Calicheamicin−Homeodomain Conjugate as an Efficient, Sequence-Specific DNA Cleavage and Mapping Tool

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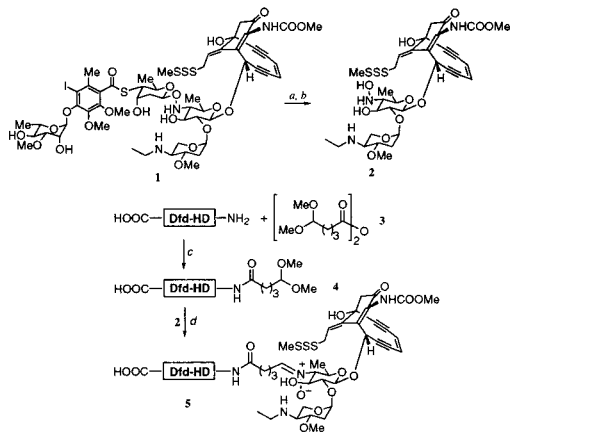
Sequence-specific recognition and manipulation of DNA on the vast scale of chromosomes or large chromosomal fragments is a principal design challenge for chemical probes aimed at mapping specific protein−DNA interactions,[1] selectively interfering with gene transcription[2] or precisely targeting a genotoxic drug molecule. In this paper we describe the conjugation of a sequence-specific DNA-binding protein to a small molecule that cleaves both strands of duplex DNA to prepare an efficient affinity cleavage agent that cuts DNA at a single base pair.

Attachment of a metal chelator, such as EDTA or 1,10-phenanthroline, to a DNA-binding protein to obtain a sequence-specific DNA cleavage agent has been reported.[1] The efficiency of DNA cleavage in these experiments often is low. A localized array of cleavage sites is generally observed, typically resulting from diffusible hydroxyl radical-induced cleavage. Calicheamicin γ_1^I (CLMγ_1^I, **1**)[3] is an enediyne antitumor antibiotic which when reductively activated in the presence of molecular oxygen can cleave both strands of DNA sequence-selectively.[4]

It was fortuitously discovered that CLMγ_1^I (**1**) can be degraded quite cleanly to CLMt (**2**) in acidic acetone.[5] Investigation of the DNA cleavage properties of this "truncated" calicheamicin revealed that its sequence selectivity was largely lost, but sufficient organization remained in its DNA-bound form that its ability to effect double-stranded scission was left substantially intact.[5] On the basis of these observations, we thought that linking CLMt to a DNA binding protein could synergistically combine the sequence-specificity of the protein and the double-stranded DNA cleavage ability of CLMt. Among DNA-binding proteins, we elected to use a homeodomain.

Proteins containing a sequence motif encoded by the homeobox constitute a large family of transcription factors that play pivotal roles in the development of eukaryotes, from fungi to humans.[6] The DNA binding unit of such proteins, called the homeodomain (HD), consists of an *N*-terminal arm that makes contacts in the minor groove and three α-helices, the third of which takes part in major groove interactions in base-specific manner. These highly conserved motifs comprise about 60 amino acids and are capable of binding to DNA as a monomer typically with nanomolar affinity. Since CLMγ_1^I is known to span the minor groove of DNA when it makes double-stranded cuts,[7] we were attracted to the possibility of attaching CLMt to the amino terminus of a homeodomain, which would direct the binding of the DNA cleavage element to the DNA minor groove in a sequence-specific manner.

