CMLS, Cell. Mol. Life Sci. 61 (2004) 2184–2188
1420-682X/04/172184-05
DOI 10.1007/s00018-004-4156-2

CMLS Cellular and Molecular Life Sciences

Visions & Reflections

Lanthanide-binding peptides and the enzymes that Might Have Been

S. Lim and S. J. Franklin*

Department of Chemistry, University of Iowa, Iowa City, Iowa 52242 (USA), Fax: +1 319 335 1270,
e-mail: sonya-franklin@uiowa.edu

Received 10 April 2004; received after revision 17 May 2004; accepted 28 May 2004

Abstract. The trivalent lanthanide ions are chemically similar to Ca(II) ions, making them useful Ca analogs for a multitude of applications. In addition, Ln(III) ions are efficient catalysts of hydrolysis due to their much stronger Lewis acidity relative to Ca(II) ions. Ln-binding peptides thus offer both the opportunity to study known Ca sites as well as to explore new biological functions with an entire family of spectroscopically rich and reactive ions. This review discusses Ln-binding peptides in three roles: (i) as models of Ca-protein structure and function, (ii) as spectroscopic tags for protein expression and characterization and (iii) as designed artificial endonucleases. The creation of hydrolytically active Ln peptides that can fold, bind, cleave and discriminate among substrates shows that the design of Ln enzymes can be accomplished, and they will serve as versatile biochemical tools to investigate protein folding, structure and nuclease function.

Key words. Lanthanides; rare earths; de novo design.

The ubiquitous nature of calcium and zinc in living systems speaks to the diverse functional utility of structural and hydrolytic metal sites in biology. It is thus perhaps surprising that the rare earth elements are not exploited biologically for similar structural and Lewis acid roles. The striking similarity of lanthanide(III) ions to calcium(II) ions in size, coordination environment and ligand preferences, along with strong Lewis acidity, suggests that this crossover could reasonably be made. Although not as rare as their moniker would imply, the low biological availability of the lanthanides no doubt precluded this evolutionary outcome. However, this presents an exciting opportunity for chemists to explore the properties, structure, selectivity and reactivity of Ln's in Ca binding sites, and to create the metalloenzymes that Might Have Been.

The trivalent Ln ions have an array of useful chemical and spectroscopic properties that include luminescence, paramagnetism (for all but La and Lu) and hydrolytic activity (due to strong Lewis acidity). These characteristics make them valuable probes to study structure, function and dynamic interactions in biomolecules. The similarity of Ln(III) chemistry to that of Ca(II) offers a spectroscopically rich Ca surrogate to enhance our understanding of naturally occurring Ca-binding structural sites, as well as illuminate the mechanisms of Lewis acid-activated hydrolytic enzymes [1–3]. There is an extensive body of work utilizing Ln(III)-substituted Ca proteins to define both local and long-range structure in signaling proteins. Additionally, there are a large number of Ln-containing small molecule coordination complexes which have been utilized as chiral shift agents and probes of biological structure (for recent reviews, see [4–6]). Here we will focus on peptide-based ligand systems as probes of structure and function, as well as the implications for de novo designed Ln metalloenzymes.

* Corresponding author.

Ln peptides as models for Ca proteins

As a complement to the direct interrogation of native Ca-binding proteins, synthetic Ln peptides from putative Ca binding sites have been important spectroscopic tools to investigate metal binding and structure for more than 20 years [7–13]. The amino acid sequences of Ln-binding peptides have primarily originated from EF-hand Ca^{2+}-binding loops (though there are exceptions) [9, 14], in which the ligands occur within a short, continuous amino acid sequence. Ln-binding peptides derived from EF-hand Ca-binding loops of calmodulin have given insight into the local structure of this motif, including secondary structure stabilization and self-association. The higher at charge-to-size ratio of the Ln(III) ions relative to Ca(II) often results in well-folded peptide motifs, even when the Ca peptide is too flexible to characterize. The relative affinities of the Ln peptides have thus helped to define the importance of charge and flexibility of specific residues in modulating affinities, metal exchange kinetics and motif dimerization. In one recent example, Bemquerer and co-workers reported Eu(III) and Tb(III) luminescence studies on synthetic peptides from plant thions. These peptides were utilized to estimate and interpret the protein binding constants and to study the chemical environment around the metal ions [9]. Earlier, Bierzynski and co-workers used the higher affinities of Ln(III) ions to enhance folding in metallated EF-hand peptides as a means to study α-helix nucleation and helix propagation [10]. They found that the C-terminal three residues of the loop are organized upon metal binding to initiate the first turn of an α-helix, which nucleates further helix structure formation. Bierzynski and co-workers also investigated metal-induced dimerization of EF-hand peptides using ^{1}H NMR and fluorescence spectroscopies [11]. In that study, the authors reported that upon Ln-ion saturation, isolated EF-hand loops dimerize in a manner analogous to the ubiquitous EF-hand pairs observed in proteins. The versatility and stability of the Ln-peptide complexes suggests these types of systems will continue to be fruitful models for Ca-binding proteins.

