CHARACTERIZATION OF NOVEL LANTHANIDE-BINDING PROTEINS

A Thesis in
Chemistry
by
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ABSTRACT

The lanthanides are a critical resource for many growing technologies such as hybrid car batteries, wind turbines, and cell phones. Along with scandium and yttrium, they comprise the “rare earth elements,” many of which, despite their name, are widespread in earth’s crust. The similar chemical properties of these elements create challenges for their economical and environmentally responsible extraction and separation; in order to keep up with technological demand for these elements, greener methods will likely need to be developed in the near future.

The discovery that certain bacteria utilize lanthanides provides a potential starting point for such methods, using molecules that have evolved to bind lanthanides selectively. Recently, our laboratory has discovered and characterized lanmodulin (LanM), a high-affinity and highly selective lanthanide-binding protein from the lanthanide-utilizing bacterium, *Methylorubrum extorquens*. With the goal of better understanding the unprecedented selectivity of this protein for rare earth elements, in this thesis, we 1) investigate three LanM homologs for their metal binding properties and 2) mutate select residues of LanM’s metal-binding sites, using a fluorescent sensor framework for rapid characterization. Our results focus on the unique metal binding characteristics of these LanM homologs and derivatives and the shifts in selectivity and cooperativity that these proteins display. In the future, these findings will be applied toward the implementation of biologically inspired extractions and separations of lanthanides in industry.
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CHAPTER 1 INTRODUCTION

 Portions of this chapter are adapted from my second-year seminar report titled “Bio-inspired extraction of lanthanides”.

 1.1. Introduction

 The rare earth elements (REEs) consist of yttrium and scandium, as well as the lanthanides (Ln), but, despite their name, are relatively abundant in earth’s crust. These elements continue to grow in demand due to their unique electronic and magnetic properties, making them indispensable to many technologies including hybrid car batteries, cell phones, and wind turbines. The challenges in acquiring the REEs lie in their separation from each other as well as their limited solubility, which impacts the economic and environmental sustainability of these extractions. Traditionally, separations of Lns utilize liquid-liquid extraction schemes using various acids and organic solvents which, having a negative environmental and economic impact, motivates exploration of greener methods than the current industrial standard. The primary sources of REEs are in phosphate or carbonate minerals, but they are also present in coal ash and acid mine drainage, although these sources contain high concentrations of competing metal ions. In order to keep up with demand for these indispensable metals, approaches to separations involving small molecules, chromatography methods, and bacterial cells are being explored.

 Recently, REEs have been discovered to be biologically significant, first observed in the characterization of pyrroloquinoline quinone (PQQ) and Ln-dependent methanol dehydrogenase (MDH) XoxF in methylo trophic bacteria. The canonical MDH, MxaFI, contains a CaII ion, a metal of similar ionic radius to the Lns, coordinated to a PQQ in its active site, which facilitates the activation of the PQQ cofactor for hydride transfer from methanol to PQQ. These Ca-dependent systems have been studied extensively for their mechanism and redox characteristics. The discovery of REEs being essential for methanotrophic life in volcanic mudpots showed that
the Ln-dependent MDH, XoxF, was perhaps more prominent in nature than the canonical Ca-dependent enzymes, which launched many studies into the interesting characteristics of these proteins\textsuperscript{19}.

These organisms that are able to utilize and rely upon the early Lns (La-Nd) are of importance to furthering the field of Ln separations. There are a few advantages of utilizing Lns over some canonical metals including availability, high charge-to-radius ratio, and their size compared to Ca\textsuperscript{II}. Studying how these organisms are able to selectively bind Lns over competing metals of similar chemical properties, is key to unlocking their potential for industrial applications to metal separations\textsuperscript{20}. In this work, current methods of aqueous separation as well as experiments attempting use of biological systems for separation will be explained in detail.

1.1.1. **Current industrial and biological separation methods**

1.1.1.1 Industrial hydrometallurgical separation of REEs

Current industrial extraction methods utilize solvent extractions of REEs from aqueous solutions which require harsh chemicals and numerous steps in order to obtain necessary separations of the metals. The inefficiency of these extractions could be ameliorated by molecules that are able to highly selectively bind and release Lns. An example process for the extraction of these metals is outlined in Figure 1.1, detailing the numerous steps and solvents needed for complete separation. This process involves acids and bases such as HNO\textsubscript{3}, H\textsubscript{2}SO\textsubscript{4}, and NH\textsubscript{4}NO\textsubscript{3}, as well as organic extractants and solvents such as Di-(2-ethylhexyl)phosphoric acid (D2EHPA) in Shellsol, which is an organophosphorus acid dissolved in an oil solvent, resulting in a solution that is harmful to humans and the environment, especially aquatic life. With refinement to less harsh extraction methods, labor and chemicals involved could be minimized, thus allowing for the costs associated with extracting, recycling, and utilizing Lns in technology to decrease.
1.1.1.2. Use of LBTs to extract REEs

In the literature, although currently there are many applications of extraction of REEs using biomolecules, but they do not utilize specific Ln-binding interactions, leading to insufficient selectivity. There are, however, a few examples of using biological systems in industry for various metal extractions. Some current industrial processes rely on microorganisms that oxidize sulfidic minerals in the ore which produces acid for dissolution of metal. For example, in the recovery of gold, microorganisms dissolve sulfidic mineral matrices surround the gold, making it easier to target the gold with other leaching agents. However, most REE ores are not rich in sulfur, meaning other protocols must be established in order to rely on biological sources for extraction and separation of these metals.

Recent developments of lanthanide binding tags (LBTs) that have a selectivity and affinity for REEs and metal binding is able to be monitored by luminescence, has allowed bioengineering of a bioadsorbent that can be implemented to bind these metals more selectively than other common transition metals. LBTs are short, EF-hand inspired Ln binding motifs that...
have displayed nanomolar to micromolar affinity for the REEs. With a strategically places Trp
residue, the LBTs are easily assayed for Tb\textsuperscript{III} binding due to its luminescent properties. Although
LBTs had been used for other biological applications including protein purification, biosensing,
and spectroscopy, they had not been studied as a tool for REE extraction of Lns until Park and
coworkers investigated the use of these tags on the S-layer of \textit{Caulobacter crescentus}\textsuperscript{11}. The S-
layer is the outer layer of many archaea and bacteria which is formed by the self-assembly of
monomeric proteins in an array\textsuperscript{22}. \textit{C. crescentus} makes an ideal organism for testing of LBT
attachment due to its S-layer containing multiple copies of \textit{RsaA}, with approximately 40,000
subunits per cell, allowing for a high density of LBT to be introduced via bioengineering\textsuperscript{11}. A
double LBT (dLBT) was inserted into the S-layer gene, \textit{rsaA}, yielding the strain of dLBTx1, and
to further improve REE adsorption, the dLBT copies were increased.

