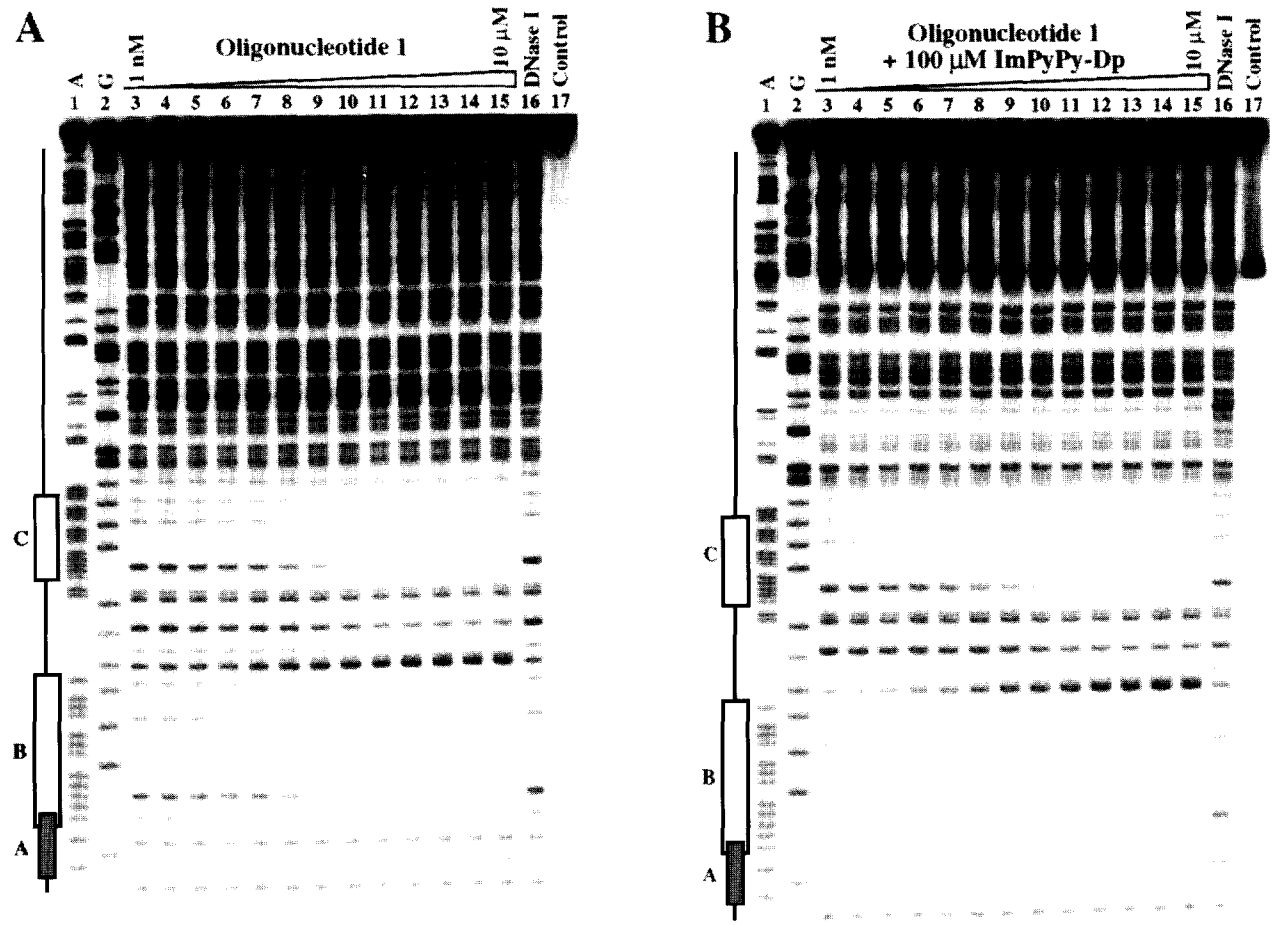

Figure 3. Storage phosphor autoradiograms of 8% denaturing polyacrylamide gels used to separate products of DNase I digestion in quantitative footprint titration experiments with oligonucleotide **1** in the absence (A) and presence of 100 μM·ImPyPy-Dp (B) on the 5′-^{32}P-labeled 255 base pair *Eco*RI/*Pvu*II restriction fragment from plasmid pMEP-2-ImN (25 mM tris-acetate, pH 7.0, 10 mM NaCl, 250 μM spermine, 100 μM calf thymus DNA in 40 μL total volume). Lane 1, A sequencing reaction; lane 2, G sequencing reaction; lanes 3–14, DNase I digestion products produced in the presence of increasing concentrations of oligonucleotide **1** alone (A) and in the presence of 100 μM ImPyPy-Dp (B): lane 3, 1 nM; lane 4, 2 nM; lane 5, 5 nM; lane 6, 10 nM; lane 7, 20 nM; lane 8, 50 nM; lane 9, 100 nM; lane 10, 200 nM; lane 11, 500 nM; lane 12, 1 μM; lane 13, 2 μM; lane 14, 5 μM; lane 15, 10 μM; lane 16, DNase I standard, lane 17, intact DNA. Location of oligonuceotide **1** binding sites **B** and **C** are indicated by a nonshaded rectangle, while the polyamide ImPyPy-Dp binding site **A** is indicated by a shaded rectangle. Note that sites **A** and **B** are protected from DNase I cleavage in (B), indicating simultaneous binding.

Table 1. Apparent first-order association constants for oligonucleotide **1** in the absence and presence of ImPyPy-Dp (M^{-1})[a,b]

ImpyPy-Dp (μM)	Proximal site **B**	Distal site **C**
0	2.7×10^7 (0.5)	2.7×10^7 (0.3)
