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Joseph A. Cotruvo, Jr.
The Pennsylvania State University

Abstract: Our BES Separation Science program project, DE-SC0021007, supported our efforts to begin to understand the mechanisms underlying selectivity of a novel class of lanthanide-binding proteins discovered by our laboratory, called lanmodulin (LanM), and to leverage these proteins for recovery and separations of trivalent rare earth elements (REEs) as well as of trivalent actinides. Overall, our work provides important insights into how higher-order (e.g., secondary, tertiary, and quaternary) protein structure modulates selectivity profiles of proteins that bind f-elements highly selectively. These results are important for advancing the concept of protein-based separations of REEs and, perhaps, of other critical minerals.

Keywords: Lanmodulin, rare earth elements, protein-based metal separation, second-sphere interactions, metal-templated protein dimerization

Key results

The lanmodulin (LanM) protein from *Methylobacterium extorquens* is the archetypal biological for REEs. It is a small, 12-kDa protein that possesses four EF hand motifs, three of which bind lanthanide ions with picomolar dissociation constants (K_ds) and selectivity over non-REEs approaching trillion fold. Studies with our collaborators have shown it to be an unusually robust protein able to bind REEs even in complex feedstocks. These preliminary results motivated us to probe its selectivity properties further and explore some of the natural diversity of LanMs. There were three major findings of this project. First, our team showed that trivalent actinide ions bind to LanM slightly tighter than REEs of similar ionic radius, and we altered the protein's selectivity for actinides over lanthanides by tuning interactions in the second coordination sphere. Second, we discovered a subset of lanmodulins that dimerize selectively in the presence of certain lanthanide ions. These proteins feature enhanced ability to distinguish between REEs, likely both from dimerization and the architecture of the lanthanide-binding sites. Third, we co-developed a technology for protein-based separations of REEs by immobilizing LanM proteins and developed a facile mechanism of separating Sc^{III} from other REE ions.

Below, we have listed the manuscripts published related to each major finding supported in whole or in part by this project. The inventions and patent applications associated with this work are listed in the invention disclosure.

1. Actinide binding to LanM and second-sphere interactions control actinide/lanthanide selectivity

1. Deblonde, G.J.-P.,* Mattocks, J.A., Wang, H., Gale, E.M., Kersting, A.B., Zavarin, M., **Cotruvo, J.A., Jr.*** Characterization of americium and curium complexes of the protein lanmodulin: a potential macromolecular mechanism for actinide mobility in the environment. *J. Am. Chem. Soc.*, **2021**, *143*, 15769-15783. doi: 10.1021/jacs.1c07103.

2. Deblonde, G.J.-P.,* Mattocks, J.A., Dong, Z., Wooddy, T., **Cotruvo, J.A., Jr.,*** Zavarin, M. Capturing an elusive but critical element: Natural protein enables actinium chemistry. *Sci. Adv.* **2021**, *7*, eabk0273. doi: 10.1126/sciadv.abk0273.

3. Mattocks, J.A., **Cotruvo, J.A., Jr.,*** Deblonde, G.J.-P.* Engineering lanmodulin's selectivity for actinides over lanthanides by controlling solvent coordination and second-sphere interactions. *Chem. Sci.* **2022**, *13*, 6054-6066. doi: 10.1039/D2SC01261H.

4. Deblonde, G.J.-P.,* Morrison, K., Mattocks, J.A., **Cotruvo, J.A. Jr.**, Zavarin, M., Kersting, A.B. Impact of a biological chelator, lanmodulin, on minor actinide aqueous speciation and transport in the environment. *Environ. Sci. Technol.* **2023**, *57*, 20830-20843. doi: 10.1021/acs.est.3c06033.

Our previous work had revealed that lanthanide(III) ions coordinated to *Mex*-LanM (at least Eu and Tb) possessed two coordinated solvent molecules, but we had not yet succeeded in crystallizing LanM to determine the molecular details of the metal-binding sites. Our collaborator, Dr. Gauthier Deblonde (LLNL), and our group demonstrated that LanM binds trivalent actinides Am and Cm with ~5-fold higher affinity than the lanthanides of most similar ionic radius, Nd and Sm.[1] Additionally, our team showed that Ac^{III} binds to LanM tighter than the lanthanide ion of most similar ionic radius, La^{III}, and that the selectivity of LanM for Ac over divalent metal ions was >11 orders of magnitude, allowing Ac-228 to be recovered and purified from minute concentrations present in legacy Th-232 stocks.[2] LanM is also tight enough to increase mobility of trivalent actinides by impacting sorption of these ions to various minerals, indicating that dedicated biological chelators for f-elements need to be considered in risk assessments of radioactively contaminated sites.[4]

