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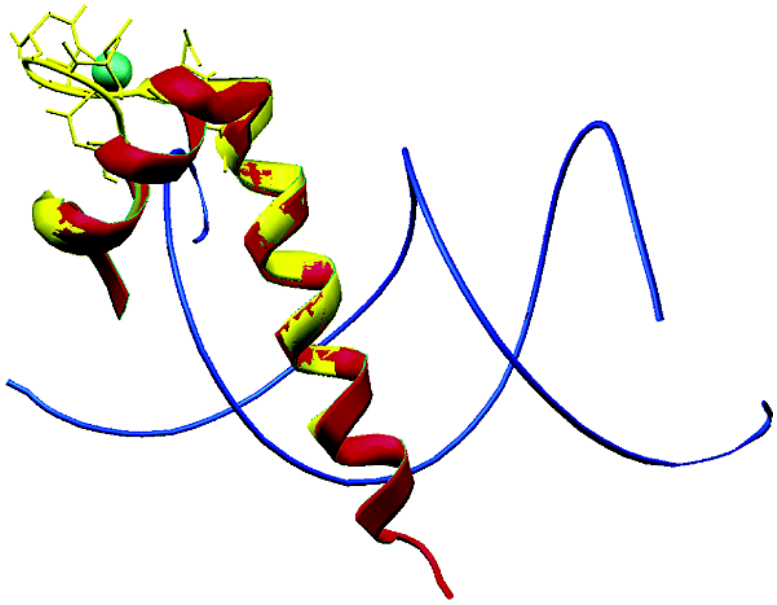

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Sequence-Selective DNA Cleavage by a Chimeric Metallopeptide

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Abstract: A chimeric metallopeptide derived from the sequences of two structurally superimposable motifs was designed as an artificial nuclease. Both DNA recognition and nuclease activity have been incorporated into a small peptide sequence. P3W, a 33-mer peptide comprising helices α2 and α3 from the engrailed homeodomain and the consensus EF-hand Ca-binding loop binds one equivalent of lanthanides or calcium and folds upon metal binding. The conditional formation constants (in the presence of 50 mM Tris) of P3W for Eu(III) ($K_a = (2.1 \pm 0.1) \times 10^5$ M^{-1}) and Ce(IV) ($K_a = (2.6 \pm 0.1) \times 10^5$ M^{-1}) are typical of isolated EF-hand peptides. Circular dichroism studies show that 1:1 CeP3W is 26% α-helical and EuP3W is up to 40% α-helical in the presence of excess metal. The predicted helicity of the folded peptide based on helix length and end effects is about 50%, showing the metallopeptides are significantly folded. EuP3W has considerably more secondary structure than our previously reported chimeras (Welch, J. T.; Sirish, M.; Lindstrom, K. M.; Franklin, S. J. *Inorg. Chem.* **2001**, *40*, 1982−1984). Eu(III)P3W and Ce(IV)P3W nick supercoiled DNA at pH 6.9, although EuP3W is more active at pH 8. CeP3W cleaves linearized, duplex DNA as well as supercoiled plasmid. The cleavage of a 5′-^{32}P-labeled 121-mer DNA fragment was followed by polyacrylamide gel electrophoresis. The cleavage products are 3′-OPO_3 termini exclusively, suggesting a regioselective or multistep mechanism. In contrast, uncomplexed Ce(IV) and Eu(III) ions produce both 3′-OPO_3 and 3′-OH, and no evidence of 4′-oxidative cleavage termini with either metal. The complementary 3′-^{32}P-labeled oligonucleotide experiment also showed both 5′-OPO_3 and 5′-OH termini were produced by the free ions, whereas CeP3W produces only 5′-OPO_3 termini. In addition to apparent regioselectivity, the metallopeptides cut DNA with modest sequence discrimination, which suggests that the HTH motif binds DNA as a folded domain and thus cleaves selected sequences. The de novo artificial nuclease LnP3W represents the first small, underivatized peptide that is both active as a nuclease and sequence selective.

Introduction

After decades of study, an understanding is emerging of cancer as a disease with its basis in regulatory gene mutations that prevent the correct self-regulation of growth.[1,2] The effect of these mutations might be mitigated if target gene sequences could be selectively cleaved or down-regulated. Recent advances in message profiling are leading to the possibility of identifying a small collection of overexpressed genes in tumerous tissues as targets for therapeutic down-regulation.[3] However, current small-molecule chemotherapy agents exhibit only subtle sequence preference based on shape complementarity and charge. These anticancer drugs are highly effective in certain contexts because of their ability to damage cancer cell DNA through covalent modification or double-stranded cleavage. These changes are difficult for the cell to repair, but there is little specificity in the sites targeted. As a consequence, there is substantial damage to nonmalignant cells as well. It is thus of great interest to develop selective chemotherapy agents that can target sequences of choice.