Since Tb is listed as one of the five REEs of highest criticality and beacuse the
aforementioned Trp excitation in the LBT allows for Tb\textsuperscript{III} luminescence, the adsorption of Tb\textsuperscript{III} to
cell surface LBTs was first examined. As previously mentioned, the authors bioengineered
multiple copies of the LBT linked together by a linker protein, and found that the dLBTx4 (four
copies of the LBT held together via linker) variant worked just as well as the dLBTx8 variant.
Therefore, most of their data were collected with the dLBTx4 construct\textsuperscript{11}. Without Ca\textsuperscript{II} present, a
metal of similar ionic radius, the control cells lacking LBTs bound very similar amounts of Tb\textsuperscript{III}
as the LBT cells\textsuperscript{11}. The cell surface possesses multiple functional groups that are able to non-
specifically adsorb REEs. Because the LBT is more selective for Ln binding than the cell surface,
in further experiments, Park and coworkers add Ca\textsuperscript{II} in order to minimize this background binding
of Lns which could help simulate a “true” extraction environment where there are many
interfering metals in the REE ore.
Since low pHs are used for extraction and solubilization of Lns, an important aspect of new methods inspired from biology is pH dependence of the biomolecules. As the pH was decreased to pH 4, the fraction of Tb recovered dropped below 50%, suggesting that this approach perhaps is not viable industrially\textsuperscript{11}. Nonetheless, the authors moved forward with metal selectivity experiments at pH 6 (the pH at which they had observed the highest fraction of Tb\textsuperscript{III} bound) which can be seen in Figure 1.2. The LBT cells showed high selectivity for Lns, with adsorption capacities in the micromolar regime and minimal adsorption of most non-REE metal below 10 mM\textsuperscript{11}. The most significant outlier to this was copper which showed an adsorption capacity of about 100 µM. Although copper was determined to have little effect on overall Tb\textsuperscript{III} binding, this competitive adsorption of abundant non-REEs could alter the economic outcome and practicality of extraction. The authors observed a trend in affinity for the Lns; as radius of the metal decreases, the affinity increases, which matches the previous \textit{in vitro} characterization of the LBT. However, the relatively small increase in affinity will not aid in separation of these metals, but it could be something to explore further down in application of LBTs.

![Figure 1.2. Competition binding experiments with dLBTx4 cells loaded with 10 µM Tb\textsuperscript{III} followed by titration of various metal ions up to 10 mM at pH 6. Reproduced from ref 11.](image-url)
The authors then tested three chelators: citrate, acetate, and gluconate, in order to test the desorption properties of their system. With dLBTx4 cells preloaded with 10 µM Tb\textsuperscript{III}, each chelator was titrated in and tested by luminescence to determine how much Ln was desorbed. Acetate and gluconate, up to 50 mM, were not able to desorb Tb\textsuperscript{III} as efficiently as citrate which gave complete desorption as determined by luminescence and ICP-MS analysis. The authors used acid leachates from soil samples to test their setup on potentially relevant industrial samples by first acid leaching the samples, followed by pH adjustment to 6, and finally adsorbing with dLBTx4 cells. The dLBTx4 cells were able to leach almost all of the REEs in the samples, but the authors did not attempt any subsequent selective desorption across the series. But, concerns of low affinity and the requirement for pH adjustment makes this workability of the LBT in an industrial setting questionable.

A major limitation of the above work is the low Ln binding capacity above background, due to low RsaA levels. Therefore, the relatively abundant surface protein of \textit{E. coli}, OmpA, was used as an anchor protein for the LBTs, as visualized in Figure 1.3. In comparison to \textit{C. crescentus}, \textit{E. coli} displayed a larger difference in Tb adsorbed with the LBT than the control cells, consistent with the increase of 5-fold more OmpA molecules per \textit{E. coli} compared to RsaA molecules per \textit{C. crescentus} as well as the LBT copy number (dLBTx8) being larger in this new system\textsuperscript{12}. The adsorption capacity of 28 mg/g dry cell weight of both of these systems is comparable to other reported adsorbents, suggesting that this could be a useful system for metal extraction compared to currently used methods\textsuperscript{12}. In order to test the efficiency of these LBT systems, the authors performed biosorption assays with leachates of sediment samples from the Bull Hill Mine in Wyoming. These assays were carried out at pH 6, which, as stated before, may not be the most appropriate industrially relevant pH and the solubility of Lns is low at a higher pH.
After the acid leachates were pH adjusted, it was determined that the REEs remained soluble and comprised ~30% by mass of the total metal content. Other metals in the leachate included Mn\textsuperscript{II}, Ba\textsuperscript{II}, Zn\textsuperscript{II}, and Sr\textsuperscript{II} predominately, but Mn comprised 50% of the total mass of metal content. Both the \textit{E. coli} cells expressing OmpA-LBT and \textit{C. crescentus} exhibited similar adsorption rates of ~90% of REEs\textsuperscript{12}. Less than 10% of non-REE cations were adsorbed by these LBT tagged cells, suggesting selective binding by both cell types. Also, the LBT cells for both organisms displayed a larger adsorption of REEs as compared to the control cells, although a higher difference for the \textit{E. coli} cells, meaning that the LBTs bound the metal in a selective manner. Upon addition of citrate, which had previously been shown as an effective desorban\textsuperscript{t} for REEs, the extracted solutions displayed greater than 90% REE content by mass for both LBT and non-LBT cells. \textbf{Figure 1.4} is a graphical representation of these data, showing the adsorbed and desorbed metal totals for each tagged cell type, with their respective controls, suggesting that these systems potentially could be useful in leachate extraction of REEs. The Bull Hill Mine ore represents a high-grade feedstock, containing a high amount of Lns which serves as an ideal

