Bioorganic & Medicinal Chemistry 19 (2011) 1361–1365

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Discrimination of phosphorylated double stranded DNA by naphthalene diimide having zinc(II) dipicolylamine complexes

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ARTICLE INFO

Article history:
Received 19 October 2010
Accepted 27 October 2010
Available online 9 November 2010

Keywords:
DNA damage
Phosphorylate DNA
Naphthalene diimide
Zinc–dipicolylamine (Zn–Dpa) coordination complex
Threading intercalation

ABSTRACT

Discrimination of phosphomonoesters and phosphodiesters of DNA was attempted with naphthalene diimide carrying two zinc–dipicolylamine (Dpa) units (**1**). The binding constant of **1** for a self-complementary octanucleotide was $1.3 \times 10^6\ M^{-1}$, while the value for the phosphorylated counterpart was $4.8 \times 10^6\ M^{-1}$. This fourfold increase in the binding constant seems to stem from higher affinity of the terminal monophosphate over the phosphodiesters of DNA as the fourth ligand for the metal in **1**. Likewise, the binding constant of **1** for DNase I-treated calf thymus DNA (average size 200 bp) was twice as large as that for untreated DNA (1 kb), possibly because the terminal phosphate groups are five times abundant in the former. These findings provide a clue to developing a system where phosphomonoesters generated upon DNA nicking are discriminated specifically from intact phosphodiesters.

1. Introduction

Organic phosphates play an important role in biological systems. They are present in various forms such as mono- di- and triphosphates as well as pyro- and tripolyphosphates. Rise and fall of individual forms are closely associated with life itself. For example, the phosphodiesters which constitute the DNA backbone are vulnerable to such stresses as radiation and reactive chemicals to result in nicking or rupture of the diester bond to expose phosphomonoesters.[1] Since this and other DNA damages are often responsible for various diseases,[2–5] it is important in medicine to detect and quantitate the phosphomonoesters generated. This is currently done by DNA footprinting in which the target DNA is extended and then analyzed by gel electrophoresis and autoradiography.[6] A simpler alternative is obviously desirable. Recently, zinc(II) dipicolylamine complexes (Zn–Dpa) attracted considerable attention in phosphate analysis.[7–9] We herein address discrimination of phosphomono- and diesters by incorporating Zn–Dpa complexes into intercalator naphthalene diimide in a model system comprising calf thymus DNA and synthetic oligonucleotides.

2. Experimental

2.1. Materials

Oligonucleotides were custom synthesized by Genenet (Fukuoka, Japan). Common chemicals including biochemical reagents were obtained from commercial sources.

2.2. Synthesis

N,*N*-Bis[3-(3-aminopropyl)methylaminopropyl]naphthalene-1,4,5,8-tetracarboxylic acid diimide (**3**) was synthesized by the route reported previously.[10]

2.2.1. *N*,*N*′-Bis[3-[3-(2,2′-dipicolyl)methylaminopropyl]-methylaminopropyl]naphthalene-1,4,5,8-tetracarboxylic acid diimide (2)

A suspension of **3** (0.10 g, 0.10 mmol) in dry dichloromethane (10 ml) was solubilized by addition of triethylamine (0.11 ml, 0.80 mmol). 2-Pyridinecarboxaldehyde (0.095 ml, 1.0 mmol) was added and the mixture was stirred at room temperature under nitrogen atmosphere. After 30 min, sodium triacetoxy borohydride (0.42 g, 2.0 mmol) was added and the mixture was stirred at room temperature for 18 h and 2 M NaOH (0.5 ml) was added. After neutralization to pH 6.0 with 1 M HCl, the product was extracted with chloroform. The organic phase was washed with water and dried over magnesium sulfate. Evaporation of the solvent under reduced pressure left **2** as a brown viscous oil in 34% yield (30 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.81–1.94 (4H, m), 2.18–2.30 (4H, m), 2.50–2.70 (4H, m), 2.57 (6H, s), 2.80–2.90 (4H, m), 2.90–3.10 (4H, m), 3.73 (8H, s), 4.12–4.28 (4H, m), 7.08 (4H, m), 7.33 (4H, d J = 7.5 Hz), 7.60 (4H, m), 8.46 (4H, d, J = 4.1 Hz), 8.60 (4H, s) ppm. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.00–2.20 (8H, m), 2.80 (6H, s), 2.88–2.97 (4H, m), 3.08–3.18 (4H, m), 3.20–3.40 (4H, m), 4.10–4.22 (4H, m), 7.31–7.41 (4H, m), 7.47 (4H, d, J = 7.8 Hz), 7.82–7.94 (4H, m), 8.61 (4H, d, J = 4.4 Hz), 8.70 (4H, s) ppm. MS m/z $[M+H]^+$ 888.99 (theory for $C_{52}H_{58}N_{10}O_4 + H^+ = 888.08$).