To design a sequence-specific DNA cleavage agent, both DNA-recognition and -reactive elements must be integrated. Transcription factors and repressor proteins are capable of selectively recognizing given duplex DNA sequences but do not cut DNA.[4] In contrast, hydrolytic cleavage of DNA by Lewis acid metal ions and complexes is well-known, but occurs with limited sequence discrimination. Although DNA is a robust molecule that is quite resistant to hydrolysis at neutral pH, lanthanide ions have been shown to be particularly efficient Lewis acids for promoting DNA cleavage.[5−7] We recognized that the supersecondary structure of the helix−turn−helix (HTH), a DNA-binding motif, and the EF-hand, a calcium-binding motif, are superimposable, (Figure 1) and exploited this similarity to design small peptides that bind lanthanides, retain the HTH fold, and cleave DNA.[8−10] These peptides represented the first examples of catalytically active EF-hands. Here we

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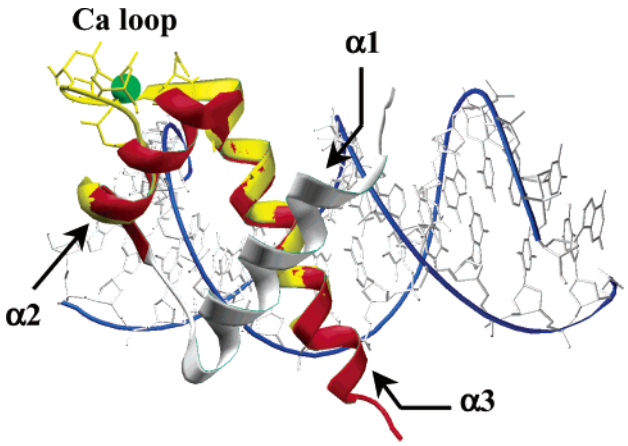

Figure 1. Computer-generated model of the interaction of chimeric peptides with DNA based on the HTH and EF-hand motifs from engrailed (red) and calmodulin (yellow), respectively. Crystal structures of engrailed homeodomain, cocrystallized with DNA (2HDD; gray and red ribbon, with blue DNA)[23] and one EF-hand of calmodulin (1OSA; yellow ribbon, with coordinated Ca(II)),[22] are overlaid to show the similarity of the folds. The peptides comprise α2 and α3 of engrailed and the Ca-binding loop of calmodulin.

report that this cleavage both results in regioselective products and is sequence dependent. Like the 3- and 4-α-helix bundle structures that are the foundation for a diversity of engineered metalloproteins,[11−13] the HTH motif retains a defined and predictable structure. Therefore the HTH is a robust structural scaffold for de novo protein design and the development of minimalist enzyme models. These de novo-designed HTH/EF-hand chimeric peptides are an initial step in the development of targeted DNA-binding metalloproteins as an alternative to antisense therapy or as a tool for manipulation of gene expression.

Materials and Methods

Materials. Chimeric peptides were designed on the basis of alignments of known crystal structures, using the freeware program SwissPDBViewer.[14] The design of the peptide P3 has been discussed in detail elsewhere.[8] Peptides (>95% pure) were obtained from New England Peptide (P3W) or the Caltech Peptide Synthesis Facility (P3). Sample peptide concentrations were based on extinction coefficients calculated from concentrations determined by peptide digestion (University of Iowa Peptide Facility; P3 $\epsilon_{280} = 254\ M^{-1}\ cm^{-1}$; P3W $\epsilon_{280} = 7289\ M^{-1}\ cm^{-1}$). $EuCl_3 \cdot 6H_2O$ and $(NH_4)_2Ce(NO_3)_6$ (anhydrous) were obtained from Sigma-Aldrich, and fresh stock solutions were made by weight before each use. Enzymes and buffers were obtained from New England Biolabs, and all other reagents were obtained from Sigma-Aldrich and used without further purification. All solutions were made with deionized distilled water (MilliQ 18 mΩ).

Plasmid DNA Cleavage. Plasmid pUC19 (New England Biolabs) was exchanged to Tris buffer containing no EDTA by affinity chromatography (Qiagen Mini-prep DNA spin column kit). EuP3W, CaP3W, and CeP3W (1:1) solutions were freshly prepared before use in Tris buffer. Samples containing 1.0 μg of plasmid, 50 mM Tris, pH 6.9 or pH 8.0 (at 37 °C) were incubated at 37 °C for 24 h with Eu(III) or Ce(IV) salts, or 1:1 metallopeptides. Prior to loading on the gel, samples were treated with Na^+-loaded Amberlyst cation-exchange resin to remove cations and peptide.[15] Products were analyzed by agarose gel electrophoresis (1%, in 1× Tris-borate running buffer), stained with ethidium bromide, visualized by UV-transillumination, and photographed with a Kodak system 120 digital camera. Quantified supercoiled band intensities were normalized for weaker ethidium bromide staining by multiplying by 1.22.[16]