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.3.png}
\caption{dLB Tx8 copies were attached to the cell surface of \textit{E. coli} using an anchor protein. Reproduced from ref 11.}
\end{figure}
system to initially prove the concept of REE extraction using this system. But, ideally, this setup would work with low-grade feedstocks, with REE concentrations an order of magnitude lower and competing metals with concentrations orders of magnitude higher. With low-grade feedstocks, REE adsorption ranged from 42% to 92% for LBT cells compared to control cells (19% to 70%), which suggests that the LBT only slightly contributes to metal binding\textsuperscript{12}. There was minimal adsorption for other competing metals such as Ca\textsuperscript{II}, Ba\textsuperscript{II}, Zn\textsuperscript{II}, Na\textsuperscript{I}, K\textsuperscript{I}, Mn\textsuperscript{II}, and Rb\textsuperscript{I}. One drawback observed while testing these low-grade feedstocks was that Cu\textsuperscript{II} was an effective competitor for REE binding to the LBTs which is consistent with previous work as discussed previously. The authors suggest using a preprocessing step to reduce Cu\textsuperscript{II} levels in source materials with a high Cu\textsuperscript{II} content however, the authors did not test anything of this nature, and they did not comment further on how this process could be completed and how it could impact the application of their \textit{E. coli} system to larger scale processing of REEs. Overall, LBTs have shown some promise in the field of extractions and separations of REEs using biomolecules, but suffer from their requirement for a high pH and unknown performance with multiple cycles of use which was only slightly mentioned by these manuscripts.
1.1.2. *Methylorubrum extorquens* lanmodulin

In order to expand upon these previous attempts at using Ln-binding biomolecules for extraction and separations of Lns, the Cotruvo lan discovered one of the first Ln-binding proteins in methylotrophic bacteria, lanmodulin (LanM), originally found in *Methylorubrum extorquens* (*Mex*) as a protein that copurified with XoxF, the Ln-dependent MDH \(^26\). Because of this copurification and the unique features of its EF hands, LanM was studied further for its Ln-binding properties as it was thought it could have a role in Ln bioavailability in methylotrophic bacteria. *Mex* LanM contains metal coordination motifs, EF hands, typically associated with Ca-
binding proteins, such as calmodulin (CaM), that usually bind with nanomolar to millimolar affinity\textsuperscript{27}. These EF hands possess several unique features relative to the typical Ca binding motifs leading to their unique metal binding properties. First off, Mex LanM contains all typical Asp and Asn metal binding residues, but also features an extra Asp residue in the ninth position of the EF hand. Secondly, three out of four EF hands (EF1-3) contain an Asp residue in the first position, whereas EF4 contains an Asn residue in its first position. Asn is rarely, if ever, observed at the first position in a functional EF hand like it is in EF4 of this protein. Additionally, Mex LanM contains a Pro residue at the second position of each EF hand, a highly unusual feature of EF hands, only encountered in <0.5\% of predicted Ca binding proteins\textsuperscript{26}. Finally, each EF hand of this protein has short 12 or 13 residue sequences between each EF hand, much smaller than the typical 24 or 25 residues of canonical EF hand proteins. These unique EF hand sequences can be seen in Figure 1.5, highlighting the differences between the canonical motifs. All of these observations about EF hands are reflected in the early characterization of Mex LanM\textsuperscript{26,28}.

<table>
<thead>
<tr>
<th>Lanmodulin</th>
<th>Calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1 35          DPKDGTLIDLKE 46</td>
<td>EF1 21        DKGDGTTTTKE 32</td>
</tr>
<tr>
<td>EF2 59          DPKDGTLDAKE 70</td>
<td>EF2 57        DADGNTIDFPE 68</td>
</tr>
<tr>
<td>EF3 84          DPDNGTLDKKK 95</td>
<td>EF3 94        DKDGNYISAAE 105</td>
</tr>
<tr>
<td>EF4 108        NPNDNGTIDARE 119</td>
<td>EF4 130       DIDGDQVNYEE 141</td>
</tr>
</tbody>
</table>

Figure 1.5. Comparison of EF hand residues in LanM and CaM, with metal binding residues in blue and red respectively.
Mex LanM was shown to have millions-fold selectivity for Lns over Ca, the metal counterpart in various other MDHs and EF hand containing proteins, and this amazing selectivity was thought to perhaps be useful in future Ln separations, allowing for extraction of REEs from non-REEs and, hopefully,

separation within the Ln series. Mex LanM is an intrinsically disordered protein in its apo form which is evident by abnormal migration on size-exclusion chromatography (SEC); the protein migrates as if it was a 46 kDa protein rather than the ~12 kDa expected. Because the apo protein spectrum in the far-UV circular dichroism (CD) spectrum is consistent with a structure of little helical content, this migration was attributed to its unfolded state rather than oligomerization. In the holo form, Mex LanM becomes highly ordered, showing alpha helix formation in the far-UV CD spectrum with a ~200% increase in molar ellipticity at 222 nm with up to ~4 equivalents of Lns. However, with addition of up to 8 equivalents of Ca, there is minimal increase in alpha helical character, meaning that Mex LanM undergoes this conformational change selectively in the presence of Lns. To determine the metal-binding stoichiometry, the Ln-responsive dye xylene orange (XO) assay was used as a competitor; the protein competes with the XO for metal binding, until the tight binding sites are all bound, then the XO binds metal and a color change occurs. Since XO binds Lns with micromolar affinity, this assay reports on binding with sites that bind tightly with a greater than micromolar affinity. Ln-XO complex formation is inhibited until ~3 equivalents of Lns are added, suggesting that only three of the four EF hands bind metal tighter than XO.

Due to the aforementioned conformational change, Mex LanM can be studied via CD to investigate Ln binding using metal chelated solutions to buffer the Lns in the proper

<table>
<thead>
<tr>
<th>Metal</th>
<th>$K_{d, app}$ (pM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>La$^{III}$</td>
<td>5.3</td>
<td>4.3</td>
</tr>
<tr>
<td>17</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Nd$^{III}$</td>
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<tr>
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<td>3.0</td>
</tr>
<tr>
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</tr>
<tr>
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<td>3.9</td>
</tr>
<tr>
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<td>2.4</td>
</tr>
<tr>
<td>Y$^{III}$</td>
<td>17</td>
<td>3.4</td>
</tr>
</tbody>
</table>
concentration range\textsuperscript{29}. The chelators used, such as Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and Ethylenediamine-N,N'-disuccinic acid (EDDS) buffer Lns, in the picomolar regime, at which, the full change in ellipticity is observed, indicating that Ln binding at these free metal concentrations account for \textit{Mex} LanM’s full conformational change\textsuperscript{26}. \textbf{Table 1.1} summarizes the apparent $K_d$ values ($K_{d,\text{app}}$) values and Hill coefficients (n, indicating cooperativity) for this protein to various REEs. The early Lns induce this conformational change at the lowest concentrations (compared to the later Lns) and are the only ions in the series that have been observed to be biologically relevant so far\textsuperscript{26}. This suggests that \textit{Mex} LanM is specifically tuned to respond to the early Lns \textit{in vivo}, perhaps acting as a cellular sensor. On the other hand, \textit{Mex} LanM’s response to Ca was too weak to be determined by buffered titrations, so unbuffered Ca assays were carried yielding a $K_{d,\text{app}}$ in the micromolar range. This result showed the amazing selectivity \textit{Mex} LanM displays for Lns as opposed to the canonical EF hand-binding ion, Ca, which led to further investigation into this protein toward the goal of industrial application into Ln separation.