50	1.9×10^7 (0.2)	2.1×10^7 (0.1)
100	1.9×10^7 (0.2)	2.0×10^7 (0.2)

[a]Values reported are the mean values of at least three footprint titration experiments, with the standard deviation for each data set indicated in parentheses.
[b]The assays were performed at 22 °C at pH 7.0 in the presence of 25 mM tris acetate, 10 mM NaCl, 250 μM spermine and 100 μM bp calf thymus DNA.

puckers instead of C2′-*exo* puckers seen in B DNA.[14] However, NMR data indicate that not all of the sugars in the three strands are in the C3′-*endo* configuration.[8,15] In addition, recent fiber type X-ray diffraction patterns of triple helical DNA[16] as well as IR spectroscopy studies[10c] indicate that triple helical DNA has B type helices rather than A type.

Our data supports a B-type conformation, because the minor groove binding ligands ImPyPy-Dp and distamycin bind to the duplex DNA target simultaneously with a third strand oligonucleotide binding. It is known that netropsin and distamycin do not bind appreciably to A-form DNA.[17] Furthermore, ImPyPy-Dp binds only to B DNA as shown by NMR studies.[5] In our systems both ImPyPy-Dp and distamycin bind simultaneously with the triple helix strongly suggesting that at least the duplex, if not all three strands, exist in the B-form conformation. Our data also suggest that the triple helix is compatible with slightly different minor groove widths since the ImPyPy-Dp 2:1 binding site is wider than the distamycin 1:1 binding site.[5,6,11]

