De Novo Nucleases Based on HTH and EF-Hand Chimeras

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Received January 19, 2001

Small molecules incorporating lanthanide ions have been known since the early 1990s to hydrolytically cleave DNA.[1] However, it remains of great interest to design synthetic nucleases with the potential to cleave sequences of choice. Artificial enzymes, which could target a single promoter region in the genome, would have both biochemical utility and pharmaceutical applications as cleavage agents.[2] Toward this end, we have taken an approach to synthetic nuclease design that could exploit the exquisite specificity achieved by DNA binding proteins to deliver a hydrolytic metal to a given sequence for selective cleavage. We report here a metal-binding peptide that exhibits hydrolytic nuclease activity, demonstrating the feasibility of employing an EF-hand motif to facilitate Lewis acid catalysis.

Several research groups have been successful in de novo metalloprotein design, incorporating new metal-binding functionality into known protein scaffolds.[3] Our design approach utilizes a known turn in a new context. We have at our disposal a biological motif with nearly idealized lanthanide-binding properties, representing a defined unit that promotes the supersecondary turn structure and serves as a hydrolytic active site. Our chimeric motif comprises a transcription factor DNA-binding domain and the topologically equivalent Ca-binding EF-hand motif. The remarkable similarity of the helix orientation in these two unrelated protein turns has been used to design a peptide system with the DNA-binding and metal-binding legacies of the parent structures. We have found that these hybrid peptides bind lanthanide ions, have metal-dependent structure, and catalyze phosphate hydrolysis of both activated phosphate esters and supercoiled duplex DNA.

Chimeric Design. The design of the chimeric 33- and 34-residue peptides was based on overlays of Engrailed and Calmodulin crystal structures (Figure 1). The HTH and EF-hand motifs consist of two helices at approximate right angles to one another and thus exhibit a similar turn topology. Our design incorporates helices from the DNA-binding protein and the metal-binding turn from the Ca protein. P3 comprises α2 and α3 of Engrailed and the consensus EF-hand sequence, whereas P4a comprises α2 and α3 of Engrailed, minus the last turn(s) of α2 and the β-turn, and contains Calmodulin loop III.[4] This latter design incorporates a greater fraction of the EF-hand turn (single underline) than does P3 and results in a shift in register of the Ca-binding loop to the N-terminal side for P4a. The design of P3 has been described in more detail elsewhere.[5]

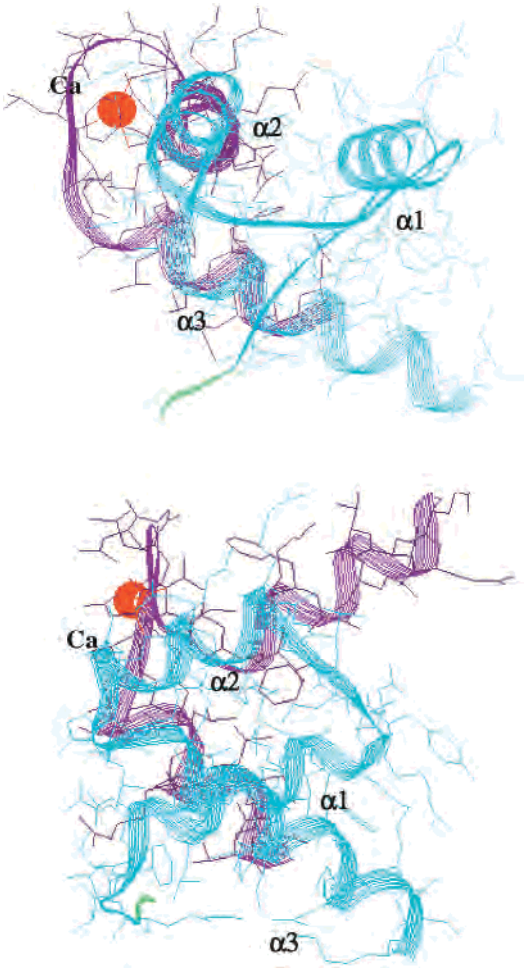

Figure 1. Two views of the overlay of Engrailed HTH region (α2−α3) and one EF-hand of Calmodulin. The C-terminal α3 is the homeodomain recognition helix, which binds in the DNA major grove. Engrailed (1ENH) is shown in blue, Calmodulin (1OSA; third Ca site) in purple, and the Ca(II) ion as a red sphere.

Metal Binding and Solution Structure. The designed chimeras bind metals with an increase in secondary solution structure, as evidenced by CD and NMR titration studies.[5] The binding affinity of P3 for Eu(III) was characterized by isothermal titration microcalorimetry.[6] The dissociation constant for EuP3 was found to be $K_1 = 10 \pm 4\ \mu$M, from which the amount of bound and free Eu(III) in solution was calculated (Table 1). Although there is only one binding site per peptide, the binding behavior of this

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(4) Sequence from rat testes Calmodulin; a small peptide loop of this sequence has been shown to bind Ca(II) and Ln(III) with a nativelike fold (ref 7b).