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doi:10.1016/j.bmc.2010.10.056

2.2.2. *N,N′*-Bis[3-[3-(2,2′-dipicolyl)methylaminopropyl]-methylaminopropyl]naphthalene-1,4,5,8-tetracarboxylic acid diimide Zn(II) complex (1)

To a solution of **2** (20 mg, 0.020 mmol) in methanol (2 ml) was added dropwise $Zn(NO_3)_2$ (10 mg, 0.040 mmol) in water (1 ml). After stirring for 30 min at room temperature, the solvent was evaporated to leave a solid (30 mg). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.70–1.87 (8H, m), 2.70–2.80 (4H, m), 3.12 (6H, s), 3.25–3.48 (8H, m), 3.94–4.02 (4H, m), 7.73 (4H, dd, J = 0.7, 7.4 Hz), 7.75 (4H, d, J = 6.7 Hz), 8.10 (4H, t, J = 7.4, 7.4 Hz), 8.64 (4H, d, J = 2.2 Hz), 8.70 (4H, s) ppm. Mp 162–165 °C.

2.3. Preparation of calf thymus DNA and synthetic oligonucleotides

Calf thymus DNA (Sigma–Aldrich, St. Louis, MO) was used after sonication according to the method reported previously.[11] DNase I-treated calf thymus DNA was prepared as follows: After 1 mM calf thymus DNA was digested with 330 U of DNase I (Nippon Gene, Tokyo, Japan) in 900 μl of 1× buffer composed of 100 mM sodium acetate (pH 5.2) and 5 mM magnesium chloride, for a various period of time at 15 °C. Phenol/chloroform containing isoamyl alcohol and saturated TE was added to the reaction solution to quench the reaction, and the DNase I-treated DNA was recovered from the aqueous phase, followed by ethanol precipitation. The concentration of calf thymus DNA was estimated from the molar extinction coefficient based on nucleic bases of 6412 $cm^{-1}\,M^{-1}$ at 260 nm.[12] The concentration of self-complementary octanucleotides 5′-GCACGTGC-3′ and 5′-PO_4^{2-}-GCACGTGC-3′ was estimated from the molar extinction coefficient of 81,980 $cm^{-1}\,M^{-1}$ at 260 nm.

2.4. Apparatus and experimental procedures

Melting points are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC250P or AVANCE 400 spectrometer operating at 250 or 400 MHz for proton, respectively, with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were taken on a Voyager™ Linear-SA (PerSeptive Biosystems, Foster City, CA) by the time-of-flight mode with a-cyano-4-hydroxycinnamic acid as matrix. Electronic absorption spectra were recorded with a Hitachi 3300 spectrophotometer equipped with an SPR 10 temperature controller. The spectra were not corrected. Circular dichroism (CD) spectra were recorded over the 220–500 nm range on a Jasco J820 spectropolarimeter under the following conditions: response, 2 s; sensitivity, 100 mdeg; scan speed, 20 nm/min; resolution, 0.1 nm; band width, 2.0 nm.

2.5. Binding equilibria

The binding affinity of the ligand for sonicated calf thymus DNA untreated or treated with DNase I for 240 s was determined by Scatchard analysis using the condition probability method of McGhee and von Hippel shown as follows:[13] $r/L = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1}$, where r is the moles of the ligand bound per DNA base pair, L is the free ligand concentration, K is the observed binding constant, and site size n is the number of base pairs excluded by the ligand. The binding constants for oligonucleotides were estimated on the basis of a model where 1:1 form successively.