Oligonucleotide DNA Cleavage. pBR322 plasmid was cleaved at *BspH*I sites, 5′-^{32}P-labeled with T4 kinase protocol (New England Biolabs) and then further digested with *Ban*I. The 121 bp *BspH*I−*Ban*I oligonucleotide fragment was gel-purified and quantified by scintillation counting of the Cherenkov radiation (≤1 μM). Other pBR322 fragments (not shown) were analyzed in a similar manner. *Eco*R1/*Hae*III digested and phenol-extracted calf thymus DNA was used to raise the DNA concentration to 50 μM basepairs. Maxam−Gilbert sequencing reactions were prepared as size- and sequence markers by standard protocols.[17,18] For 3′-experiments, the 3′-strand of pUC19 restricted with *BspH*I was radiolabeled by incubating Klenow Fragment (3′ → 5′ exo-) DNA polymerase (New England Biolabs) and [^{32}P]-α-dCTP for 15 min in polymerase buffer. The reaction was quenched by adding EDTA and heating at 75° for 20 min. Unincorporated dCTP was removed with a G-50 ProbeQuant (Amersham Biotech) spin column. Subsequent *Ban*I restriction digestion gave the same fragment described above (with the opposite strand labeled).

Samples containing various concentrations of $(NH_4)_2Ce(NO_3)_6$ and peptides (50 mM Tris buffer, adjusted to pH 6.9 at 37 °C) were incubated at 37 °C for 24 h in an insulated heat block prior to analysis by polyacrylamide gel electrophoresis (20% acrylamide, 1× TBE buffer under denaturing conditions), and an equal amount of radioactivity (counts) was loaded for each sample lane. Gels were exposed to the phosphor screens at −4 °C for up to 2 days prior to imaging with a Molecular Dynamics Storm Phosphoimager system. DNase 1[19] and Fe(II)-MPE[20] reactions were prepared by published methods. 3′-Labeled DNA experiments were carried out similarly, incubating $(NH_4)_2Ce(NO_3)_6$ or $EuCl_3$, and P3W (25 mM Tris buffer, adjusted to pH 7.2 at 37 °C) at 37 °C for 24 h prior to electrophoresis.

Circular Dichroism. CD spectra were recorded on an Olis-DSM17 spectrophotometer at 25 °C under N_2 atmosphere. Helical content was calculated from $[\Theta]_{222}$ as described previously.[8,21] Spectra were collected from 350 to 200 nm, with 0.5 point/nm, in a 0.1 cm cell. Samples in 50 mM Tris buffer, pH 6.9 (25 °C) were 50 μM in peptide.

Results and Discussion

Metallopeptide Design. The similarity in supersecondary structure of two physiologically unrelated motifs was utilized to design both activity and recognition into a single peptide domain. The HTH and EF-hand motifs consist of two helices at approximate right angles to one another, stabilized by conserved hydrophobic interactions along the helical inner surfaces. This "α−α corner" topology is a common structural motif in a diversity of proteins,[11,22] including DNA-binding proteins (HTH) and signaling or regulatory proteins (EF-hands). The remarkable similarity of the shape of these unrelated folds allows the turn to be modularly substituted. The metal-binding loop of the EF-hand thus promotes the α−α corner turn within the HTH sequence.

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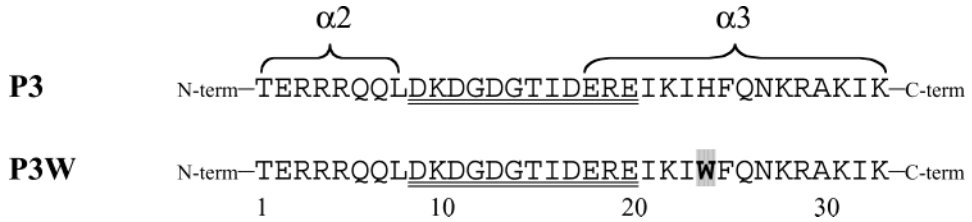

Figure 2. Sequences of P3W and P3, with helices α2 and α3 of engrailed indicated. The EF-hand Ca-binding loop is underlined, and the native Trp_{24} substituted in P3 with His_{24} is shaded.

The 33-mer peptides P3 and P3W were designed on the basis of overlaid crystal structures of the α−α corner motifs from calmodulin (1OSA)[23] and the engrailed homeodomain (1ENH).[24] The structures were oriented to align α−α corner helical axes of the two motifs (Figure 1, red and yellow motifs), from which the sequences of the chimeric peptides were derived. These peptides comprise helices α2 and α3 of engrailed, with the turn region replaced by the consensus Ca-binding EF-hand loop (Figure 2). Important hydrophobic interactions known to stabilize EF-hand structures[25] were retained at key positions before and after the loop (L_8, I_{21}, and W_{24} for peptide P3W), although the residues were derived from the engrailed sequence. The native α3 sequence of P3W preserves the hydrophobic core of the HTH motif and results in a well-structured metallopeptide, whereas P3, which has a Trp_{24} to His_{24} substitution, adopts a less defined tertiary structure.[9]