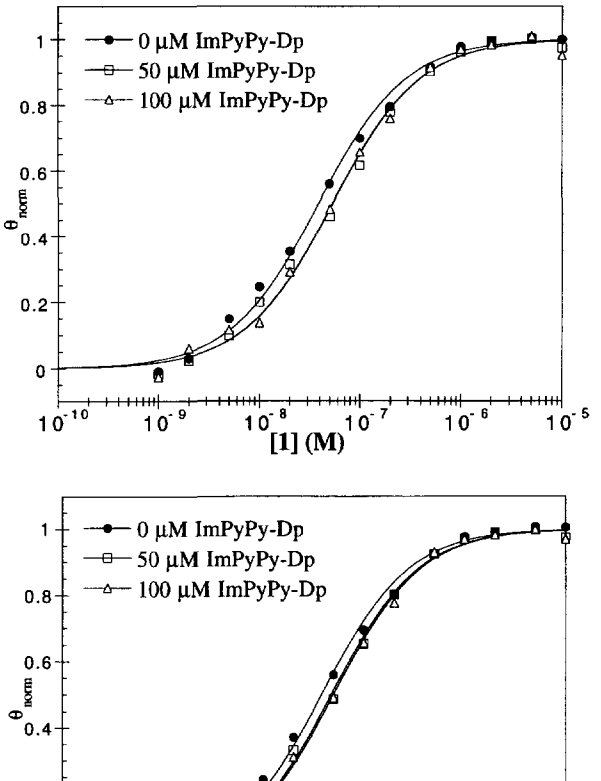

Figure 4. Data for the quantitative DNase I footprint titration experiments for oligonucleotide **1** in complex with the designated proximal **B** and distal **C** triplex sites (top and bottom, respectively). The θ_{norm} points were obtained using photostimulable storage phosphur autoradiography and processed as described in the experimental section. The data points for oligonucleotide **1** in the presence of no ImPyPy-Dp, 50 μM ImPyPy-Dp and 100 μM ImPyPy-Dp are indicated by filled circles (●), open squares (□), and open triangles (△), respectively. The solid curves are the best-fit Langmuir binding titration isotherms obtained from nonlinear least squares algorithm using equation (2).

Conclusion

In summary, binding of an oligonucleotide to double helical DNA by triple helix formation in the major groove is compatible with the polyamide ImPyPy-Dp binding as an antiparallel dimer in the minor groove of *an overlapping sequence*. Remarkably, no binding cooperativity between the two partners is observed under the experimental conditions described, implying that triple helix formation is unaffected by different minor groove widths. This data suggests that a new class of hybrid molecules, polyamide–oligonucleotide conjugates, which interact with both the minor and major grooves of DNA, could be designed for sequence-specific recognition of DNA. This should further expand the sequence repertoire available for targeting DNA by artificial methods.

Experimental

Materials

Doubly distilled water was further purified using the Milli Q filtration system from Millipore. Spermine-tetrachloride was purchased from Sigma and dissolved in water to a concentration of 2.5 mM. Sonicated, deproteinized calf thymus DNA was obtained from Pharmacia and dissolved in water to a concentration of 2 mM in base pairs. Enzymes were purchased from Boehringer–Mannheim or New England Biolabs and used with the buffers supplied. DNase I was obtained from Pharmacia. Deoxyadenosine-5′-[γ-^{32}P]-triphosphate was obtained from Amersham. Phosphoramidites were supplied by Cruachem. pUC 19 plasmid was purchased from Boehringer–Mannheim Biochemical. Epicurian XL-1 Blue Supercompetent cells were obtained from Stratagene. UV–vis spectra were recorded on a Hewlett–Packard Diode Array spectrophotometer. Cerenkov radioactivity was measured with a Beckman LS 3801 Scintillation Counter. Gels were dried using a Bio-Rad Model 483 Slab Dryer. Storage phosphor autoradiography was performed using a

Molecular Dynamics 400S Phosphorimager and ImageQuant software. Kodak S0230 storage phosphor screens were purchased from Molecular Dynamics.

Methods

Preparation of Plasmid DNA. The plasmid pMEP-2-ImN was prepared by ligation of the duplex formed between 5′-GATCCAGACAAAAAGAAAGAAAGACGATCGATCGAAGCAAAAAGAAAGAAAGAA-3′ and 5′-AGCTTTCTTTCTTTCTTTTTGCTTCGATCGATCGTCTTTCTTTCTTTTTGTCTG-3′ with T4 DNA ligase into pUC 19, previously digested with *Bam*HI and *Hind*III. The resulting ligation products were used to transform competent cells. Colonies were selected for α-complementation on 25 mL Luria–Bertani agar plates containing 50 μg/mL ampicillin and treated with IPTG and XGAL solutions. Large-scale plasmid purifications were carried out using Qiagen purification kits according to the provided protocol. The resulting DNA was sequenced using a Sequenase Version 2.0 sequencing kit (United States Biolabs) according to the manufacturer's protocol. Concentration of plasmid DNA was determined at an absorbance of 260 nm using the relation 1 OD unit = 50 μg/mL duplex DNA.