1.1.2.1. \textit{Mex LanM} NMR Structure

The structure of \textit{Mex} LanM, as seen in \textbf{Figure 1.6A}\textsuperscript{30} was determined by nuclear magnetic resonance (NMR) spectroscopy uncovering the selective conformational change that LanM undergoes in the presence of Lns\textsuperscript{28}. This was the first structural analysis of a native Ln-binding protein that is not a PQQ-dependent alcohol dehydrogenase, suggesting that this structure could guide efforts to develop biologically inspired methods for Ln separations. Y\textsuperscript{III} is chosen for structural characterization of holo-LanM because it has a similar ionic radius and coordination chemistry to Lns, but is diamagnetic, facilitating the NMR determination. Its $K_{d,\text{app}}$ is also similar to the physiologically relevant Lns, so it should give an accurate representation of the protein bound by any of the Ln ions.
Upon structural analysis, it was seen that *Mex* LanM forms a compact helical bundle containing three central helices, a hydrophobic core, and the EF loops positioned at the periphery. Interestingly, unlike CaM, a canonical EF-hand protein, which pairs its EF hands 1 and 2 together and 3 and 4 together, *Mex* LanM pairs EF 1 with EF 4 and EF 2 with EF 3. This was further confirmed with paramagnetic relaxation enhancement experiments. Hydrogen bonding, seen in Figure 1.6C, and hydrophobic packing play key roles in these EF hand pair interactions, presumably leading to the cooperativity observed by this protein upon metal binding. Although efforts to date have been unsuccessful at obtaining a crystal structure, it would be useful for determining the finer details of the coordination environment, but the NMR structure does suffice in its visual representation of *Mex* LanM’s metal binding, seen in Figure 1.6B, allowing for a more meticulous mutagenesis of various EF residues in this work.
1.1.2.2. Mex LanM utilized in a FRET sensor for lanthanides

Due to Mex LanM’s selective conformational change in the presence of Lns, it was used to construct a genetically encoded sensor for these ions, retaining its picomolar affinity and selectivity\textsuperscript{31}. Challenges in the detection of REEs necessitates a high affinity method sensor for these elements which would guide efforts towards recycling and extractions from REE sources to the highest concentration metal deposits. The sensor, LaMP1, was constructed with Mex LanM flanked by ECFP and citrine, a donor-acceptor Förster resonance energy transfer (FRET) pair, which undergoes a ratiometric response to Lns. This sensor design can be seen in Figure 1.7, and is used in this work to explore Mex LanM’s EF hands. LaMP1 displayed a strong, 7-fold response to all REEs with $K_{d,app}$’s in the picomolar range, just as Mex LanM by itself, and the CD response of LaMP1 matches its FRET output, showing that the flanking fluorescent proteins do not alter the metal binding characteristics\textsuperscript{31}. In the presence of Fe$^{III}$, Al$^{III}$, Mn$^{III}$, and Cu$^{II}$, all common competing metals in potentially relevant samples such as mine leachates and electronic waste, LaMP1 showed little or no response. Even at higher concentrations, where the sensor showed
/modest response, La\textsuperscript{III} was able to outcompete the other metals. These experiments showed the potential viability of this sensor in the detection of REEs\textsuperscript{31}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{A) Sensor design of LaMP1 with LanM flanked by ECFP and citrine. B) Stoichiometric titration of LaMP1 with 0-4 equivalents of La\textsuperscript{III}, exhibiting a ratiometric response. Adapted from ref 31.}
\end{figure}

Along with the industrial, practical, and technological advantages that LaMP1 offered, this sensor also was used to start probing the in vivo mechanism of Ln uptake in M. extorquens. For example, LaMP1 was used to probe to probe Ln levels in the cell, more specifically, probe why lighter Lns are able to support Ln-dependent growth but not the later Lns. Cells that were grown in the presence of Ca\textsuperscript{II} in the absence of Lns exhibited a FRET ratio similar to apo-LaMP1 in vitro. Upon addition of 2 µM Ln to the media the FRET ratio was increased selectively by La, Ce, Pr, and Nd within 2 hours, whereas the later Lns did not show a significant response.
compared to the Ca control\textsuperscript{11}. This suggests that the earlier Lns are imported in the cytosol, while the later ones are not, providing key initial insight into the mechanism of Ln uptake and utilization.

\textit{1.1.2.3. Initial probing of LanM for REE extraction}

With \textit{Mex} LanM’s high affinity and remarkable selectivity for Lns, it was then probed for its potential use at extracting REEs from industrial feedstocks. Since the previously described LBTs failed at pHs lower than 6, it was hoped that \textit{Mex} LanM could potentially solve this problem with its binding approximately 4 orders of magnitude higher than the LBT, if it is in fact stable at low pH. \textit{Mex} LanM displayed excellent selectivity over competing metals in feedstock solutions, when added to solutions containing from ~10 to 20,000 ppb REEs and ~100 to 3,000,000 ppb non-REEs in a solution with a pH of 3.7, there were almost no REEs left in filtrates and all non-REE is left in the filtrate solutions upon this treatment with \textit{Mex} LanM (\textbf{Figure 1.8})\textsuperscript{14}. Paired with this excellent selectivity, \textit{Mex} LanM also displayed the ability to tightly bind Lns down to pH 2.5. This performance has yet to be seen in any synthetic or natural biomolecule for the extraction of REEs. An immobilization strategy, however, would be useful for industrial applications, where this work showed a proof of concept in solution.

\textbf{Figure 1.8.} Extraction of REEs from non-REEs using LanM in solution at a pH of 3.7. Reproduced from ref 14.
1.2. Overview of this thesis

Thus far, a multitude of biomolecules have had varying success in extracting REEs from non-REEs under vastly differing conditions, but, LanM has proven to be the best, most selective biological REE extractant to date. The aim of this work is to understand how different LanMs function as well as how the different residues in EF hands affect overall metal binding, with the ultimate goal of being able to utilize these properties to separate Lns from one another.