Solution Structure and Lanthanide Binding. The designed peptides P3 and P3W bind lanthanide ions via the consensus EF-hand site and fold to a nativelike structure. Both peptides P3 and P3W bind lanthanides with micromolar affinities under the sequencing gel electrophoresis conditions. The conditional binding affinity of P3W for Eu(III) and Ce(IV) was determined by fluorescence spectroscopy at pH 6.9, 50 mM Tris, following the intensity of the Trp_{24} emission at 350 nm as described previously.[26] A nonlinear least-squares fit of the intensity data for each metallopeptide supports a 1:1 Ln:peptide associative model. The conditional formation constants for Ce(IV) and Eu(III) with P3W were found to be $K_a = (2.6 \pm 0.1) \times 10^5$ M^{-1} and $K_a = (2.1 \pm 0.1) \times 10^5$ M^{-1}, respectively, which is very similar to the affinity of P3 for Eu(III) ($K_a = (1.2 \pm 0.5) \times 10^5$ M^{-1}) reported previously.[10,27] These values have been corroborated by luminescence titration experiments for EuP3 and EuP3W (W. D. Horrocks, Jr.; S. J. Franklin, unpublished data). As is typical for EF-hand sites, the affinity of P3W for Ca(II) ($K_a = (2.8 \pm 0.7) \times 10^4$ M^{-1}) is less than for the higher valent lanthanide ions.[25,28]

The chimeric metallopeptides exhibit solution behavior characteristic of folded domains. Previously we showed that P3 has little structure in the absence of lanthanides, but increased secondary structure upon metal binding.[8] In contrast, circular dichroism spectroscopy shows that P3W has a modest amount of secondary structure even as the apopeptide and folds further to a flexible, but more organized tertiary structure upon metal binding (Figure 3). The favorable impact of Trp_{24} (P3W) vs His_{24} (P3) on peptide stability and structure is noteworthy, and highlights the importance of hydrophobic side chains along the inner surfaces of the α−α corner motif in generating a structured domain. The shape and magnitude of the circular dichroism signals are typical of the increasing secondary structure changes upon metal binding observed for peptides derived from EF-hands.[29,30] Additionally, the CD spectrum of wild-type engrailed has the same relative CD band intensities, with a minimum at 208 nm and a weaker shoulder at 225 nm.[31] Thus, the CD signals of the metallopeptides are consistent with the spectra of the α−α corner parental motifs, and LnP3W is more folded than LnP3.

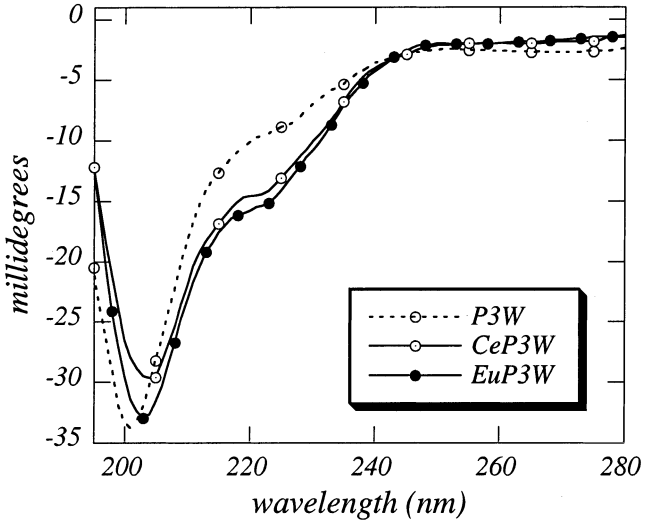

Figure 3. Circular dichroism spectra of peptide P3W (50 μM) in the presence of 3 equivalents of $EuCl_3$ or $(NH_4)_2Ce(NO_3)_6$. Spectra were collected on an Olis/DSM17 Spectrophotometer, at 25 °C and pH 6.9, in 50 mM Tris buffer, in a 0.1-mm cell. Under these conditions, estimates of α-helicity based on molar ellipticity $[\Theta]_{222}$ are 18% (P3W), 28% (CeP3W), and 30% (EuP3W).

An estimate of α-helical secondary structure based on molar ellipticity $[\Theta]_{222}$ from CD data[21] indicates that at pH 6.9 the CeP3W metallopeptide is 28% α-helical, and EuP3W is 30% helical (and up to 40% helical in the metal saturated form). These calculated estimates of secondary structure assume that all observed ellipticity at 222 nm is due to α-helical structure and that the fully folded species is 100% helical. Because this is not the case for either the EF-hand motif or HTH motif on which the peptide is based, the maximum amount of α-helicity predicted for a rigid fold would be 73% (24 of 33 residues occur in a putative helix). Further corrections based on the impact of helical length and end effects result in a predicted helicity of

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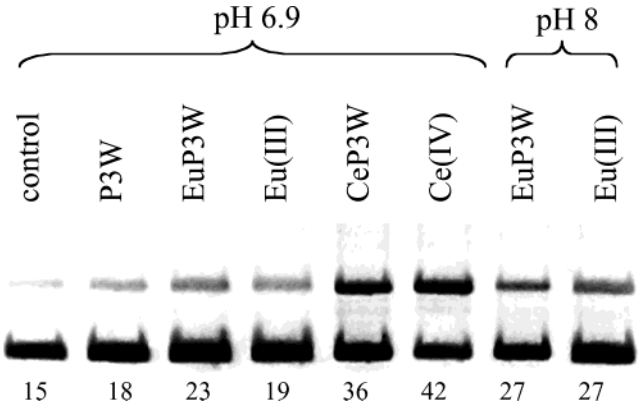