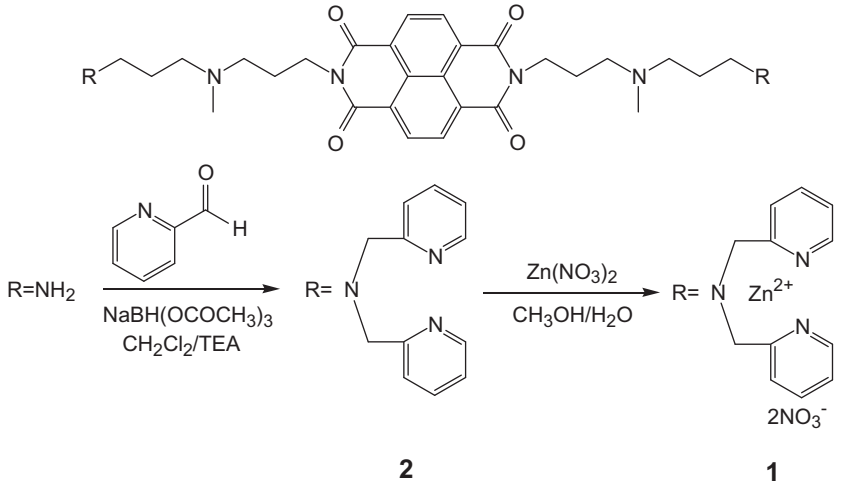
3. Results

3.1. Complex formation of 2 with Zn^{2+}

Zn–Dpa complex **1** was prepared as reported previously (Scheme 1)[14] and characterized, among others, by the downfield shifts of the pyridine protons of **1** from those of **2** in ^{1}H NMR (Fig. S1). Complex formation was monitored also by the fluorescence change of **2** upon addition of Zn^{2+} in 50 mM HEPES (pH 7.2) containing 0.10 M NaCl. Thus, the fluorescence intensity at 398 nm increased linearly up to 1:2 of **1** to Zn^{2+} when excited at 360 nm and then leveled off (data not shown), proving that the stable complex **1** was formed quantitatively between **2** and Zn^{2+}.

3.2. Binding behavior of 1 with calf thymus DNA

Introduction of two Zn–Dpa complexes to the side chain termini barely affected the basic intercalating properties of naphthalene diimide. Thus, the absorption band of **1** in 50 mM HEPES (pH 7.2) containing 0.10 M NaCl at 383 nm underwent as much as 60% of hypochromic shift with a small red shift upon addition of up to a 11-fold excess of sonicated calf thymus DNA (ctDNA) (Fig. S2). This behavior is characteristic of DNA threading intercalation by naphthalene diimide derivatives.[15–18] Binding affinity of **1** for ctDNA was determined by exploiting this phenomenon. The binding constant (K) and binding site size (n) thus obtained by fitting Scatchard plots with McGhee and von Hipple equation were



Scheme 1.

$1.8 \pm 0.22 \times 10^6\ M^{-1}$ and 4.3, respectively. Binding affinity of **2** was also determined analogously to be $6.7 \pm 0.25 \times 10^4\ M^{-1}$ and 3.0. Namely, **1** carrying the Zn–Dpa complexes has about 30 times higher affinity for DNA than metal-free **2**, demonstrating that the metal complexes contribute significantly to stabilization of the DNA-**1** intercalation complex. A putative model for the DNA-**1** complex is depicted in Figure 1, where the metal is supposed to interact more or less with the phosphate groups of DNA. In addition, the metal complexes as a whole or their ligands in particular may interact with the nucleobases through stacking.

The stabilizing effect of the Zn–Dpa complexes on the intercalation complex of **1** was supported by kinetic data, too. Association of **1** and **2** with sonicated ctDNA and dissociation of the resulting complex were studied in the same buffer. Typical decay curves are exemplified in Fig. S3. The association and dissociation rate constants in the DNA binding for **2** were $2.1 \times 10^5\ M^{-1}\ s^{-1}$ and $3.6\ s^{-1}$, respectively. These values are reasonable for naphthalene diimide type threading intercalators.[16] The corresponding values for **1** were $6.5 \times 10^4\ M^{-1}\ s^{-1}$ and $0.4\ s^{-1}$. That is, association of **1** occurs three times more slowly than that of **2**. On the other hand, the dissociation rate constant of **1** was nine times smaller than that of **2**. Two explanations may be invoked for this phenomenon: the interaction of the Zn–Dpa with the phosphodiesters or the anchorage of the metal complexes on DNA stabilizes the intercalation complex.

3.3. Characterization of the intercalation complex of 1 with double stranded DNA

To further clarify the binding mode of **1** with dsDNA, the following experiments were carried out. A negative Cotton effect was induced for **1** at 340–469 nm with an ellipticity of −1370 deg cm^2/decimol upon addition of an 11-fold excess of dsDNA (Fig. S4). It was reported that a negative Cotton effect was induced on bis-intercalator **3** (Fig. S5) with an ellipticity of −2560 deg cm^2/decimal in the presence of a 10-fold excess of DNA.[19] When these values were divided by their respective molar extinction coefficients (ε = 27,000 and 40,200 $M^{-1}\ cm^{-1}$), the ratio was 0.95 and 0.38 for **3** and **1**, respectively. This may suggest that these two molecules bind to DNA in a similar mode. Meanwhile, the ratio for **2** was 0.080, a value considerably smaller than that for **1**, suggesting that the dissymmetric environment in which **1** and **2** reside is subtly different.