Ln peptides as spectroscopic and magnetic tags

The rich spectroscopic and magnetic repertoire of the lanthanides provide unique opportunities for metallopeptide complexes (both free and appended to macromolecules) in various biochemical applications. Ln peptides have shown utility in measuring nuclear magnetic resonance (NMR) coupling constants, as fluorescent tags for protein expression, and offer new directions in magnetic resonance imaging (MRI) contrast agents.

The electronic properties of Ln(III) have been utilized in a number of peptide-based Ln-binding tags. The bright fluorescence emission of Tb(III) has been exploited to develop Ln-binding tags as a sensitive detection method for coexpressed protein or for defining internal protein distances. Kaback and co-workers reported such a tag and its use in distance determination by Tb $\rightarrow$ flurophore energy transfer in the transmembrane protein LacY [15]. Unlike earlier studies where both Tb chelators and flurophores were chemically appended to given Cys residues, here only one type of Cys label is required, resulting in much greater sample homogeneity. These studies showed the Tb-binding tag had no adverse impact on the protein function, which suggested that luminescence resonance energy transfer (LRET) with an appended Ln tag could be a general approach for studying distance and structural changes in membrane proteins.

Recently, Imperiali and co-workers optimized both Tb affinity and fluorescence emission intensity of an EF-hand via a combinatorial peptide synthesis and selection approach [16]. The optimized peptide tag binds Tb(III) with low nanomolar affinity, and has a long emission lifetime due in part to the exclusion of inner-sphere water molecules. The intensity of the emission is significantly enhanced by energy transfer from Trp and Tyr fluorescent donor groups in the loop [17]. Thus, the coexpressed Ln peptide renders the protein of interest easily detectable during purification [18]. Additionally, the Ln-peptides can facilitate NMR structure determination. Because of the large anisotropic magnetic susceptibility of most of the lanthanides, these ions are strongly affected by an external magnetic field. Therefore, Ln complexes tend to align in the magnetic field of an NMR instrument, which can be exploited to orient macromolecules to enhance resolution and information content of spectra. Imperiali and co-workers have demonstrated this principle with ubiquitin coexpressed with a Ln-binding tag. Residual dipolar coupling constants were measured for the aligned proteins, which were then used as constraints in determining tertiary structure [19].

Another application of Ln-peptide magnetic properties is in MRI. Our designed EF-hand peptides (vida infra) were found to be very efficient MRI contrast agents as the Gd(III) adducts [20]. In collaboration with Caravan and co-workers we found that a Gd(III) bound 33-mer peptide had a relaxivity of 21.2 mM^{-1} s^{-1} at 60 MHz (37 °C), and an unusual field dependence that maximized relaxivity at higher field. Furthermore, the relaxivity was enhanced 100% upon DNA binding. Although the affinity for Gd(III) was too low for biological applications, the Gd(III) bound peptides were shown to be more efficient relaxation agents than currently utilized small molecule complexes, and suggested a new embedded chelator approach to designing 'switchable' contrast agents based on peptides or proteins.

Ln peptides as designed metalloenzymes

In addition to tools to probe Ca structural sites, the Ln ions are efficient Lewis acid catalysts of hydrolysis, which drove our interest in engineering a peptide-based Ln-metalloenzyme. We chose the design of an artificial endonuclease as our target system, as a foil against which to test whether Ln-polypeptides could fold, bind, cleave and discriminate among substrates like a true enzyme. Additionally, the ability to tune DNA binding and cleavage targets will allow a systematic look at nuclease activity generally, which is still incompletely understood.