Figure 4. Agarose gel electrophoresis of supercoiled pUC19 plasmid DNA treated with peptide P3W and europium or cerium (50 mM Tris, pH as indicated). Lane 1: pUC19 plasmid control. Lane 2: 10 μM P3W (free). Lane 3: 10 μM EuP3W (1:1). Lane 4: 10 μM $EuCl_3$. Lane 5: 10 μM CeP3W (1:1). Lane 6: 10 μM $(NH_4)_2Ce(NO_3)_6$. Lane 7: 10 μM EuP3W (1:1). Lane 8: 10 μM $EuCl_3$. The open circular (upper) band is quantified below as a percentage of total DNA for this representative gel ($\pm$4% between repeated gels). The control lane at pH 8.0 (not shown) has the same background damage within $\pm$1% of shown.

approximately 50% for a (time-averaged) fully folded metallopeptide.[32,33] As a comparison, the folded, wild-type engrailed homeodomain is reported to be 60% helical by CD spectroscopy.[31] Notably, an NMR solution structure model based on chemical shift index and NOE constraints has shown that the LaP3W complex retains the helix−loop−helix structure of both parent motifs.[9] These data suggest that the P3W metallopeptides are significantly folded and the chimeras can interact with DNA as a domain with tertiary structure.

DNA Cleavage Produces Phosphate Termini. The Eu(III) and Ce(IV) complexes of P3W catalyze the cleavage of supercoiled DNA, producing primarily single cuts (open circular product) and demonstrating that an exposed EF-hand is catalytically competent. As observed for EuP3,[10] in repeated gels EuP3W nicks supercoiled DNA slightly more efficiently than the uncomplexed Eu(III) ions (Figure 4, lanes 3 and 4, respectively), although EuP3W is more active at pH near 8 (lane 7). Both CeP3W and free Ce(IV) ions (lanes 5 and 6) are more active than the Eu(III) catalysts, even near neutral pH. This is consistent with the Lewis acidity, and therefore nucleophilic activity predicted based on the pK_a of inner sphere water molecules for each complex.[5,34,35]

The greater reactivity of Ce(IV) chimeras toward supercoiled DNA prompted us to focus on these complexes initially for sequencing reactions. ^{32}P-labeled DNA oligonucleotides (83−153 bp fragments) were incubated with $(NH_4)_2Ce(NO_3)_6$ and varying concentrations of peptides P3 or P3W at 37 °C for 24 h and analyzed by polyacrylamide gel electrophoresis. The strain inherent in supercoiled plasmid is not required to achieve cleavage, as we observe cutting of linearized ^{32}P-labeled DNA oligonucleotides as well. Although the Eu(III) experiments gave less product, the hydrolytic mechanism of cleavage is well established[6,7,36,37] and gives a nice reference to compare the more active Ce(IV) catalysts. The Eu(III) and Ce(IV) metallopeptides have essentially identical product profiles in the gels, although Eu(III) cleavage is much weaker. We find the cleavage gives single products (phosphates), likely occurring via a hydrolytic rather than an oxidative mechanism, and that peptide−DNA binding protects or suppresses cleavage by free metal.

The hydrolytic mechanism of DNA cleavage by Ce(IV) salts has recently been demonstrated by several groups.[35,38−41] Komiyama and co-workers showed that aqueous Ce(IV) salts hydrolytically cleave deoxyribodinucleotides, by analyzing the products by HPLC. They further demonstrated that DNA oligonucleotides were also hydrolytically cleaved with no dependence on molecular oxygen, generating termini that could be enzymatically manipulated. The cleavage was determined to be catalyzed by tetravalent, and not trivalent cerium. This analysis was elaborated by Branum and Que,[38,40] who demonstrated that ligand chelates of Ce(IV), as well as aqueous Ce(IV) salts, cleave DNA via a hydrolytic rather than oxidative mechanism, generating only hydroxyl and phosphate termini.

Hydrolysis by free Ce(IV) ion[42] produces both 3′- and 5′-phosphate termini, as there is no stereoselectivity in the hydrolysis mechanism. For the 5′-^{32}P-labeled fragment shown in Figure 5, two bands per base are generated by Ce(IV) ions (lane 6) under our experimental conditions. As observed by Que and by Komiyama, the resultant termini comigrate with the Maxam−Gilbert fragments (lanes 1−3; p = 3′-OPO_3) and DNase I fragments (lane 4; h = 3′-OH).[43] Both 3′-products are readily observed, although the bands due to 3′-OH termini are more intense. Oxidative cleavage by Fe(II) methidiumpropyl-EDTA (MPE) and peroxide generates both 3′-OPO_3 and 3′-phosphoglycolate termini (and corresponding 5′-products) (lane 5, g = 3′-phosphoglycolate).[44] One band (p), but not both, of the free Ce(IV) products comigrates with these 4′-oxidative products, which confirms the 3′-OPO_3 band assignment and the non-oxidative mechanism of uncomplexed Ce(IV) ion cleavage.