Scheme 1



Conditions: a, acetone, PPTS, reflux; *b*, CH_3CN/H_2O, 0.1% TFA; *c*, aq. buffer, pH 7.2; *d*, H_2O, 0.1% TFA

CLMγ_1^I (**1**) is sensitive to oxidation, reduction, acid, base, and elevated temperatures. Selective derivatization, therefore, is a chemically challenging task. Extensive investigation of potential conjugation strategies led us to the direct and general approach of nitrone formation to couple a representative homeodomain, from the *Drosophila* deformed (Dfd) protein,[8] to CLMt (**2**) (Scheme 1).[9] Unlike terminal or side-chain amines, the side chains of histidine, tyrosine, and cysteine residues in a protein do not form stable products with anhydrides under controlled aqueous conditions.[10] Because of the differences in pK_a and nucleophilicity between the *N*-terminal amino group and side-chain amino groups, we were able to prepare the desired modified Dfd homeodomain **4** by selective acylation of the Dfd terminal amine with anhydride **3**.[9c,10] Monoacylation at the *N*-terminus of Dfd was verified by MALDI-TOF mass spectrometry and enzymatic digestion experiments.[11] Upon treatment with an aqueous solution of CLMt and adjustment to pH 2−3 with 4% aqueous TFA, the free aldehyde-containing linker-Dfd formed in situ and then reacted with CLMt to give the desired nitrone adduct **5** in about 40% yield.

Electrophoretic mobility shift assay (EMSA) experiments confirmed that the Dfd-linker-CLMt adduct **5** is active for DNA

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(8) (a) Dfd sequence: H_2N−PKRQRTAYTRHQILELEKEFHYNRY−LTRRRRIEIAHTLVLSERQIKIWFQNRRMKWKKDNKLPNTKNVRKK−COOH (b) Ekker, S. C.; von Kessler, D. P.; Beachy, P. A. *EMBO J.* **1992**, *11*, 4059−4072.

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(11) MALDI mass spectra gave the following data: for the modified Dfd-HD **4**, 9233.8 ($M + H^+$, found) and 9233.8 (calcd), and for the modified *N*-terminal dipeptide $(MeO)_2CHCH_2CH_2CH_2CO$-Pro-Lys from trypsin digestion, 388.0 ($M + H^+$, found) and 388.5 (calcd).

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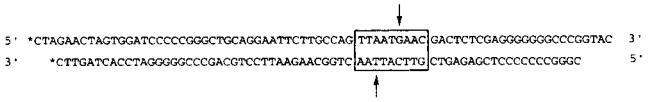

Figure 1. Sequence of DNA fragments used for cleavage experiments. Arrows mark the cleavage sites. The optimal DNA binding site for the Dfd homeodomain[8b] is boxed.

binding. Treatment of plasmid DNA[12] with **5** led to double-stranded DNA cleavage, as revealed by agarose gel electrophoresis. Our data show that adduct **5** converts the supercoiled plasmid DNA to nicked (single-stranded cleavage) and linear (double-stranded cleavage) DNA in an approximate ratio of 3:2.[13]

The ability of adduct **5** to effect sequence-specific DNA cleavage was examined using a 3′-radiolabeled (68 bp, bottom strand, Figure 1) and a 5′-radiolabeled (75 bp, top strand, Figure 1) DNA restriction fragment (*XbaI/KpnI*) prepared from plasmid ptDopt, which contains the optimized Dfd binding site[8b] (boxed in Figure 1). Two bands resulted from bottom strand cleavage (Figure 2A, lane 3). Treatment of cleaved DNA with 0.1 M piperidine caused loss of the upper band, and concomitant enhancement of the lower band (Figure 2A, lane 5). The lower band comigrates with DNA cleaved by Maxam−Gilbert sequencing chemistry at the thymine that is indicated by the arrow in Figure 1. These results indicate that bottom-strand cleavage is due to abstraction of a 5′-H from this thymine, the upper of the two bands in Figure 2A, lane 3 contains a DNA fragment having a 5′-aldehyde terminus,[4c] and the lower band contains a strand terminated by phosphate (as in Maxam−Gilbert chemistry). Under normal cleavage conditions without base treatment, the 5′-aldehyde-containing fragment is only partially converted to the 5′-phosphate-ended fragment.