In recent years, hydrolytic cleavage of phosphate diesters and DNA by Ln complexes has received considerable attention [21, 22]. The efficient hydrolytic cleavage of DNA by Ln complexes is a result of fast ligand exchange rates ($\sim 10^8$ s^{-1}) and high Lewis acidity [23]. Although there has been significant progress in developing small-molecule Ln DNA cleaving agents, the majority of these are not capable of sequence targeting. Therefore, incorporating suitable recognition systems within the hydrolase is an important consideration in developing new site-specific cleavage agents. Peptides are ideal candidates for this goal, as they are small, synthetically accessible and have the functional flexibility of proteins, provided they are well folded. We therefore sought to combine the capacity for specific DNA sequence recognition and calcium binding to generate a Ln peptide metalloenzyme. In this vein, we have developed a series of chimeric Ln peptides with both DNA-binding and hydrolytic activity.

A series of 32–34-mer peptides was designed based on the overlay of engrailed homeodomain and one Ca-binding loop from calmodulin [24]. The similarity of the super-secondary structure allowed us to replace the turn of the classic DNA-binding helix-turn-helix motif (HTH) with the metal-binding turn of the EF-hand as depicted in figure 1, thus retaining both the metal-binding site (sphere) and the DNA-binding interface (zigzag line). The model in figure 2 represents an overlay of engrailed homeodomain and calmodulin crystal structures, and illustrates the strikingly similar topologies guiding the design (Protein Data Bank: 3HDD and 1EXR) [25, 26].

The designed chimeric peptides were found to bind one equivalent of metal ion and to fold upon binding. The affinities of the peptides for Ca(II) and Ln(III) ions were measured by fluorescence spectroscopy, following the subtle environmental changes at the Trp residue upon metal binding [24, 27]. The chimeras' affinities for Ln(III) are similar to those of isolated EF-hand peptides (10^{-5}–10^{-6} M), with Ca(II) affinities 10–100 fold weaker as expected.

The structures of the chimeras were investigated using circular dichroism (CD) and NMR spectroscopy [28]. These studies demonstrated that the designed Ln peptides adopt the same secondary and super-secondary structures as the parental motifs. Upon metal binding, the overall secondary structure content of the peptides was enhanced to varying degrees, and in the best case, resulted in helical content near the maximum values predicted based on the design (~50%). This metal-dependant folding can be explained by maximizing critical interhelical hydrophobic interactions upon organizing the metal-binding turn, as well as the formation of an α-helix nucleation site at the C-terminal end of the metal sites as observed for native EF-hand peptides. The solution structure of one metal-bound chimeric peptide (LaP3W, where P3W is a 33-mer peptide of the design depicted in figure 1) was elucidated using NMR spectroscopy and found to be qualitatively similar to the parental EF-hand motif [28]. The metal binding site was confirmed by paramagnetic shifts of loop protons in the EuP3W ^{1}H-NMR spectrum. Although the terminal regions of the peptide are conformationally flexible (not unusual for small peptides) and difficult to assign due to peak overlap, an analysis of chemical shifts shows that these regions on average sample helical secondary structure.

In a complementary study, the coordination environment of the Eu binding site was probed directly using luminescence spectroscopy [29]. Eu-titrations confirmed the low micromolar affinities found earlier by fluorescence titrations. The Eu(III) environment was consistent with six oxygen donors from the peptide with two inner-sphere water molecules. Importantly, DNA binding by the metallopeptides did not compromise the metal-binding loop, which maintained only two water ligands when associated with nucleotides. These results suggested that both the overall HTH fold and the native EF-hand metal-binding environment were preserved in the designed peptides. The ability to bind metals and fold into a defined structure is a critical step in our metalloprotein design, but