We have also investigated the 5′-termini products by radiolabeling the 3′-terminus of the same region (opposite strand; Supporting Information Figure S2). In this case, Maxam−Gilbert, DNaseI, and oxidative Fe-MPE/H_2O_2 cleavage all produce 5′-OPO_3 termini (p). Ce(IV) alone again leaves 5′-OPO_3 (p; major band) and 5′-OH (h; minor band), which complements both the observed termini and their intensities across the cut (Figure 5). Eu(III), which is well-known to cleave only by hydrolysis, gives the same OPO_3 and OH products at both 5′- and 3′-termini as observed for Ce(IV). This again corroborates the hydrolytic mechanism of uncomplexed Ce(IV) DNA cleavage.

In the presence of CeP3W and CeP3, only a single product is observed at each cleaved base step (Figure 5, lane 7). The products of chimera-catalyzed cleavage comigrate with the bands in Maxam−Gilbert sequencing lanes (5′-labeled DNA), which

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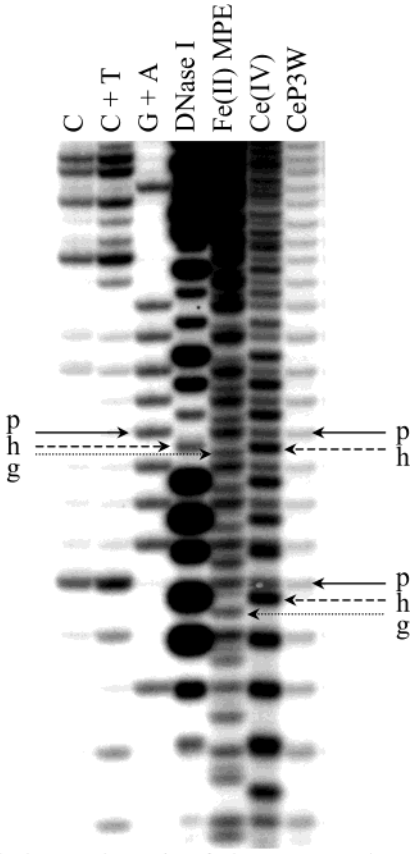

Figure 5. Polyacrylamide gel electrophoresis of cleavage products of a 5′-end-labled 121 bp DNA oligonucleotide fragment. Cleavage by Ce(IV) ions produces only the hydrolytic products 3′-OPO_3 and 3′-OH. The chimera CeP3W cleaves DNA, producing only 3′-OPO_3 termini. Lanes 1−3: Maxam−Gilbert sequencing lanes (p = 3′-OPO_3 termini). Lane 4: DNase I treated DNA (h = 3′-OH termini). Lane 5: Fe(II)MPE treated DNA (p = 3′-OPO_3 and g = 3′-phosphoglycolate termini). Lane 6: DNA incubated at 37 °C, pH 6.9 for 24 h with 30 μM $(NH_4)_2Ce(NO_3)_6$. Lane 7: DNA incubated at 37 °C, pH 6.9 for 24 h with 30 μM CeP3W.