Cleavage of the complementary (top) strand (5′-radiolabeled) by adduct **5** (Figure 2B) was also highly sequence-specific, and occurred at only one nucleotide, the guanine indicated by the arrow in Figure 1. The cleavage sites on the two strands are offset by three nucleotides to the 3′ direction, consistent with minor groove binding of the cleavage agent and, indeed, with the known DNA cleavage pattern of CLMγ_1^I itself.[4c,7c] The site of cleavage also fits the orientation of Dfd as it is known to be bound to the Dfd-optimal site, as determined by previous hydroxyl radical footprinting and missing nucleoside experiments.[14] In particular, the amino terminus of the Dfd homeodomain is extended along the minor groove placing the CLMt portion at a site corresponding to the two nucleotides at which we observed cleavage.

The precise and efficient cleavage of DNA by adduct **5** is presumably due to the orientation imposed on CLMt by the attached Dfd homeodomain and, secondarily, by binding of CLMt itself in the minor groove to correctly localize a benzenoid diradical. The single-cleavage site we observed stands in sharp contrast to the multiple cleavages generated by diffusible, reactive species produced by conjugates of metal chelators with DNA-binding proteins.[1] Single-site, double-stranded DNA cleavage by an enediyne-based DNA cleaving agent presents unique advantages for mapping the interaction of a homeodomain with large genomic DNA fragments accurately and with high signal-to-noise.

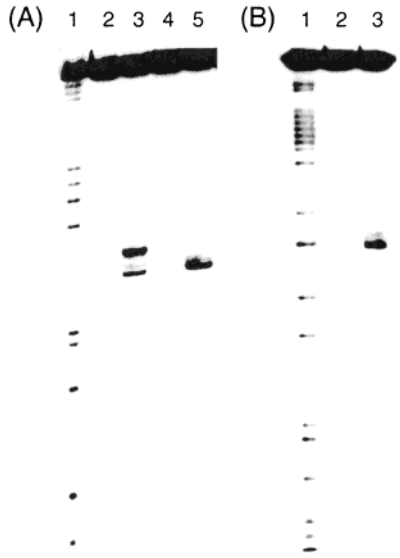

Figure 2. (A) Denaturing polyacrylamide gel electrophoresis of the products of cleavage of a 3′-radiolabeled DNA restriction fragment (see Figure 1) by Dfd-CLMt adduct **5**. Cleavage was performed at 37 °C for 30 min: lane 1, products of the Maxam−Gilbert guanine-specific sequencing reaction; lane 2, DNA control, without piperidine treatment; lane 3, cleavage by 1.8 μM Dfd-CLMt adduct, without piperidine treatment; lane 4, DNA control, treated with 0.1 M piperidine; lane 5, cleavage by 1.8 μM Dfd-CLMt adduct followed by treatment with 0.1 M piperidine. (B) Denaturing polyacrylamide gel electrophoresis of the products of cleavage of a 5′-radiolabeled DNA restriction fragment (see Figure 1) by Dfd-CLMt adduct **5**. Cleavage was performed at 37 °C for 30 min: lane 1, products of the Maxam−Gilbert guanine-specific sequencing reaction; lane 2, DNA control; lane 3, cleavage by 1.8 μM Dfd-CLMt adduct.

The nitrone conjugation strategy is a general and practical microscale (100 nmole) approach for the attachment of CLMt to other sequence-specific DNA binding molecules. Enediyne-based DNA cleaving agents could be applied to genome mapping, sequencing, and gene cloning. Conjugates between CLMt and sequence-specific DNA binding molecules that can permeate human cells[2] may have applications in human medicine where enediyne-based DNA cleavage can be initiated by the (10^3−10^4)-fold higher concentration of glutathione inside cells.[15]

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(12) We used plasmid ptDopt for these experiments. This plasmid was constructed by inserting one copy of the Dfd optimal DNA binding site[8b] (see Figure 1) into pBluescript.

(13) Efficiency of total DNA cleavage is >3% assuming adduct **5** is present as 100% reactive enediyne.

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