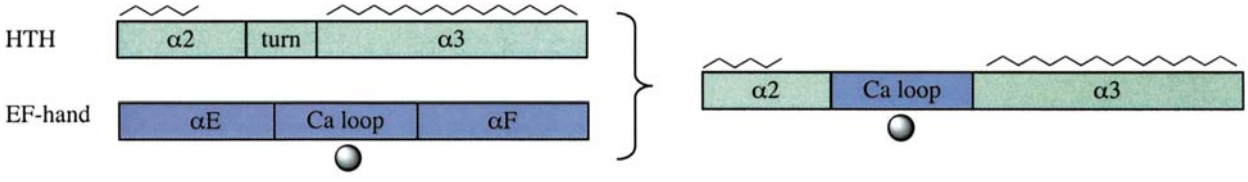

Figure 1. Schematic model of Ln-binding metallopeptide enzymes. The chimeric peptide (left) comprises the helices of the DNA-binding HTH motif (α2, α3), and the turn of the Ca-binding EF-hand motif (Ca loop). The site of Ln binding is indicated by the sphere, and the presumed site of DNA interaction is indicated with the zigzag lines.

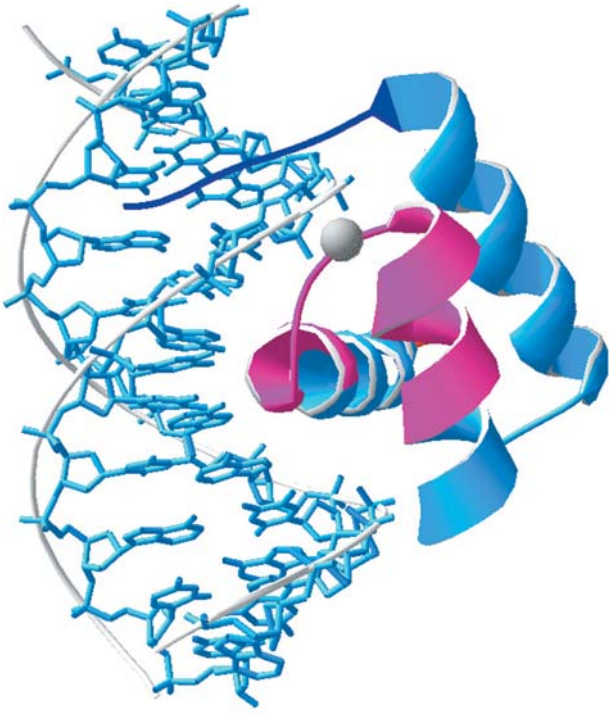

Figure 2. Structural model of the helix-turn-helix/EF-hand chimera, based on overlayed crystal structures of *engrailed* homeodomain (blue; [26]) and *calmodulin* (pink; [25]). The chimeric peptides comprise helices α2 and α3 (in the major groove of DNA), but omit the N-terminal tail (in the minor groove) and α1.

demonstrating DNA binding, nuclease activity and selectivity are additional key objectives. Although the Ca-EF-hand sites of calmodulin are strictly structural sites in vivo, we have found that lanthanide-bound chimeric peptides cleave DNA and phosphate esters. We have shown cleavage of supercoiled plasmid DNA to give open-circular plasmid product [27, 30], as well as investigated hydrolysis rates spectrophotometrically with the model compound bis-nitrophenylphosphate (BNPP). Phosphate hydrolysis is mediated by water molecules coordinated to the metal, and occurs with significant rate enhancements over uncomplexed (or weakly buffer bound) Ln ion. A series of DNA and BNPP cleavage experiments were performed that showed that DNA cleavage rates were dependent on pH, identity of the Ln ion and sequence differences within the metal-bound chimeric peptides themselves [31]. The DNA binding affinities of the designed metallopeptides were also investigated using agarose gel electrophoresis assay, and were typically in 1 ~ 20 µM range both with and without metal ion [30, 32]. Among the most intriguing observations is that DNA binding and cleavage occurred with some degree of sequence specificity [32]. A polyacrylamide gel electrophoresis (PAGE) sequencing gel assay was used to show Ce(IV)-P3W cleaved DNA with modest sequence discrimination, cutting at sites differing from the target sequence of the parental DNA binding domain. Recently the family of DNA sequence targets recognized by the designed peptide was investigated by an electrophoretic mobility shift assay (EMSA), which selects binding sites from a random library of DNA sequences. These data show that the family of 5–6 bp recognition sites were consistent with the cleavage targets, but again differed somewhat from that of homeodomain parent [unpublished results]. Although the designed peptides do not have the N-terminal tail or helix α1 of the native homeodomain that makes important minor groove contacts for sequence recognition and enhanced affinity [33], the metallopeptides still apparently interact with DNA as a defined structural unit. This suggests that the selectivity, activity and affinity of the Ln peptides can be tuned, which could lead to an increased mechanistic understanding of nucleases generally.