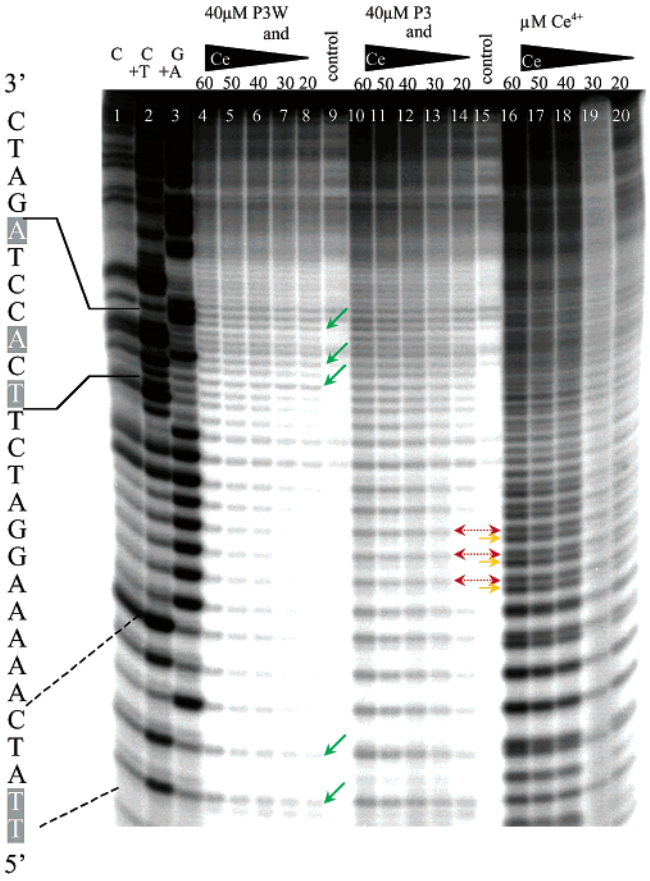

Figure 6. Polyacrylamide gel electrophoresis: the effect of metal−peptide concentrations on DNA cleavage. ^{32}P-Labeled oligonucleotides were incubated with 40 μM of peptides P3 or P3W and increasing concentrations of $(NH_4)_2Ce(NO_3)_6$ (from 20 to 60 μM, as indicated) at 37 °C for 24 h, pH = 6.9, 50 mM Tris buffer. Samples with no peptide or metal served as controls, and Maxam−Gilbert sequencing reactions as sequence markers. Bands that are notably enhanced over controls are indicated with green arrows for P3W lanes. Horizontal red hashed and orange solid arrows indicated 3′-phosphate and 3′-hydroxyl termini, respectively. Both bands were observed in the metal-only lanes, but only 3′-phosphate products were observed in the peptide-containing lanes.

suggests the metallopeptides are generating 3′-OPO_3 termini exclusively. In contrast, Que's bimetallic polyaminocarboxylate nuclease generated exclusively 3′-OH termini.[45] However, the complementary cleavage product (3′-labeled DNA) shows that Ce(IV)P3W also produces only 5′-OPO_3 termini, instead of the expected 5′-OH, implying the loss of the intervening sugar moiety (by either hydrolysis or oxidative cleavage), rather than a simple single-strand cut. It is possible that the peptide has a relatively long residence time on the DNA, allowing a second *exo*-nuclease step, once the initial *endo*-nuclease cleavage has been accomplished. Investigations to determine the reason for the unusual phosphate termini produced under various conditions are ongoing.

Peptide binding apparently also protects the DNA from further random cleavage by excess free metal in analogy to footprinting studies, although at higher metal:peptide ratios (≥4:1), the double band cleavage pattern generated by uncomplexed Ce(IV) hydrolysis is again observed. These random interactions are likely enhanced by the low-salt conditions, chosen to maximize DNA−peptide interactions, and prevent competition by Ca(II) or Mg(II) for the peptide.[46]

Sequence-Selective DNA Cleavage. In addition to single OPO_3 products, the chimeric peptides direct sequence-dependent cleavage. As shown in Figure 6, CeP3W-catalyzed cutting is not sequence-neutral, but rather selected sequences are preferentially cleaved (arrows). This nonrandom cleavage pattern suggests that the chimera occupies a set of higher affinity sites for greater average residence times, resulting in more damage at those positions. Sequence discrimination is observed to a lesser extent for CeP3 (Figure 6, lanes 10−14). CeP3 lacks important internal hydrophobic contacts (from Trp_{24}) that stabilize the HTH structure and is thus a less organized domain than CeP3W.[9]

These cleavage patterns are consistent with preferential binding by a folded domain at selected sites, such as those indicated by the arrows and shaded sequences in Figure 6. On this particular fragment, cleavage at the 5′-thymidine of the 5′-TCACCT-3′ sequence (black arrow) is notably more intense.

(45) Because the products have 3′-OPO_3 termini, present in both oxidative and hydrolytic reactions, it cannot be ruled out that the cleavage mechanism by Ce(IV) in the presence of peptide is oxidative, although cleavage by the free Ce(IV) ion and by Eu(III) is not. Furthermore, the analysis of the EuP3W termini is complicated by the relatively weak cleavage compared to Ce(IV). Consequently, cleavage intensities are barely over background, and although the products of EuP3W hydrolysis appear to be OPO_3 in both directions (paralleling the Ce(IV) results), the data are inconclusive.

(46) No change in background occurs during the course of the reaction, as 0 h and 24 h control lanes look identical.

However, the length and sequence of the global consensus recognition site remains to be determined.

It is notable that a very different apparent sequence dependence by EuP3W can be imposed than the T/C-rich sequence targeted under neutral cleavage conditions. Incubating the reactants under slightly acidic conditions ($< pH = 6.5$) partially depurinates the DNA. The metallopeptides (both EuP3W and CeP3W) then target and preferentially hydrolyze these abasic A and G steps (again leaving OPO_3 termini; see Supporting Information Figure S3).

The P3W chimera represents a "half domain" relative to the parental engrailed DNA-binding homeodomain, as the N-terminal tail and first helix have been omitted (Figure 1, gray ribbon). Nonetheless, the peptide is capable of DNA-binding with sequence discrimination, although the recognition site is not necessarily expected to be that of the parent. Engrailed recognizes the 6-bp sequence 5′-TAATTA-3′ as a monomer, with strong binding affinity (less than 1 nM).[31] The consensus general homeodomain binding site is of the form 5′-TAATNNN-3′, comprising 6−7 base pairs, and in many cases encompasses a "core" 5′-TAAT-3′ sequence.[47] Homeodomains typically interact with DNA via base contacts to both the major groove (the recognition helix, α3) and the minor groove (N-terminal tail and α1), along with phosphate backbone contacts from both α2 and α3 (Figure 1). The recognition helix contacts the major groove within the 3′-region of the recognized sequence (5′-TAATNN-3′), and the N-terminal tail contacts the minor groove along the 5′ region of the sequence (5′-TAATNN-3′).[47] In the native engrailed homeodomain, α2 is on the "backside" of the domain, and does not make direct base contacts. In contrast, our EF-hand/HTH chimeric peptides do not include the N-terminal tail and α1 (Figure 1, gray helix), so specific interactions with the 5′-TAA sequence of the engrailed recognition site are less likely. However, the engrailed homeobox target sequence is an overlapping palindrome, so the 3′-region that the recognition helix contacts is 5′-ATTA-3′, the coincidental complement to the "core" sequence. Several fragments of pUC19 were cleaved and labeled near 5′-TAAT-3′ sequences (data not shown), but no preferential cleavage at these sites was observed.