The viscosity of a [poly(dA-dT)]$_2$ solution was determined in the presence of **1** or **4** (Fig. S5). It increased linearly with an increase in the amount of **1** or **4** nearly up to the equimolar ratio of DNA to ligand to yield a slope of 2.3 ± 0.12 and 1.3 ± 0.08, respectively (Fig. S6), indicating that the **1**-bound DNA assumes a more rigid conformation than the **4**-bound one.[20] Presumably, binding of the Zn–Dpa complexes deprives DNA of conformational flexibility.

Finally, topoisomerase I assay was carried out in the presence of **1** or **4**. It was found that unwinding of plasmid DNA caused by topoisomerase I was reversed by **1** (Fig. S7). Reversal of plasmid DNA unwinding was maximal at 5 μM **1**, while it was at 50 μM **4**, demonstrating that **1** can unwind DNA more effectively than **4**. This result is consistent with that of viscosity measurements (see above), confirming that **1**-bound DNA assumes a more rigid conformation, presumably because the Zn complexes serve as an anchor to prevent naphthalene diimide from dissociating from DNA.

3.4. Affinity of 1 for 5′-phosphorylated dsDNA

Binding affinity of **1** was studied with 5′-phosphorylated and un-phosphorylated self-complementary octanucleotides 5′-PO_3^{2-}-GCACGTGC-3′ and 5′-GCACGTGC-3′. They have a double stranded DNA structure with or without phosphate at the 5′-terminus and hereafter the former and latter are called pODN and ODN, respectively. Compound **1** underwent large hypochromic and small red shifts upon addition of pODN or ODN, just like with natural DNA. Hypochromicity leveled off nearly at one half molar or an equimolar ratio of pODN or ODN to **1**, respectively (Fig. 2). The data were analyzed on the basis of a model where **1** binds to DNA successively (Scheme 2) and the latter has multiple binding sites (Eq. 1) in which A_f and A_b represent the absorbance of free and DNA-bound **1**, respectively, and $[\mathbf{1}]_0$ the analytical concentration of **1**, C the concentration of free ODN or pODN) with an R greater than 0.99.[21] The binding constant K and site size s were $1.3 \pm 0.23 \times 10^6\ M^{-1}$ and 1.5, respectively, for ODN and $4.8 \pm 0.83 \times 10^6\ M^{-1}$ and 1.2 for pODN. These data imply that **1** binds to either ODN or pODN by essentially the same mode, that is, 1–2 molecules per every five base pairs. Incidentally, two molecules of nogalamycin were found to bind to self-complementary hexanucleotide 5′-TGTACA-3′ by X-ray diffraction.[22] Nonetheless, **1** exhibits nearly four times higher affinity for pODN. It is certain that the terminal phosphate of pODN contributes considerably to the binding of **1**.

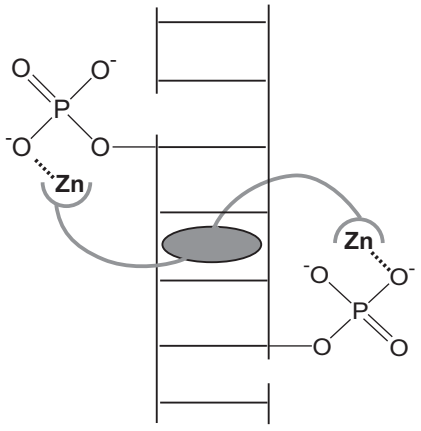


Figure 1. A putative model for the complex of **1** with nicked sites on dsDNA.

