Sequence Specificity of DNA Cleavage by Bisnetropsin-Linked Hydroxamic Acid–Metal Complexes: Highly Specific Cleavage by Ferrous Complexes

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Bisnetropsin-linked hydroxamic acids (BNHA) were shown to cleave DNA strands at a single residue with a preferred binding orientation in the presence of ferrous ion. In contrast, the corresponding cerium (III) complexes cleaved DNA with low sequence specificity.

Key words DNA cleavage; hydroxamic acid; netropsin; ferrous ion; lanthanide metal; specific cleavage

Sequence-specific DNA cleavage by designed ligands is of continuing interest to organic chemists. Despite considerable effort, only a few small organic molecules are known to induce single site DNA cleavage at specific sequences.[1] Our research is directed towards the development of ligands which specifically cleave DNA using the hydroxamic acid–metal system.[2] Based on the high affinity of netropsin for the AT rich minor groove,[3] we have recently synthesized bisnetropsin-linked hydroxamic acids which contain two *N*-methylpyrrole dipeptide units coupled by β-alanine (BNHA2) or γ-aminobutyrate (BNHA3) groups.[2c] These molecules cleave DNA in the presence of transition or lanthanide metal ions, possibly through oxidative or hydrolytic mechanisms, respectively.[2c,d] The hydroxamic acid–transition metal complexing moiety should show a different mode of DNA binding to the corresponding lanthanide case, since the latter cation is known to strongly bind to the DNA phosphate group like typical "hard" acids.[4] It is therefore expected that transition metal complexes of BNHAs would show different cleavage selectivities compared to the lanthanide ones. To clarify this point, we have compared the DNA cleavage patterns of Fe(II) complexes of BNHAs with the corresponding Ce(III) ones, using the sequencing gel technique. We report herein the unique DNA cleavage properties of BNHAs in the presence of metal ions.

A series of DNA-cleaving hydroxamic acids are shown in Fig. 1. According to the $n+1$ rule,[5] the bisnetropsin binding moieties of BNHAs should bind to the 8-base pair site. Taking this binding-site size into consideration, we selected the *Eco* RI-*Rsa* I DNA fragment, which has 8 or 10 base pair AT contiguous sites, for cleavage reactions of metal complexes of BNHAs. This 517 base pair restriction fragment was labeled with ^{32}P on the 5′- or 3′- ends separately. The cleavage patterns produced on these substrates were resolved using DNA sequencing gels, then visualized by autoradiography (Fig. 2). The sequenced portion of this DNA fragment contains 8 or 10 base pair AT contiguous sites (site 1 and 2, respectively). The cleavage specificities of a number of DNA-cleaving oligopyrrole derivatives developed by the Dervan group have been analyzed on this sequenced portion of the same DNA fragment.[6] Preliminary deoxyribonuclease (DNase) I footprinting demonstrated that BNHA2 caused inhibition of the enzyme cleavage at a one order of magnitude lower concentration at both sites 1 and 2, than does BNHA3 (data not shown). Metal complexes of phenanthridine-linked hydroxamic acid (PHA) cleaved DNA with hardly any sequence specificity in both the ferrous (Fig. 2 (a), lane 4 and Fig. 2 (b), lane 4) and cerium (Fig. 2 (a), lane 5 and Fig. 2 (b), lane 5) systems. This lack of specificity may simply result from non-sequence selective binding of the phenanthridine ring. Although the ferrous complex of the distamycin-linked hydroxamic acid (DHA) produced distinct cleavage in the high mobility region of the sequencing gel (Fig. 2 (a), lanes 6), this complex produced no cleavage pattern at both sites 1 and 2 despite the presence of the distamycin moiety (Fig. 2 (a), lane 6 and Fig. 2 (b), lane 6). Noticeable is the highly specific cleavage observed for the ferrous complexes of BNHAs.[7] As can be seen from lane 8 in Fig. 2 (a), the ferrous complex of BNHA2 produced a distinct cleavage only at the 3′ side residue of site 1. Similar single site cleavage was also observed for this complex at the 3′ side residue of site 2 (Fig. 2 (b), lane 8). On the other hand, the ferrous complex of BNHA3 produced strong and sharp cleavage at the 5′ or 3′ side residue of site 2 (Fig. 2 (a), lane 10 and Fig. 2 (b), lane 10, respectively). Interestingly, no specific cleavage was produced at site 1 by this complex. These differences in site-specificities for DNA cleavage appear to depend on the length of the linker between the two netropsin units. In marked contrast to the ferrous system, site-specific cleavage was not observed for the cerium complexes of BNHA2 (Fig. 2 (a), lane 9 and Fig. 2 (b), lane 9) and BNHA3 (Fig. 2 (a), lane 11 and Fig. 2 (b), lane 11). Figure 3 shows the histogram of the DNA cleavage patterns produced by the ferrous complexes of BNHAs, within the region containing sites 1 and 2. The significant finding from this result is as follows: i) the ferrous complex of BNHA2 causes single site cleavage