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(6) A CD titration of P3 with Eu(III) showed more than one equilibrium event, from which estimates of metal binding ($K_1 \leq 20\ \mu$M) and dimerization ($K_{dim(obs)} \approx 70\ \mu$M) equilibria were made. Isothermal titration microcalorimetry of EuP3 (125 μM) gave similar results for K_1 ($K_1 = 10 \pm 4\ \mu$M) and showed another equilibrium at higher metal concentrations. Full thermodynamic characterization of the system is in progress.

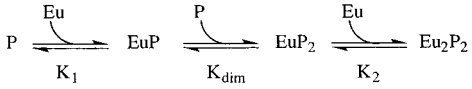
10.1021/ic0155075 CCC: $20.00
Published on Web 03/17/2001

Table 1. Pseudo First-Order Rates of BNPP Cleavage as a Function of Eu−Peptide, at 37 °C[a]

concentration (μM Eu/μM P3)	[Eu_{free}] (calc; μM)	[EuP3] (calc; μM)	[$EuP3_2$] (calc; μM)	rate (k_{obs}) ($s^{-1} \times 10^7$)
10/10	6.2	3.6	0.2	6.0
25/25	11.6	11.6	1.8	7.3
25/50	5.6	14.1	5.3	20.6
50/50	18.9	25.9	5.2	28.8
concentration (μM Eu/μM P4a)	[Eu_{free}]	[EuP4a]	[$EuP4a_2$]	rate (k_{obs}) ($s^{-1} \times 10^7$)
10/10	4.2	5.8		11.3
25/25	7.2	17.8		56.3
25/50	2.4	22.6		62.0
50/50	10.8	39.2		149.5
50/100	2.7	47.3		212.0
concentration (μM $EuCl_3$)	[Eu_{free}]	[EuP]	[EuP_2]	rate (k_{obs}) ($s^{-1} \times 10^7$)
10	10			1.4
20	20			2.7

[a] BNPP concentration = 500 μM; pH = 7.7, 10 mM Tris buffer. Calculated [EuP], [EuP_2], and [Eu_{free}] values are based on the measured dissociation constants for EuP3 (K_1 = 10 μM, $K_{dim(obs)}$ = 70 μM) and EuP4a (K_1 = 3 μM). Error limits: k_{obs} = ± 2%.

chimera is not simply biomolecular.[6] In analogy to isolated EF-hand peptides, which form well-defined back-to-back dimers in solution,[7] EuP3 also dimerizes at higher concentrations, as evidenced by microcalorimetry and CD titration studies.[6,8] In contrast, chimera P4a shows no appreciable dimerization to 300 μM metal−peptide. The binding affinity of P4a for Eu(III) was determined to be $K_1 = 3 \pm 0.3$ μM by fluorescence quenching of W_{25} as a function of added metal. The decrease in fluorescence was well-modeled by a single-exponential assigned to metal binding (K_1), and the inclusion of a second equilibrium step (K_{dim}) did not improve the fit.



Hydrolytic Phosphate Cleavage. Because the EF-hand is physiologically strictly a structural motif, an isolated Ca-binding loop's ability to affect hydrolytic cleavage was addressed. The hydrolysis of bisnitrophenyl phosphate (BNPP) was followed spectrophotometrically under turnover conditions, and absorbance vs time was plotted to give pseudo-first-order rate constants. The absorbance increase at 400 nm due to liberated 4-nitrophenolate was observed over the initial 10−14 h of the reaction (≤10% BNPP converted). No measurable hydrolysis of BNPP was observed in the absence of metal or peptide.[9]

The chimeric metallopeptides catalyze BNPP hydrolysis with rate constants on the order of $k = 10^{-5}$−10^{-6} s^{-1} (Table 1),

P3 x x x x x x
N-term–TERRRQQLDKDGDGTIDERELKIHFQNKRAKIK–C-term

P4a
N-term-TERRRFDKDGNGYISAAELRHVKIWFQNKRAKIK–C-term

Figure 2. Sequence of the chimeric P3 and P4a peptides. Parent protein sequence is indicated by double underlining (homeodomain) or single underlining (EF-hand), and the 12 residues of the Ca-binding loop are shaded. On the basis of the known crystal structures of the parent motifs, predicted sites of Eu^{3+} binding are indicated by an **x**.

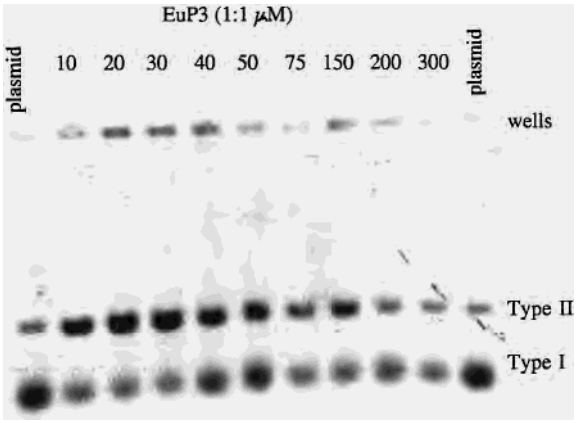


Figure 3. Agarose gel electrophoresis of supercoiled pUC19 plasmid, with increasing concentrations of EuP3 (1:1 ratio).[14]