Implications and directions

The spectroscopic and magnetic properties of Ln-binding peptides have proven to have wide application as models and tools to study biomolecules. These include structural studies of Ca proteins, spectroscopic and magnetic probes for proteins, and the design of an artificial Ln hydrolase. The design of Ln-binding chimeric endonucleases represents a new combination of reactivity and function, and a versatile system to investigate protein folding, structure and function. Furthermore, our work shows that it is feasible to create the Ln enzymes that Might Have Been, and that they may have long-range potential in biochemical and clinical applications. Although the broad application of Ln peptides to biological problems is still a relatively young field, its rich potential suggests these systems will continue to have significant impact in the coming years.

Acknowledgements. S.J.F. wishes to acknowledge and thank the coworkers who have performed the research on designed Ln metalloenzymes (referenced herein). This work was supported by NSF CAREER Award CHE-0093000.

1 Lanthanides are ideal calcium analogs due to the chemical similarities of the ions, including ionic radii, coordination environments and ligand preferences. The ionic radius of Ca(II) ion is 0.99 Å, and the ionic radii of lanthanides range from 0.86 to 1.22 Å. Coordination numbers of calcium ion vary from 6 to 8, and those of lanthanide ions typically range from 8 to 9. In addition, both calcium and lanthanide ions have a strong preference for hard oxygen donor ligands, and can adopt flexible geometries mainly governed by steric factors. As such, the lanthanide ions can substitute for calcium in proteins with minimal structural changes.

2 Cotton F. A. and Wilkinson G. (1988) Advanced Inorganic Chemistry, Wiley, New York

3 Snyder E. E., Buoscio B. W. and Falke J. J. (1990) Calcium(II) site specificity: effect of size and charge on metal ion binding to and EF-hand-like site. Biochemistry **29:** 3937–3943