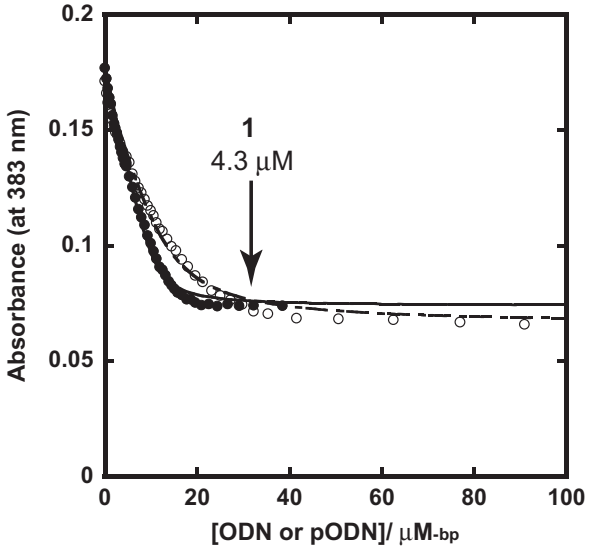


Figure 2. Absorbance changes for 4.3 μM **1** with 0–90.9 μM ODN (open circles) and 0–38.5 μM pODN (filled circles) in 50 mM HEPES (pH 7.2) and 0.10 M NaCl. The curves are drawn based on Eq. 2 with the parameters described in the text.

$$\text{ODN or pODN} + \mathbf{1} \underset{}{\overset{K}{\rightleftharpoons}} \text{ODN-}\mathbf{1}_S \text{ or pODN-}\mathbf{1}_S$$

Scheme 2.

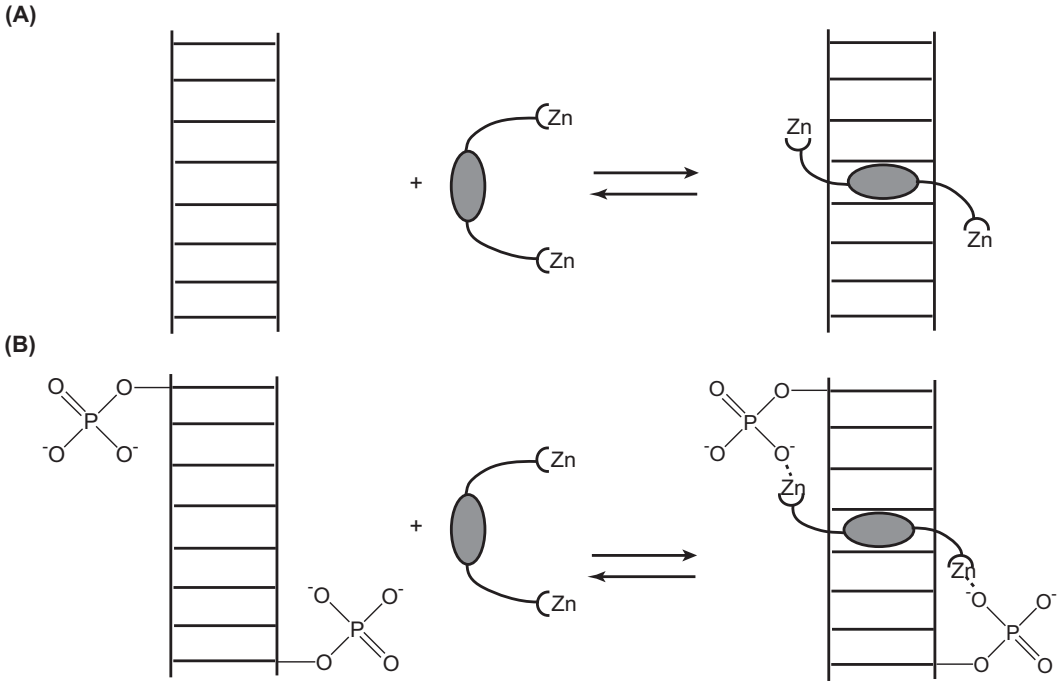


Figure 3. Possible 1:1 binding model for **1** and ODN (A) or pODN (B).

Presumably, the single monophosphate rather than one of the seven phosphodiesters of ODN serves as the preferred fourth ligand for Zn in **1** (Fig. 3), thereby offering extra stabilization to the intercalation complex of **1** with pODN.