In chapter 2, we study a new LanM from a different organism for its metal binding characteristics. Through BLAST search with Mex LanM, hundreds of potential LanM proteins were found and a few organisms were looked at in more depth due to some unique EF hand characteristics. In particular, LanM from Xanthomonas axonopodis pv vasculorum (Xax), a plant pathogen, was studied in depth for its metal binding characteristics, with preliminary separation data exhibiting a modest separation of $\text{La}^{III}$ from $\text{Dy}^{III}$ but less effective separation of $\text{Nd}^{III}$ from $\text{Dy}^{III}$. Nonetheless, Xax LanM has provided another insight into how nature sequesters and binds Lns, furthering the field by adding to the library of Ln binding proteins.

In chapter 3, utilizing the previously mentioned LaMP1 sensor, many variants of Mex LanM were able to be created, allowing for ease of characterization for each new variation of the protein. These studies provided many details of how some of the EF hands work, more particularly how some residues of these EF hands influence Ln binding. These studies can be used to further the understanding of how the EF proteins work as well as provide a starting point for perhaps building “model” EF hands for molding the selectivity desired. Also, using a modified version of LaMP1, this sensor was successfully cloned into plants for further study of the uptake and utilization of Lns in these new systems.

In chapter 4, we lay out all miscellaneous protocols and experiments undertaken for various projects.
CHAPTER 2 CHARACTERIZATION OF XANTHOMONAS AXONOPODIS LANM

2.1. Introduction

*Mex* LanM has provided a good baseline for the studies needed in order to further the use of biomolecules in the field of LnIII extraction, and, thus far, has shown some promise in the separation of these. In this chapter, we explore the diversity of Ln coordination in Nature, with hopes that this new protein could potentially be useful in separating Lns from each other. Through BLAST search using *Mex* LanM as query, a library of potential LanM homologs was acquired. Within this search, we looked for proteins that had similar, but unique, EF hand properties as well as low overall sequence homology to *Mex* LanM, maintaining the short sequences between EF hands that is distinctive for LanM compared to other EF containing proteins. *Xax* LanM quickly became of interest due to its 33% identity to *Mex* LanM as well as its unique EF hands (Figures 2.1 and 2.2). *Xax* LanM is entirely missing EF 3, which means it must adopt a substantially different structure than the previously studied *Mex* LanM. Its EF hands also lack a Pro residue in the second position as well as missing a carboxylate residue at the ninth position. These unique features led to an investigation into this protein and how these unique EF properties affect metal binding.

* M. extorquens  X. axonopodis
  EF1  DPDKDGTIDLKE  DTDGDGRVSLDE
  EF2  DPDKDGLDAKE  DTDHGVLQGDE
  EF3  DPDNDGTLDKKE  (missing)
  EF4  NPNDNGTIDARE  DANGDGYSARE

Figure 2.1. EF hand comparison between *Mex* LanM and *Xax* LanM. Carboxylate residues likely to coordinate metal are shown in blue, missing carboxylates at position nine are shown in red.
*X. axonopodis* is a gram-negative, slender, rod-shaped bacterium that causes citrus canker by attacking young, developing organs of the tree. This disease manifests itself as dark spots on leaves and fruits, making these pieces unable to be sold for consumption. This organism contains a putative Ln-dependent alcohol dehydrogenase (WP_171891058), which is interesting because, to our knowledge, Ln utilization has not been seen in plant pathogens to date. Therefore, understanding the mechanisms of Ln utilization in this organism could be of interest in the agricultural field.

![Sequence Comparison](image)

**Figure 2.2.** Full sequence comparison for *Mex* LanM and *Xax* LanM.

Following the approach used in the characterization of the first LanM (*Mex*), we carried out an in-depth study of the metal binding and separation potential of *Xax* LanM. Through CD, ICP-MS, analytical ultracentrifugation (AUC), SEC, and size exclusion chromatography in line with multi angle light scattering (SEC-MALS), we successfully characterized the metal binding of this protein with its unique oligomerization. *Xax* LanM dimerizes in the presence of the late Lns, but remains a monomer with the early Lns and Ca\(^{II}\). This protein retains picomolar affinity for Lns, with a higher affinity for the late Lns over the early, and has a high nanomolar affinity for Ca\(^{II}\). Through this data, we hypothesize that perhaps *Xax* LanM is used in Ln/Ca\(^{II}\) sensing in the cell, as well as distinguishing the early Lns for the late through its selective dimerization. Overall, this protein behaves vastly different from *Mex* LanM, and displays unique properties due to its sequence.
2.2. Experimental

The following Xax constructs were made in this work.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence start</th>
</tr>
</thead>
<tbody>
<tr>
<td>6His-Xax</td>
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</tr>
<tr>
<td>SUMO XAX</td>
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<tr>
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<td>AQAQQVQV</td>
</tr>
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<td>3His-Xax</td>
<td>HHHAQAQQVQV</td>
</tr>
<tr>
<td>GXax</td>
<td>GAQAQQVQV</td>
</tr>
</tbody>
</table>

2.2.1. Expression of Xax LanM

Xax LanM was acquired from Twist Bioscience (pET-29b-Xax) and transformed into chemically competent E. coli BL21 (DE3) cells which were plated on LB-agar plates with 50 µg/mL kanamycin (Kan) and grown at 37°C for ~10 hours. A single colony was used to inoculate 100 mL of LB (50 µg/mL Kan) for ~16 hours at 37°C with shaking at 200 rpm. After overnight incubation, 40 mL of starter culture was used to inoculate 2 L of LB (50 µg/mL Kan) and grown at 37°C with shaking at 200 rpm. At OD$_{600}$ ~0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and cells were incubated for an additional 3 hours at 37°C. The cells were pelleted by centrifugation for 7 min at 7000 x g at 4°C yielding ~1.5 g of cell paste per L of culture. Typical scale for expression of Xax LanM was 4 L.

2.2.2. Purification of Xax LanM from inclusion bodies

The cell paste was resuspended in 5 mL/g of Buffer A [50 mM Tris, 1 mM EDTA, 10 mM KCl, pH 8], 2 U/mL DNAsae, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), and complete mini protease inhibitor Tablets (1 per 10 mL). Cells were lysed on ice using sonication with pulse sequence of 20 s on/40 s off with 50% amplitude for 20 mins and debris were pelleted by
centrifugation at 40,000 x g for 20 min at 4°C. The supernatant was decanted and the pellet was used for inclusion body extraction.