comparable to rate constants with other Ln catalysts.[10] This represents a rate increase of approximately 10^6 over the uncatalyzed reaction, showing that the metal in the Ca-binding motif is indeed hydrolytically active (at 37 °C, pH = 7, BNPP is estimated to be hydrolyzed at a rate of 6×10^{-11} s^{-1}).[11] The observed phosphate hydrolysis rates show a linear dependence on catalyst concentration at low concentrations, which can be fit to a second-order rate relationship for both EuP3 and EuP4a ($k = 1 \times 10^{-1}$ and $k = 3 \times 10^{-1}$ M^{-1} s^{-1}, respectively). However, the linear relationship breaks down at concentrations greater than 25 μM Eu−peptide (calculated [EuP]), suggesting that at higher metallopeptide concentrations, additional equilibria have a significant effect on the cleavage rates. Notably, the concentrations of free Eu(III) (based on KINSIM[12] calculations employing experimental K_1 dissociation constants) are not sufficient to explain the cutting rates in this regime.

These metallopeptides have affinity for supercoiled, double-stranded DNA.[5] To test their catalytic ability toward DNA as well as model compounds, the conversion of supercoiled plasmid (type I) to open circular (type II), linear (type III), or smaller fragments was monitored by agarose gel electrophoresis.[13] The concentration-dependent formation of open circular plasmid was observed in the presence of EuP3 (Figure 3). Over a 10−300 μM EuP3

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(8) $K_{dim(obs)}$ is the product of K_{dim} and K_2 because the CD titration monitors only changes due to [Eu(III)], not [peptide]. In the EF-hand peptide systems (ref 7), the second metal site in the dimer has low affinity ($K_2 \geq 1$ mM), and thus, free Eu(III), EuP3 monomer, and a singly occupied dimer ($EuP3_2$) are the species predicted to be present at concentrations below 100 μM total peptide.

(9) The cleavage of BNPP alone was found to be $\leq 1 \times 10^{-8}$ s^{-1}, which is taken as the lower error limit on our measurements. Free P3 and P4a peptides exhibit measurable rates under these conditions perhaps because of general base hydrolysis: 10 μM P3, $k_{obs} = 0.7 \times 10^{-7}$ s^{-1}; 10 μM P4a, $k_{obs} = 1.3 \times 10^{-7}$ s^{-1}. Corrections to k_{obs} for free peptide cleavage have not been included in Table 1 but could account for up to 10% of the observed rates.

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(13) Because these chimeric peptides bind strongly to DNA, preventing the observation of products, the peptides were chelated with cation resin prior to electrophoresis. Hettich, R.; Schneider, H.-J. *J. Am. Chem. Soc.* **1997**, *119*, 5638−5647.

(14) EuP3 stock solutions contained 10 mM phosphate buffer. Gel samples contained 1.0 μg plasmid (~50 μM b.p.), 10 mM Tris, pH = 7.5, with increasing concentrations of EuP3 (1:1 ratio). Samples were incubated for 48 h at 37 °C prior to quenching with EDTA. Agarose gels (1%) were run at 70 V for 2 h, 1 × Tris-borate running buffer.

gradient, nicking occurred from 10 to 150 μM, with the greatest amount of cleavage at 30 μM EuP3. Neither $EuCl_3$ nor free P3 promotes appreciable DNA cleavage at these concentrations, though strong cleavage by $EuCl_3$ occurs at ≥ 100 μM (Supporting Information). A truncated EF-hand peptide (20-mer without Engrailed helices) also cleaves supercoiled DNA, though the EF-hand loop has little DNA affinity (Supporting Information). These results suggest that a monomeric EF-hand is catalytically active toward DNA, as it is toward our model system. However, higher concentrations of EuP3 are less effective, suggesting that aggregation to nativelike dimer structures ($EuP3_2$ or Eu_2P3_2) results in a noncatalytic form of the metallopeptide. Additionally, tight peptide−DNA binding may inhibit catalyst release and slow rates.

We have demonstrated that HTH/EF-hand chimeras bind metals, have metal-dependent solution structure, and cleave DNA. These results are a significant step in establishing the feasibility of this novel nuclease design. We are currently investigating the sequence selectivity of this DNA cleavage.

Acknowledgment. The authors thank Profs. Kenneth P. Murphy and Amnon Kohen for invaluable discussions, and the California Institute of Technology Peptide Facility and Anaspec, Inc. for peptide syntheses. We thank Youngbae Kim for experimental assistance. This work was supported by a Department of Education G.A.A.N.N. fellowship (J.T.W.), and the University of Iowa Carver Research Initiative.

Supporting Information Available: Agarose gel electrophoresis showing DNA cleavage by $EuCl_3$, free P3, and EuP3. Agarose gel electrophoresis showing low DNA affinity, but DNA cleavage by a truncated EF-hand peptide (without Engrailed helices). This material is available free of charge via the Internet at http://pubs.acs.org.

IC0155075