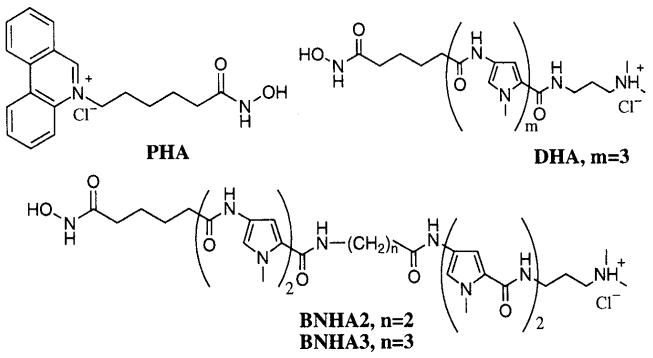

Fig. 1. DNA Cleaving Hydroxamic Acids

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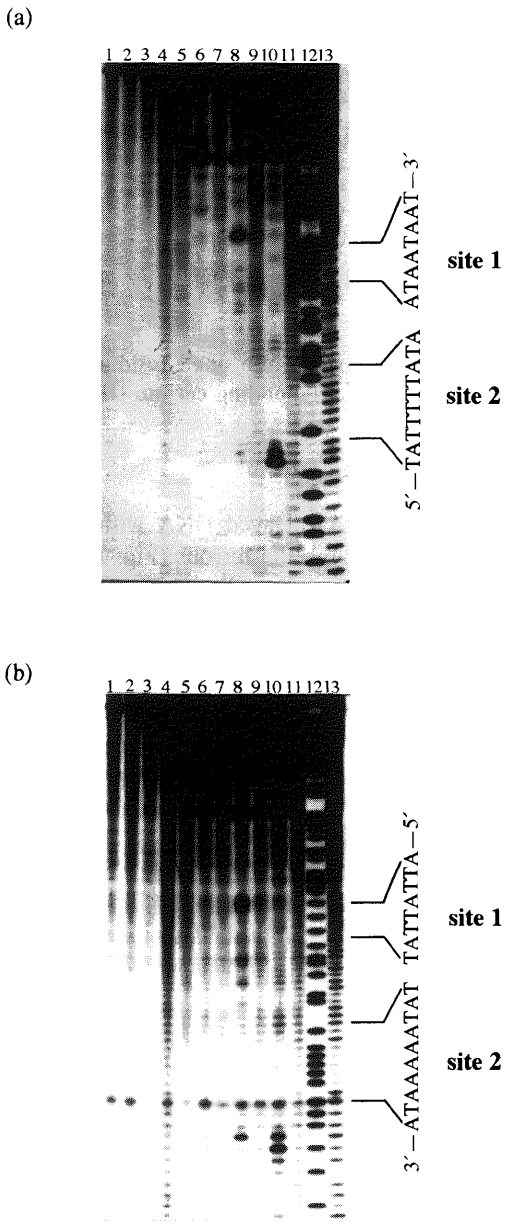

Fig. 2. Autoradiograms of a 10% Polyacrylamide Gel Showing Cleavage of ^{32}P-Labeled pBR 322 DNA Fragment (*Eco* RI-*Rsa* I Fragment, 517 bp) by Hydroxamic Acid–Metal Complexes