The pellet was resuspended in 5 mL/g original cell paste of Buffer B [100 mM Tris, 4% Triton X-100, 2 M urea, pH 8] by vortex. The suspension was centrifuged at 17,000 x g for 20 min at 4°C, the supernatant was decanted, and the pellet was saved. This step was repeated once more. The pellet was then resuspended in the same amount of ddH$_2$O and centrifuged at 17,000 x g for 20 min at 4°C and the solution was decanted and the pellet was saved. This step was repeated once more. Inclusion bodies were stored at -20°C until used immediately. Typical inclusion body yields were ~500 mg per L of cell culture.

Inclusion bodies were solubilized in 10 mL/g of original cell paste in 50 mM sodium phosphate, 4 M urea, pH 8 overnight on a nutator at room temperature. The next day, the insoluble materials were pelleted at 10,000 x g and the supernatant was concentrated using an Amicon Ultra 10-kDa MWCO centrifugal filter device to 5 mL. Protein from inclusion bodies was relatively pure, but high and low molecular weight contaminants were removed and buffer was exchanged into Buffer C [20 mM MOPS, 100 mM KCl, 10% glycerol, pH 7.2] by size-exclusion chromatography on a HiLoad 16/600 Superdex 75 pg column. After column equilibration protein was loaded using a 5 mL capillary loop, which was washed with 7.5 mL Buffer C and protein was eluted with 1.2 CV Buffer C at 0.75 mL/min with 1.5 mL fractions collected in peak fractionation mode. Xax LanM-containing fractions were collected and ~20 mL of 50 µM and all constructs but G Xax were stored at -80°C. For G Xax, unstable protein was precipitated in cold room overnight, followed by addition of 2 mM Ca$^{II}$ for additional precipitation. Following Ca$^{II}$ addition, protein was dialyzed in Buffer C + 10 g/L Chelex 100 for 3 h. Before precipitation, protein yields were ~10 mg per L cell culture and after precipitation, protein yields were ~5 mg per L cell culture. Protein was then stored in the cold room and stable for at least three weeks at a time.
2.2.3. **Circular dichroism spectroscopy**

CD spectra of \( Xax \) LanM were collected using a Jasco J-1500 CD spectrometer, thermostatted at 25°C, and a 1 mm pathlength quartz CD cuvette (Jasco J/0556). Samples were scanned from 260 to 195 nm with 1 nm bandwidth, 0.5 nm data pitch, 50 nm/min scan rate, and 4 s average time. Two scans were acquired and averaged for each spectrum. For stoichiometric titrations, 15 µM \( Xax \) LanM was added to the cuvette in 200 µL of Chelex-treated Buffer C and La\textsuperscript{III} was titrated in 0.25 equivalents at a time. A blank spectrum was subtracted from each sample spectra.

2.2.4. **Stoichiometry and separation determination by ICP-MS**

Along with CD determinations, stoichiometry was determined by inductively coupled plasma mass spectrometry (ICP-MS) as well. \( Xax \) LanM (150 µL of ~50 µM) was incubated with 3 equivalents of Ln\textsuperscript{III} for 10 min at room temperature. \( Xax \) LanM was then applied to a Zeba spin column (7K MWCO, Fisher scientific), following manufacturer’s protocol, and column flowthrough was analyzed by UV-visible spectrophotometry for concentration and 30 µL were sent in 7 mL of 2% analytical grade HNO\textsubscript{3} (VWR, Aristar Ultra) for ICP-MS analysis. For separations, metals were mixed together 1:1 (15 mM each) and then incubated with 100 µL of 50 µM \( Xax \) LanM for ~1 h. \( Xax \) LanM was applied to the Zeba spin column as above and column flowthrough was analyzed by UV-visible spectrophotometry for concentration and ICP-MS as above.

2.2.5. **Preparation of buffered Ln\textsuperscript{III}-EGTA solutions**

The procedure for preparing buffered Ln\textsuperscript{III} solutions is described in detail in a Methods in Enzymology chapter\textsuperscript{33}. Briefly, ~1 M ethylene glycol-bis(β-aminoethyl ether)-\( N,N,N',N' \)-tetraacetic acid (EGTA) was prepared in Milli-Q water with 2 equivalents of KOH and was brought to pH 7.2. A ~1 M solution of Ln\textsuperscript{III} was made in Milli-Q water with a few drops of 6 M HCl to help dissolution. Both EGTA and Ln\textsuperscript{III} were diluted to ~10 mM in Buffer D [20 mM MES,
20 mM acetate, 100 mM KCl, pH 6.1] treated with 10 g/L Chelex for 3 hours. A cuvette contained 470 µL of Buffer H [20 mM MES, 100 mM KCl, pH 6.05], 25 µL ~10 mM EGTA, and 5 µL of ~7 mM XO. The ~10 mM Ln^{III} solution was titrated in 1 µL increments and monitored for A_{579 nm}. Ln^{III}:EGTA equivalency was taken at the point which the increase in A_{579 nm} exceeded 2% of the overall change. The final solution (20 mL) was prepared with 10 mL 2x Buffer E [30 mM MOPS, 100 mM KCl, pH 7.2], 200 µL ~1 M EGTA, and the appropriate amount of ~1 M Ln^{III} stock as determined by the titration followed by pH adjustment to 7.2. Since this standardization failed for the later Ln^{III}s, they were sent for ICP-MS analysis in order to determine the correct amount to add to buffered solution.

2.2.6. \( K_d \) determinations by CD

The procedure for preparing and analyzing samples is described in detail in Methods in Enzymology chapter 33. Briefly, a solution of Xax LanM was diluted into high and low Ln^{III} buffer separately to 15 µM. These solutions were mixed to give various free concentrations of free metal, as calculated by equation 5 in reference 32, with each sample being 200 µL. The samples were incubated for 1 hour at room temperature to allow equilibration prior to data collection. Data were collected as described above and the ellipticity (\( \theta \)) at 222 nm was plotted versus the free metal concentration. Data were fitted to the Hill equation in Origin 2018 (OriginLab Corporation).