We sought to probe the importance of the 9th position Asp residue in each of LanM's EF hands in metal binding, and perhaps increase the actinide selectivity of the protein by installing softer N and S donor atoms at this position. We generated five variants, substituting the aspartate residue at the 9th position of EF1-EF3 with asparagine, histidine, alanine, methionine, and selenomethionine. We carried out spectroscopic measurements with lanthanides (Nd^{III} and Eu^{III}) and actinides ($^{243}Am^{III}$ and $^{248}Cm^{III}$) and found that the two metal-coordinated water molecules are integral to the metal sites and, contrary to the behavior of many small chelators, affinity for f-elements and pH stability of the complexes are compromised by bulky 9th position residues that decrease coordinated water.[3] The results with the $3D_9N$ and $3D_9A$ variants also strongly argued that the native Asp (and presumably also the Asn in $3D_9N$) does not coordinate the metal directly but instead likely hydrogen bonds to coordinated solvent. This observation was later supported by the Nd^{III}-LanM crystal structure, where the 9th position residue also plays an important role hydrogen bonding to the helix exiting the EF-hand. Unexpectedly, we found that the $3D_9N$ variant selectively destabilized the lanthanide complex relative to the actinide complex, roughly doubling the SF for Cm^{III}/Eu^{III} (maximum of 11 vs. 5) compared to the wild-type protein. Therefore, the second-sphere interactions between the residue at this position and coordinated solvent are critical for LanM's selectivity. More broadly, it demonstrates how proteins possess a number of tunable interactions compared to most small-molecule ligands, and these interactions can be leveraged for separations.

2. Dimerization as a mechanism to shift and amplify selectivity profiles

5. Dong, Z., Mattocks, J.A., Deblonde, G.J.-P., Hu, D., Jiao, Y., **Cotruvo, J.A., Jr.,*** Park, D.M.* Bridging hydrometallurgy and biochemistry: A protein-based process for recovery and separation of rare earth elements. *ACS Cent. Sci.* **2021**, *7*, 1798-1808. doi: 10.1021/acscentsci.1c00724.

6. Mattocks, J.A., Jung, J.J., Lin, C.-Y., Dong, Z., Yennawar, N.H., Featherston, E.R., Kang-Yun, C.S., Hamilton, T.A., Park, D.M.,* Boal, A.K.,* **Cotruvo, J.A., Jr.*** Enhanced rare-earth separation with a metal-sensitive lanmodulin dimer. *Nature* **2023**, *618*, 87-93. doi: 10.1038/s41586-023-05945-5.

7. Larrinaga, W.B., **Cotruvo, J.A., Jr.*** A tale of two dimers: lanthanide recognition at biomolecular interfaces. *Trends Biochem. Sci.* **2025**, *50*, 989-1000. doi: 10.1016/j.tibs.2025.08.008. (Invited review)

8. Martin, K.E., Mattocks, J.A., Śmiłowicz, D., Alucio-Sarduy, E., Whetter, J.N., Engle, J.W., **Cotruvo, J.A., Jr.,*** Boros, E.* Radiolabeling and in vivo evaluation of lanmodulin with biomedically relevant lanthanide isotopes. *RSC Chem. Biol.* **2023**, *4*, 414-421. doi: 10.1039/D3CB00020F.

We discovered LanMs that dimerize in a manner sensitive to the ionic radius to the bound REEs.[6] We identified ~700 unique protein sequences that exhibited *Mex*-LanM's trademark 4 EF-hands spaced an unusual 12-13 residues apart, with at least one EF-hand containing a Pro residue at the second position. We sought to demonstrate whether these sequence features were sufficient to define this protein family, so we selected one of the most distantly related proteins in this group (*Hans*-LanM), with only 33% sequence identity to *Mex*-LanM. *Hans*-LanM's first three EF-hands have Asn residues in place of Asp at the first position (N_1) and Glu in place of Asp at the ninth position (E_9), so we expected that the protein's inter-REE selectivity might differ from that of *Mex*-LanM. Whereas *Mex*-LanM is a monomer under all tested conditions, we found that the oligomeric state of *Hans*-LanM is sensitive to the ionic radius of its bound REE, the La^{III}-induced dimer being >100-fold tighter than the Dy^{III}-induced dimer. The apparent affinities of the La^{III} and Dy^{III} complexes with *Hans*-LanM also span an extra order of magnitude compared to *Mex*-LanM.