Autoradiograms (a) and (b) Show DNA Fragments Labeled at the (a) 5′ End or (b) 3′ End, Respectively. Lane assignment: lane 1, intact DNA; lane 2, 10 μM $FeSO_4$; lane 3, 30 μM $CeCl_3$; lane 4, 10 μM $FeSO_4$+10 μM PHA; lane 5, 30 μM $CeCl_3$+30 μM PHA; lane 6, 10 μM $FeSO_4$+10 μM DHA; lane 7, 30 μM $CeCl_3$+30 μM DHA; lane 8, 10 μM $FeSO_4$+10 μM BNHA2; lane 9, 30 μM $CeCl_3$+30 μM BNHA2; lane 10, 10 μM $FeSO_4$+10 μM BNHA3; lane 11, 30 μM $CeCl_3$+30 μM BNHA3; lane 12, Maxam–Gilbert G+A; lane 13, Maxam–Gilbert C+T.

at both the 8 and 10 AT base pair sites, with the hydroxamic acid moiety oriented to the 3′ side of the strand to be cleaved; ii) the ferrous complex of BNHA3 causes specific cleavage only at the labeled side of the 10 AT base pair site; and iii) the β-alanine (BNHA2) or γ-aminobutyrate (BNHA3) linkage is the structural element responsible for controlling the highly-specific cleavage. The cleavage mode of these ferrous complexes differs significantly from that of the affinity cleaving agent EDTA–Bis(distamycin)·Fe(II), which produces multiple cleavage patterns flanking both sides of sites 1 and 2.[6a] It is reported that the AT rich minor groove in B form DNA exhibits an unusual narrowness.[6d,8] We believe that the specific binding orientation of the ferrous

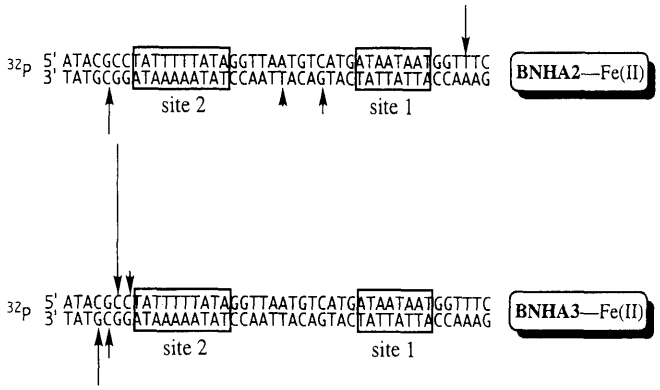

Fig. 3. Histograms of Fe(II) Complexes of BNHA2 (Fig. 2 (a) and (b), lane 8) and BNHA3 (Fig. 2 (a) and (b), lane 10) Cleavage Patterns Flanking Both Sites 1 and 2

Bars indicate sites and extent of DNA cleavage.

Table 1

Binding agents	CT DNA[a]	Poly(dA-dT)	Poly(dG-dC)
Distamycin	1.1	0.17	65
BNHA2	4.5	0.26	46
BNHA2—La(III)	0.40	0.16	3.1
BNHA3	88	17	200<
BNHA3—La(III)	1.9	0.98	7.2
La(III)	5.4	6.4	5.7

a) CT DNA calf thymus DNA.

complexes of BNHAs could be influenced by this sequence-dependent variability in the DNA structure. Not only the sequence-dependent local structure of DNA, but also the additional binding specificity provided by the metal complexing moiety, is crucial for highly specific DNA cleavage. Our previous study indicated that ferrous-mediated DNA cleavage by the DNA-binding hydroxamic acid involved a diffusible active oxygen species.[2a,b] To achieve highly specific DNA cleavage by the ferrous system of BNHAs, the hydroxamic acid–ferrous complexing moiety should be oriented in the immediate vicinity of the hydrogen of the deoxyribose in the AT rich minor groove.