Unsurprisingly, the selectivity of the peptides differs from engrailed; the peptide chimera is sequence *selective*, although not sequence *specific* like the native homeodomain. Without the anchoring tail, α3 may be rotated in major groove, altering the relative orientation of α2 and allowing contacts to bases on the 3′-side of the site that are not accessible to the full domain. However, the metallopeptide appears to be binding to DNA as a structured unit to discriminate among sequences. The influence of DNA flexibility, flanking sequences, and length of the recognition site remains to be understood.

The cleavage rates and efficiency that we observe are typical for molecular hydrolase models.[5−7] The rate of EuP3-catalyzed bis-nitrophenyl phosphate cleavage, a DNA substrate analogue, was found to be 0.1 M^{-1} s^{-1}, which represents a hydrolysis rate enhancement of approximately 10^6 over uncatalyzed cleavage.[10] Although the cleavage rates, and consequently, band intensities are lower than often observed for small-molecule oxidative or photocleavage nucleases,[48,49] variations in band intensities are readily observed at low CeP3W concentrations. These variations cannot be explained by nonspecific binding and cleavage alone. While hydrolysis rates, and thus cleavage products, are enhanced at higher metallopeptide concentrations (above 50 μM peptide), competitive, nonspecific DNA binding becomes a factor, masking specific binding and cleavage.[50] Under such conditions, nonspecific (but still apparently phosphate-directing) cleavage by the metallopeptides results in ladder-like product distributions.

The level of sequence-dependent cleavage promoted by the chimeras is unprecedented in an underivatized peptide of this size. The one earlier report of selective Ln-mediated hydrolysis involved antisense recognition, by a 19-mer DNA oligonucleotide with an appended 5′-iminodiacetate-chelated Ce(IV) ion.[51] Barton and co-workers have also reported examples of small, zinc-binding peptides (16−22 residues) that hydrolyze DNA with sequence discrimination,[52,53] although in that case, the peptide is augmented by an appended Rh-intercalator complex that delivers and orients the peptide within the DNA groove. Additionally there are several oxidatively active examples in which selective damage was conferred by larger protein domains. Isolated DNA-binding domains such as Fos and Sp1 incorporating an amino-terminal NH_2-Xaa-Xaa-His Cu(II) or Ni(II) chelating sequence have been found to bind DNA sequence-selectively, thus generating diffusible radicals adjacent to localized sequence targets.[54−56] Similarly, Sarkar and co-workers reported sequence-dependent oxidative DNA damage by an iron-loaded estrogen receptor DNA-binding domain (84-mer), which comprises two zinc finger motifs per monomer.[57]

Even without reactivity, small peptides that fold and interact with DNA are unusual. There are very few examples of small, well-folded "miniproteins," natural polypeptides 20−42 amino acids in length with defined tertiary structure,[58−63] and even fewer that bind DNA selectively. A notable example is Schepartz's 42-residue avian pancreatic peptide (aPP), which was modified to include leucine zipper basic region DNA contacts.[63] Several designed single zinc finger peptides have also been shown to have affinity for selected RNA sites.[59,64] While these peptides bind strongly and selectively to targets, they were not designed for reactivity. In an alternative approach, Berg and co-workers redesigned a zinc finger peptide (22

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residues) with an open coordination site, which could potentially have hydrolytic nuclease activity.[65] Berg found this minimalist zinc finger folded correctly, though it did not show activity for acetate or aldehyde hydrolysis, and a test of nuclease activity was not reported.

Our de novo designed lanthanide chimera represents the first underivatized small peptide that is a sequence-selective artificial nuclease. The chimeras illustrate how a robust fold can be redesigned to incorporate catalytic activity into a minimalist recognition motif. The recognition helix of the HTH motif delivers a Lewis acidic ion (Eu(III) or Ce(IV)) to the DNA backbone for cleavage with modest sequence discrimination. Whether our chimeric peptides have specific targets, for which they exhibit higher selectivity and affinity, is under investigation. The combination of folding, DNA binding, and sequence-dependent cleavage makes chimeras based on the HTH scaffold a potential foundation for de novo nuclease design, perhaps leading to new clinical and biochemical tools.

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Supporting Information Available: Polyacrylamide gel electrophoresis: DNA cleavage by 1:1 CeP3W (25−45 μM); 5′- and 3′-^{32}P-radiolabeled DNA cleavage by CeP3W and EuP3W; EuP3W cleavage products of DNA under acidic conditions (depurinated DNA) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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