The x-ray structures of *Hans*-LanM, obtained in collaboration with Prof. Amie Boal's laboratory, with La^{III} and Dy^{III} show how the small difference in radius between these ions is propagated to *Hans*-LanM's quaternary structure: a carboxylate shift in the E_9 residue (Glu91) from bidentate coordination to the larger La^{III} ion to monodentate with Dy^{III} leads to rearrangement of a second-sphere hydrogen-bonding network centered at a key Arg residue (Arg100) on the protein dimer interface. Guided by this structural work, we showed that an R100K variant is substantially weakened even for La^{III}, and the protein's affinity for La^{III} is also weakened. This result established the importance of this Arg residue (conserved in a subset of the LanM family) in protein dimerization. It also showed that an effect of (perhaps, the physiological rationale for) dimerization is to shift the selectivity of the protein from Nd^{III} to lighter lanthanide ions, without needing to substantially alter the selectivity of the EF hands per se.[7]

We had previously developed a cysteine-containing LanM for maleimide-based immobilization and showed that mild chelators like citrate preferentially desorb HREEs over LREEs from LanM to enable REE separations.[5] (A similar construct was used to test the viability of LanM as a chelator for radiolanthanides in vivo, with the help of collaborators in Prof. Eszter Boros' laboratory.[8]) The separations were carried out by our collaborators (Dr. Dan Park, LLNL) using the protein immobilized on agarose beads; 1 mL, ~2 cm columns, pH 5.0, are used for these and all column-based separations reported here for sake of comparison. Distribution coefficients (*D*) and separation factors (SF) are reported for equilibration of a multi-REE solution with the protein; they are intrinsic to the protein ligand itself, with no competing ligand (the SFs reported are not for the process but for the ligand – a longer column will yield higher purities). Immobilized *Mex*-LanM yielded modest SFs [SF(Nd/Dy) = 5.2] which required two consecutive runs of the column with pH-based desorption (HREEs desorb at higher pHs than do LREEs) to separate a 95:5 mixture of Nd:Dy mimicking a magnet waste leachate, to >99% individual element purities. Immobilized *Hans*-LanM(R100K) [SF(Nd/Dy) = 13.6] achieved the same separation to 98% purities in a single column stage, though requiring stepped concentrations of malonate for desorption.[6]

3. Scandium is the tightest binding REE, yet easily desorbed by a chelator

9. Dong, Z., Mattocks, J.A., Seidel, J.A., **Cotruvo, J.A., Jr.,*** Park, D.M.* Protein-based approach for high-purity Sc, Y, and grouped lanthanide separation. *Sep. Purif. Technol.* **2024**, *333*, 125919. doi: 10.1016/j.seppur.2023.125919.

10. Seidel, J.A., Dong, Z., Diep, P., **Cotruvo, J.A., Jr.**, Park, D.M.* EF-hand battle royale: hetero-ion complexation in lanmodulin. *JACS Au* **2024**, *4*, 4273-4284. doi: 10.1021/jacsau.4c00628.

The main focus of the work was on refinement of the *Mex*-LanM column process for separating REEs into 5 groups.[9] However, the contribution that was specifically funded by this project was our first foray into understanding the chemistry of Sc^{III}-bound LanM. Interestingly, we found that solutions of 30 mM malonate desorbed Sc^{III} selectively (>99% purity and >99% yield), without desorption of any other REEs. This spurred us to determine the apparent K_d for Sc^{III}-LanM by CD titration. These experiments yielded $K_{d,app}$ = 7.4 pM at pH 5 (**Figure 6**), which is ~3-fold tighter compared to Nd, demonstrating that LanM forms the strongest complex with Sc among the REEs. We attribute the reversed selectivity effect on-column in the presence of malonate to the greater affinity difference between Sc and other REEs for malonate relative to LanM; the stability constant for the Sc-malonate complex is two orders of magnitude higher than the strongest lanthanide-malonate complex (i.e., Lu-malonate), whereas the Sc-LanM complex is only moderately stronger than LanM complexes with other REEs (e.g., $K_{d,app}$ of 7.4 pM for Sc compared to 59 pM for Ho). Whereas the high Sc selectivity of malonate is a primary driver for the separation effect, it is likely that other effects also contribute. LanM's response to Sc shows a Hill coefficient ~1, suggesting no cooperativity, whereas the protein responds to all lanthanides cooperatively up to at least Er. The lack of cooperativity with Sc suggests a distinct protein conformation; indeed, the magnitude of the CD response is similar to that with Ca^{II} – ~2/3 of the maximal response observed with lanthanides. Further work explored a mathematical model for LanM's cooperative metal ion binding and treated the protein's ability to form complexes with different REE ions at the same time.[10] Additional work addressing the mechanism of folding of LanM will be reported in the future.

4. Advancing methods to study lanthanide-binding proteins

11. Mattocks, J.A., Tirsch, J.L., **Cotruvo, J.A., Jr.*** Determination of affinities of lanthanide-binding proteins using chelator-buffered titrations. *Methods Enzymol.* **2021**, *651*, 23-61. doi: 10.1016/bs.mie.2021.01.044.

This work[11] served the nascent community of lanthanide biochemistry by describing in detail our methods for determining the strength of lanthanide-protein interactions.