2.2.7. Oligomeric state determination by AUC

AUC was carried out by Kushol Gupta at the Johnson Foundation Structural Biology and Biophysics Core at the Perelman School of Medicine, University of Pennsylvania. 400 µL of 50 µM Xax LanM, as well as matching buffer was sent for analysis. Sedimentation velocity analytical ultracentrifugation experiments were performed at 20°C with an XL-A analytical ultracentrifuge (Beckman-Coulter, Brea, CA) and a TiAn60 rotor with two-channel charcoal-filled Epon centerpieces and quartz windows. Data were collected with detection at 280 nm.
Complete sedimentation velocity profiles were recorded every 30 seconds at 40,000 rpm. Data were fit using the c(S) distribution model of the Lamm equation as implemented in the program SEDFIT.

The partial specific volume (\(\bar{\nu}\)), solvent density (\(\rho\)), and viscosity (\(\eta\)) were derived from chemical composition by SEDNTERP. S values provided have not been corrected for \(S_{20,w}\).

Figures were created using the program GUSSI.

2.2.8. *Oligomeric state determination by SEC-MALS*

Xax LanM (100\(\mu\)L of 400 \(\mu\)M) was sent for analysis. SEC experiments were performed on 100 \(\mu\)L injections with a Superdex 75 10/300 GL column (GE Healthcare) at 0.5 mL/min at 25\(^\circ\)C in Buffer E, 0.1 micron filtered. Absolute molecular weights were determined using MALS.

The scattered light intensity of the column eluant was recorded at 18 different angles using a DAWN-HELEOS MALS detector (Wyatt Technology Corp.) operating at 658 nm after calibration with the monomer fraction of Type V BSA (Sigma). Protein concentration of the eluant was determined using an in-line Optilab T-rex interferometric refractometer (Wyatt Technology Corp.). The weight-averaged molecular weight of species within defined chromatographic peaks was calculated using the ASTRA software version 6.0 (Wyatt Technology Corp.), by construction of Debye plots (\(KC/R\theta\) versus \(\sin^2[\theta/2]\)) at 1 s data intervals. The weight-averaged molecular weight was then calculated at each point of the chromatographic trace from the Debye plot intercept, and an overall average molecular weight was calculated by averaging across the peak.

2.2.9. *Oligomeric state determination by size-exclusion chromatography*

For size-exclusion experiments, a Superdex 75 Increase 10/300 GL (24 mL volume) column was used. After wash with Buffer E + 1 mM EDTA, the column was equilibrated in Buffer C and protein was loaded using a 500 \(\mu\)L capillary loop. The loop was washed with 750
μL Buffer C and eluted with 1.2 CV Buffer C at 0.6 mL/min with 0.5 mL fractions collected in peak fractionation mode. Fractions were analyzed via SDS-PAGE and ICP-MS as above.

2.3. Results and discussion

2.3.1. Homology model of Xax LanM using Mex LanM

A homology model was generated for Xax LanM using Mex LanM as query (Figure 2.3). Although this model does not necessarily reflect what the actual structure of the protein may be, it gives insight into the effect of removing part of the protein (EF hand 3), giving a more minimal LanM that retains high Ln affinity. Also, as previously mentioned, this protein is seen to dimerize in the presence of the later Lns, and this model provides a potential dimerization interface. After EF 1, there is a pocket of hydrophobic residues that may be this interface, to minimize hydrophobic interactions with solvent. Although a crystal structure would be useful in order to visualize this dimerization, the homology model gives a basis to hypothesize this potential interface.

2.3.2. Construction and purification of final Xax LanM construct

Like Mex LanM, Xax LanM was predicted to possess an N-terminal signal peprited. Signal P suggested two potential cleavage sites, one after A25 and the other after A28. Initially we selected the more likely site, A28, and designed an N-terminally His tagged construct for
cytosolic expression. An N-term tag was selected because this was seen to have minimal effect on Mex LanM’s properties. This construct was expressed abundantly in *E. coli* but purified with low protein yields (~0.25 mg per L cell culture). Nonetheless, there was enough protein in order to carry out preliminary studies of metal binding stoichiometry. Through CD titrations, it was observed that 1.5 equivalents of LaⅢ saturated the change in secondary structure in the far-UV region, suggesting that the protein was only binding 1.5 equivalents of metal rather than the expected 3. The non-integer metal binding stoichiometry suggested the potential for metal-induced oligomerization in this protein.

Due to the poor protein yield, the expression and purification were optimized before further characterization. Various temperatures were attempted for expression, but none yielded a significant improvement in the amount of protein present in lysate, thus, a construct of SUMO tagged *Xax* LanM was cloned. SUMO is typically used to increase the expression and solubility of proteins, but, surprisingly, this construct did not yield any higher protein yields. Therefore, due to its low solubility, *Xax* LanM was then purified from inclusion bodies. Isolation and solubilization of inclusion bodies, followed by SEC on a Superdex 75 column yielded pure protein for characterization as explained previously.

Since the protein was no longer purified using Ni-NTA affinity chromatography, a new construct was created with no His tag, creating the construct starting with ‘QVQV’. After analysis with MS, it was seen that the molecular weight of the protein was not as expected, prompting an investigation into whether the alternative predicted signal peptide cleavage site might yield more stable protein. A new construct was created, with three additional amino acids: ‘AQAQVQV’. Unexpectedly, this new untagged construct did not express at all in *E. coli*, so we thought that perhaps adding three His residues, to return to the original number of amino acids in the ‘HHHHHHHQVQV’ construct, may allow for expression. This construct did express well, albeit still primarily in inclusion bodies, so we sought to determine the minimal construct that would
also yield a sufficient quantity of protein. We determined that adding a Gly residue prior to AQA allowed for robust expression, so we used the construct ‘GAQAQVQV’ for metal binding and separation experiments as this was the most minimal construct that expressed in E. coli. This purification was still done through inclusion body solubilization, but yielded ~40 mg of protein before precipitation and ~20 mg of protein after precipitation from 4 L of cell culture. Ca\textsuperscript{II} precipitation was necessary because not all of the protein that was solubilized refolded, although most of the protein is disordered, in the proper conformation, resulting in unstable protein. But, this Ca\textsuperscript{II} was removed by Chelex 100 resin for further characterization.