Preparation of labeled restriction fragment. Labeled restriction fragment from pMEP-2-ImN was generated as follows. Plasmid DNA was linearized using *Eco*RI, followed by treatment with calf alkaline phosphatase and subsequent 5′-end labeling with T4 polynucleotide kinase and γ-^{32}P-dATP. The linearized pMEP-2-ImN plasmid DNA was digested with *Pvu*II and the 255 base pair *Eco*RI/*Pvu*II restriction fragment was isolated by nondenaturing 5% polyacrylamide gel electrophoresis (PAGE). The gel bands were visualized by autoradiography, isolated, and eluted with 10 mM tris•HCl and 1 mM EDTA (TE). Polyacrylamide was removed from the resulting mixture by filtration through a 0.45 micron Centrex filter. The DNA was then isopropanol precipitated, resuspended in TE and phenol extracted several times. The DNA was further purified by ethanol precipitation. Chemical sequencing adenine- and guanine-specific reactions were carried out as previously described.[18] All DNA manipulations were performed according to standard protocols.[19]

Quantitative DNase I footprint titrations. Footprint titration reaction conditions were 25 mM tris-acetate, pH 7.0, 10 mM NaCl, 250 μM spermine, 100 μM calf thymus DNA, ~10,000 cpm of 5′-labeled DNA in 40 μL total volume. Oligonucleotide **1** was allowed to equilibrate with the labeled restriction fragment in the above buffer conditions for 24 h at 24 °C. For titrations in the presence of ImPyPy-Dp, ImPyPy-Dp was added after the 24-h equilibration and allowed to equilibrate for an additional hour. (The order of addition of oligonucleotide **1** and ImPyPy-Dp was reversed as a control. Results were identical for both orders of addition.) After equilibration, DNase I was added to give a final concentration of 38 μunits/μL in 10 mM $MgCl_2$, 10mM $CaCl_2$, and 1 μM nonspecific oligonucleotide to maintain uniform DNase I reactivity. DNase I digestion was quenched after 8 min by the addition of EDTA, glycogen and NaOAc (pH 5.2) to give final concentrations of 5 mM, 60 μg/mL, and 300 mM, respectively. The reactions were then precipitated with 2.5 volumes of ethanol. The resulting pellets were washed with 70% ethanol, redissolved in 35 μL of water and lyophilized to dryness. The residue was then resuspended in 5 μL of denaturing 80% formamide loading buffer. The DNA was denatured at 90 °C for 10 min, then placed on ice until loading. Reaction products were analyzed using 8% denaturing polyacrylamide gel (5% crosslink, 7 M urea) electrophoresis. Gels were then dried and autoradiographed and/or exposed to phosphor screens for quantitative analysis.

Footprint titration fitting procedure. The footprint titration gels were quantitated using storage phosphor technology (Molecular Dynamics), and data were analyzed by performing volume integrations of the target and reference sites using the ImageQuant v 3.1 software running on an AST Premium 386/33 computer. DNase I footprint titration experiments were analyzed according to the previously described protocol.[12,20] Briefly, θ_{app} was determined for each site using the following equation:

$$\theta_{app} = 1 - \frac{I_{tot}/I_{ref}}{I_{tot}^{o}/I_{ref}^{o}}, \quad (1)$$

where I_{tot} and I_{ref} correspond to the integrated volumes of the target and reference sites, respectively. I_{tot}^{o} and I_{ref}^{o} represent the amount of DNase I digestion at the target site and the reference site, respectively, in a DNase I control lane to which no oligonucleotide or ImPyPy-Dp has been added. The ($[O]_{tot}$, θ_{app}) data points were fit by minimizing the difference between θ_{app} and θ_{fit}, using the following equation, where θ_{min} and θ_{max} represent the experimentally determined site saturation values when the site is unoccupied or saturated, respectively:

$$\theta_{app} = \theta_{min} + (\theta_{max} - \theta_{min}) \bullet \frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}}. \quad (2)$$

Data were fit using a nonlinear least-squares fitting procedure of KaleidaGraph software (version 3.0.1, Abelbeck software) running on a Macintosh IIfx computer with K_T, θ_{max}, and θ_{min} as the adjustable parameters and without weighting the individual data points. All lanes from each gel were used unless visual inspection of the computer image from a storage phosphor screen revealed a flaw at the target or reference site or unless the θ_{app} value was greater than two standard errors away from the initial θ_{fit}. Data for experiments in which fewer than 80% of the lanes were usable were discarded. Data were normalized using the following equation:

$$\theta_{norm} = \frac{\theta_{app} - \theta_{min}}{\theta_{max} - \theta_{min}} \quad (3)$$

At least three sets of acceptable data were used in determining each association constant.

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