In order to explore the correlation between the low cleavage specificity and binding preferences of Ce(III) complexes of BNHAs, the DNA binding affinity of La(III) complexes of BNHAs[9] for DNA polymers was evaluated using an ethidium displacement assay.[10] The agent concentrations required for a 50% decrease in the fluorescence of the DNA bound-ethidium, the so-called C_{50} (μM), were determined for each complex and DNA pair, and the results are summarized in Table 1. It can be seen that the C_{50} values of BNHA2 to CT DNA and poly(dA-dT) are one order of magnitude lower than those of BNHA3. The binding affinity of this ligand is comparable to that of the *N*-methyltripyrrolepeptide distamycin. This suggests that the β-alanine linkage is effective for the binding of two netropsin units to DNA. Upon complexation with the La(III) ion, the binding of BNHA2 to poly(dA-dT) was almost unchanged. Although binding of the lanthanum complex of BNHA3 to this polymer appears to be significantly enhanced compared with that of the uncomplexed BNHA3, it was only slightly enhanced compared with free La(III) ion. The lanthanum complex of BNHA2 showed

similar binding affinity for poly(dG-dC) to the free La(III) ion. Similar binding behavior was also observed for the lanthanum complex of BNHA3 to this polymer. These results indicate that both the lanthanide chelation moieties and the two netropsin units in the lanthanide complexes of BNHAs may bind to the AT consecutive sequences of DNA in a low synergistic manner. Thus, BNHAs presumably do not effectively increase the local concentration of lanthanide cation in proximity to the phosphodiester linkage adjacent to sites 1 and 2. A more elaborate molecular design is necessary for the development of DNA-cleaving hydroxamic acid lanthanide systems with sequence recognition ability.

In conclusion, high and low specific DNA cleavages have been demonstrated by the hydroxamic acid–metal system, depending on the nature of the metal ions.

Experimental

Chemicals Plasmid pBR 322 DNA was isolated from *Escherichia coli* strain JM109 by alkali lysis and was purified by precipitation with polyethylene glycol.[11] [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dATP (6000 Ci/mmol) were obtained from NEN-Dupont. The microfine glass beads and chaotropic salt buffer were from Bio 101. All enzymes utilized were from commercial sources.

Preparation of 5′- and 3′-Labeled Restriction DNA Fragments The restriction *Eco* RI–*Rsa* I fragment was labeled at the *Eco* RI site. The 5′-end was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP after treatment with calf intestinal alkaline phosphatase. The 3′-end was labeled using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. Digestion with the second enzyme *Rsa* I yielded the singly end-labeled 167 and 517 bp fragments which were purified by 4% agarose gel. The band of interest was identified by autoradiography, excised and isolated using the glass matrix method.[12]

Cleavage Reactions of Labeled DNA Fragments by Hydroxamic Acid Complexes The reaction mixtures contained 3×10^4 cpm of the ^{32}P-end-labeled DNA fragment (5′- or 3′- labeled) and the desired concentration of the hydroxamic acid–metal complexes in 40 mM Tris–HCl, pH 8.0, buffer. In the ferrous system, dithiothreitol (DTT) and sonicated calf thymus DNA were added to a final concentration of 100 and 10 μM (bp), respectively. The cleavage reactions were carried out at 37 °C for 4 h (ferrous system) or 12 h (cerium system) and stopped by the addition of stopping buffer (0.3 M AcONa, pH 7.0, 0.1 mM EDTA and 25 μg/ml tRNA) followed by ethanol precipitation. The precipitated DNA was washed with 70% cold ethanol and dried *in vacuo*. The recovered DNA was dissolved in 5 μl of loading buffer (80% v/v formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). All DNA samples were heated at 90 °C for 3 min and loaded on a 10% polyacrylamide 8 M urea gel. Electrophoresis was performed at 1500 V for approximately 2.5 h. Autoradiography of the gel was carried out at −80 °C overnight on Fuji medical X-ray film, and the autoradiogram was scanned at 485 nm with a Shimadzu CS-9000.

Ethidium Displacement Assay The fluorescence decrease in the ethidium displacement assay was monitored using a Shimadzu RF-5000 spectrofluorophotometer at room temperature. The C_{50} values of the DNA-binding agents were determined by employing the DNA polymer (1.0 μM in nucleotide) in 10 mM Tris–HCl, 1 mM NaCl, pH 8.0, containing 1.26 μM ethidium as described in the literature.[10]

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