$$\mathrm{Abs} = A_f + (A_b - A_f)\frac{(a - (a^2 - \frac{2K^2[1]_0C)}{s})^{1/2}}{1 + 2K[1]_0} \quad (1)$$

$$a = 1 + K[1]_0 + \frac{KC}{2s} \quad (2)$$

$$C = [\mathrm{ODN\ or\ pODN}] \quad (3)$$

3.5. Affinity of 1 for phosphorylated calf thymus DNA

As described above, **1** discriminated phosphorylated synthetic DNA from the un-phosphorylated counterpart to some extent, discrimination by **1** was tested with natural DNA (Fig. 4). Thus, sonicated ctDNA of average size of 1 kb was digested with endonuclease DNase I for fragmentation. The average size of the resulting DNA fragments obtained under a given condition was 200 bp. Binding capabilities of **1** for this and undigested ctDNA were then compared at the same DNA concentration. Thus, the binding constant of **1** for the digested DNA was $4.0 \pm 0.39 \times 10^6$ M^{-1}, that is, twice as large as that for ctDNA (see above). Though modest, the larger binding constant for the DNase I-treated DNA may be associated with the presence of a larger amount of terminal phosphomonoesters. The fact that the difference between the two types of DNA was smaller than that observed for the synthetic DNA may be associated with the difference in the size of DNA used (8 vs 200 bp); as the DNA size increases, more ligand molecules bind to DNA and the fraction of the ligand interacting with the terminal phosphates is marginalized.

4. Discussion

In biological settings, many compounds, irrespective of their size, exist in their phosphorylated forms. Thus, mono-, di- and triphosphates as well as pyro- and tripolyphosphates are widely distributed in the cell and they play individual roles. Discrimination and specific analysis of these species are important to unravel their action in biological systems. Although a number of methods are available to enable these analyses, an ever-lasting demand for simpler techniques never ceases. Those based on the Zn–Dpa complex attracted considerable attention in recent years. Thus, phosphorylated peptides were successfully analyzed with anthracene having two Zn–Dpa units **5** (Fig. S5),[6,7] and pyrophosphate was discriminated specifically from adenosine triphosphate and orthophosphate with naphthalene diimide carrying two Zn-Dpa units **6** (Fig. S5).[8] Furthermore, an intracellular pH change was monitored with fluorescein carrying Dpa **7** (Fig. S5).[23] We herein addressed discrimination of phosphomono- and phosphodiesters with naphthalene diimide carrying two Zn-Dpa units **1**, similar to **5**. In the study with an octanucleotide, it was found that **1** exhibited higher affinity for the phosphorylated form than the un-phosphorylated counterpart. Presumably, the oxy anions of phosphomonoesters

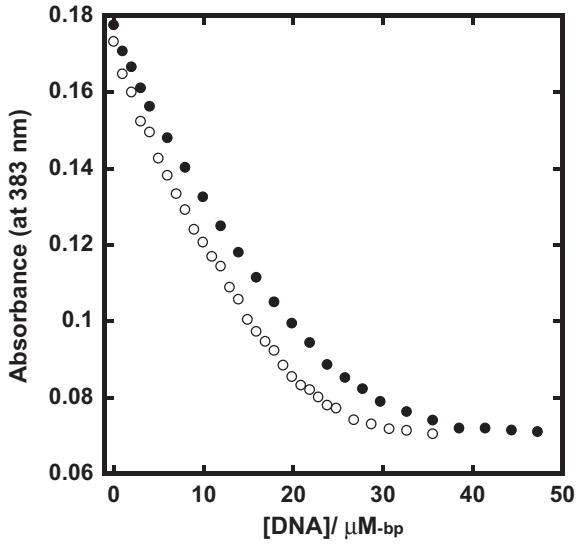


Figure 4. Absorbance changes for 4.3 μM **1** with 0–35.6 μM sonicated calf thymus DNA treated with DNase I (open circles) and 0–47.2 μM calf thymus DNA (filled circles) in 50 mM HEPES (pH 7.2) and 0.10 M NaCl.

are more basic than that of phosphodiesters and three oxygen atoms are available for metal coordination in phosphomonoesters, while there are two in phosphodiesters. It was found that the binding of **1** to an octanucleotide was enhanced to some extent where its terminus is phosphorylated. It is reminded that there are seven phosphodiesters and a single monophophates in the phosphorylated octanucleotide. Furthermore, in natural (calf thymus) DNA the binding of **1** was enhanced slightly where the DNA was shortened fivefold. The effect of the phosphoryl group(s) was modest at best, yet this system may serve as a model to analyze nicked DNA with this technique. We believe it possible to enhance the effect of phosphates by proper design of the ligand.

In conclusion, the Zn-Dpa complex is a versatile scaffold to develop reagents for analysis of phosphorylated compounds in biological systems. There is plenty of room for improvements in the design by combining such functional units as chromophores and intercalators and changing the linker length to meet individual need.

Acknowledgements

This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. S.W. is grateful for financial support from the Japan Society for the Promotion of Science.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.056.

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