4 Tsukube H. and Shinoda S. (2002) Lanthanide complexes in molecular recognition and chirality sensing of biological substrates. Chem. Rev. **102:** 2389–2403
5 Caravan P., Ellison J. J., McMurry T. J. and Lauffer R. B. (1999) Gadolinium(III) chelates as MRI contrast agents: structure, dynamics and applications. Chem. Rev. **99:** 2292–2352
6 Aspinall H. C. (2002) Chiral lanthanide complexes: coordination chemistry and applications. Chem. Rev. **102:** 1807–1850
7 Borin G., Ruzza P., Rossi M., Calderan A., Marchiori F. and Peggion E. (1989) Conformation and ion binding properties of peptides related to calcium binding domain III of bovine brain calmodulin. Biopolymers **28:** 353–369
8 Gariépy J., Sykes B. D. and Hodges R. S. (1983) Lanthanide-induced peptide folding: variations in lanthanide affinity and induced peptide conformation. Biochemistry **22:** 1765–1772
9 Bemquerer M. P., Bloch C., Brito H. F., Teotonio E. E. S. and Miranda M. T. M. (2002) Steady-state luminescence investigation of the binding of Eu(III) and Tb(III) ions with synthetic peptides derived from plant thionins. J. Inorg. Biochem. **91:** 363–370
10 Siedlecka M., Goch G., Ejchart A., Sticht H. and Bierzynski A. (1999) α-Helix nucleation by a calcium-binding peptide loop. Proc. Natl. Acad. Sci. USA **96:** 903–908
11 Wójcik J., Góral J., Pawlowski K. and Bierzynski A. (1997) Isolated calcium-binding loops of EF-hand proteins can dimerize to form a native-like structure. Biochemistry **36:** 680– 687
12 Clark I. D., Brown C. M., Sikorska-Walker M., MacManus J. P. and Szabo A. G. (1993) Self-association of Ca^{2+}-binding peptides induced by lanthanide ions: a fluorescence study. Analy. Biochem. **213:** 296–302
13 Kuhlman B., Boice J. A., Wu W. J., Fairman R. and Raleigh D. P. (1997) Calcium binding peptides from α-lactalbumin: implications for protein folding and stability. Biochemistry **36:** 4607–4615
14 Kohn W. D., Kay C. M., Sykes B. D. and Hodges R. S. (1998) Metal ion induced folding of a de novo designed coiled-coil peptide. J. Am. Chem. Soc. **120:** 1124–1132
15 Vázquez-Ibar J. L., Weinglass A. B. and Kaback H. R. (2002) Engineering a terbium-binding site into an integral membrane protein for luminescence energy transfer. Proc. Natl. Acad. Sci. USA **99:** 3487–3492
16 Nitz M., Franz K. J., Maglathlin R. L. and Imperiali B. (2003) A powerful combinatorial screen to identify high-affinity terbium(III)-binding peptides. ChemBioChem **4:** 272–276
17 Hogue C. W. V., MacManus J. P., Banville D. and Szabo A. G. (1992) Comparison of terbium(III) luminscence enhancement in mutants of EF hand calcium binding proteins. J. Biol. Chem. **267:** 13340–13347
18 Franz K. J., Nitz M. and Imperiali B. (2003) Lanthanide-binding tags as versatile protein coexpression probes. ChemBioChem **4:** 265–271
19 Wöhnert J., Franz K. J., Nitz M., Imperiali B. and Schwalbe H. (2003) Protein alignment by a coexpressed lanthanide-binding tag for the measurement of residual dipolar couplings. J. Am. Chem. Soc. **125:** 13338–13339
20 Caravan P., Greenwood J. M., Welch J. T. and Franklin S. J. (2003) Gadolinium-binding helix-turn-helix peptides: DNA-dependent MRI contrast agents. Chem. Commun. **20:** 2574–2575
21 Franklin S. J. (2001) Lanthanide mediated DNA hydrolysis. Curr. Opin. Chem. Biol. **5:** 201–208
22 Komiyama M. (1999) Hydrolysis of DNA and RNA by lanthanide ions: mechanistic studies leading to new applications. Chem. Commun. **16:** 1443–1451
23 Hegg E. L. and Burstyn J. N. (1998) Toward the development of metal-based synthetic nucleases and peptidases: a rationale and progress report in applying the principles of coordination chemistry. Coord. Chem. Rev. **173:** 133–165
24 Kim Y., Welch J. T., Lindstrom K. M. and Franklin S. J. (2001) Chimeric HTH motifs based on EF-hands. J. Biol. Inorg. Chem. **6:** 173–181
25 Wilson M. A. and Brunger A. T. (2000) The 1.0 Å crystal structure of Ca^{2+}-bound calmodulin: an analysis of disorder and implications for functionally relevant plasticity. J. Mol. Biol. **301:** 1237–1256
26 Fraenkel E., Rould M. A., Chambers K. A. and Pabo C. O. (1998) Engrailed homeodomain-DNA complex at 2.2 Å resolution: a detailed view of the interface and comparison with other engrailed structures. J. Mol. Biol. **284:** 351–361
27 Sirish M. and Franklin S. J. (2002) Hydrolytically active Eu(III) and Ce(IV) EF-hand peptides. J. Inorg. Biochem. **91:** 253–258
28 Welch J. T., Kearney W. R. and Franklin S. J. (2003) Lanthanide-binding HTH peptides: solution structure of a designed metallonuclease. Proc. Natl. Acad. Sci. USA **100:** 3725–3730
29 Jain S., Welch J. T., Horrocks W. DeW. Jr and Franklin S. J. (2003) Europium luminescence of EF-hand HTH chimeras: impact of pH and DNA-binding on europium coordination. Inorg. Chem. **42:** 8098–8104
30 Welch J. T., Sirish M., Lindstrom K. M. and Franklin S. J. (2001) De Novo nucleases based on HTH and EF-hand chimeras. Inorg. Chem. **40:** 1982–1984
31 Kim Y. and Franklin S. J. (2002) pH dependent phosphate hydrolysis by a lanthanide EF-hand peptide. Inorg. Chim. Acta **341:** 107–112
32 Kovacic R. T., Welch J. T. and Franklin S. J. (2003) Sequence preference in DNA cleavage by a chimeric metallopeptide. J. Am. Chem. Soc. **125:** 6656–6662
33 Ades S. E. and Sauer R. T. (1994) Differential DNA-binding specificity of the engrailed homeodomain: the role of residue 50. Biochemistry **33:** 9187–9194