2.3.4. Binding stoichiometry of Xax LanM

After the initial finding via CD that Xax LanM binds 1.5 equivalents of La\textsuperscript{III} we sought out ICP-MS as a secondary means of confirming this unusual binding stoichiometry. We chose five Lns to further characterize to give a sense of trends within the series: La\textsuperscript{III}, Nd\textsuperscript{III}, Gd\textsuperscript{III}, Dy\textsuperscript{III}, and Yb\textsuperscript{III}. We also analyzed binding of Ca\textsuperscript{II}, the metal bound by canonical EF hand s, as previously mentioned. In all cases, at pH7.2, we observed a metal binding stoichiometry between 1 and 1.5 equivalents, with the averages reported in Table 2.1. We also explored the stoichiometry of Ln binding with Ca\textsuperscript{II} present, where 3 equivalents of Ca\textsuperscript{II} were added to Xax LanM followed by 1.5 equivalents of La\textsuperscript{III} or Dy\textsuperscript{III}. The Lns were able to displace Ca\textsuperscript{II} for binding, but only at one site, exhibiting an interesting stoichiometry of 1.07 Ca\textsuperscript{II} bound with 1.08 La\textsuperscript{III} bound or 0.95 Ca\textsuperscript{II} bound with 1.40 Dy\textsuperscript{III} bound. As the protein has 3 EF hands, these data

<table>
<thead>
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<th>Metal Added</th>
<th>Equiv Ca\textsuperscript{II}</th>
<th>Equiv Ln\textsuperscript{III}</th>
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<td>La\textsuperscript{III}</td>
<td>-</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Nd\textsuperscript{III}</td>
<td>-</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Gd\textsuperscript{III}</td>
<td>-</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Dy\textsuperscript{III}</td>
<td>-</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Yb\textsuperscript{III}</td>
<td>-</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Ca\textsuperscript{II}</td>
<td>1.52 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>Ca\textsuperscript{II} + La\textsuperscript{III}</td>
<td>1.07 ± 0.04</td>
<td>1.08 ± 0.03</td>
</tr>
<tr>
<td>Ca\textsuperscript{II} + Dy\textsuperscript{III}</td>
<td>0.95 ± 0.02</td>
<td>1.40 ± 0.02</td>
</tr>
</tbody>
</table>
suggested either that Xax LanM was dimerizing the presence of metal or that one more site(s) was/were partially occupied under these conditions.

2.3.5. **Metal affinity characterization**

We also assessed the $K_{d,\text{app}}$ and Hill coefficient, $n$ for binding of metals to Xax LanM. Interestingly, all of the five Ln$^{\text{III}}$ ions tested had very large Hill coefficients (Figure 2.4, Table 2.2). As the stoichiometric titrations indicated, Xax LanM was only binding at 1-2 metal binding sites, so our explanation for this cooperativity is the dimerization of the protein, as investigated further below. One thing to note is that these buffered Ln titrations cannot be done in the presence of Ca$^{\text{II}}$, meaning that these titrations may not take into consideration the Ca$^{\text{II}}$ selective site.

**Table 2.2.** Apparent $K_d$s and Hill coefficients for titrations of Xax LanM with Lns and Ca$^{\text{II}}$.

<table>
<thead>
<tr>
<th>Metal</th>
<th>$K_{d,\text{app}}$ (M)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>La$^{\text{III}}$</td>
<td>$(5.6 \pm 0.1) \times 10^{-11}$</td>
<td>$10.6 \pm 1.2$</td>
</tr>
<tr>
<td>Nd$^{\text{III}}$</td>
<td>$(6.0 \pm 0.1) \times 10^{-12}$</td>
<td>$14.1 \pm 1.8$</td>
</tr>
<tr>
<td>Gd$^{\text{III}}$</td>
<td>$(4.9 \pm 0.3) \times 10^{-13}$</td>
<td>$15.2 \pm 1.5$</td>
</tr>
<tr>
<td>Dy$^{\text{III}}$</td>
<td>$(3.2 \pm 0.3) \times 10^{-13}$</td>
<td>$6.7 \pm 2.0$</td>
</tr>
<tr>
<td>Yb$^{\text{III}}$</td>
<td>$(6.7 \pm 2.0) \times 10^{-14}$</td>
<td>$11.4 \pm 1.7$</td>
</tr>
<tr>
<td>Ca$^{\text{II}}$</td>
<td>$(5.2 \pm 4.4) \times 10^{-7}$</td>
<td>$1.7 \pm 0.2$</td>
</tr>
</tbody>
</table>
Figure 2.4. Representative Ln-EGTA titration for various metals with Xax LanM. $K_{d,app}$ and n values are an average of three trials. A) Titration with La$^{III}$. B) Titration with Nd$^{III}$. C) Titration with Gd$^{III}$. D) Titration with Dy$^{III}$. E) Titration with Yb$^{III}$. F) Titration with Ca$^{II}$. 
The $K_{\text{d,app}}$ values for $Xax$ LanM ranged from 63.0 fM for Yb$^{\text{III}}$ to 56.3 pM for La$^{\text{III}}$. Interestingly, the trend in affinity of $Xax$ LanM, increasing affinity with decreasing ionic radius, is distinct from that of $Mex$ LanM (Figure 2.5)\textsuperscript{26}. The difference in $K_{\text{d,app}}$ within the series motivated some preliminary REE separation experiments. To this end, we added a premixed solution of three equivalents of each metal to $Xax$ LanM of La$^{\text{III}}$/Dy$^{\text{III}}$ and Nd$^{\text{III}}$/Dy$^{\text{III}}$ to separate tubes containing $Xax$ LanM at pH 7.2, followed by a Zeba spin column and ICP-MS analysis. This protein was able to modestly separate La$^{\text{III}}$ from Dy$^{\text{III}}$ binding only 0.59 equivalents of La$^{\text{III}}$ while binding 1.56 equivalents of Dy$^{\text{III}}$. However, it was not able to separate Nd$^{\text{III}}$ from Dy$^{\text{III}}$, binding 0.90 equivalents of Nd$^{\text{III}}$ and 1.01 equivalents of Dy$^{\text{III}}$. Based on just $K_{\text{d,app}}, Xax$ LanM should, theoretically, be able to separate these metals from each other. In these cases, the Ca$^{\text{II}}$ selective site may be occupied by Lns, as that EF hand may have little Ln selectivity. To avoid this, for example, a lower pH could be used to avoid Ln binding in the potential non-specific site. These lower pH experiments were not undertaken in this work, but could help the selective separation of metal based on the $K_{\text{d,app}}$s that are observed.

![Figure 2.5](image-url)  

**Figure 2.5.** Apparent $K_d$ values for Ln-LanM complexes for A) $Xax$ and B) $Mex$ with assumed coordination number of 9. $Mex$ LanM graph reproduced from ref 26.

We determined the $K_{\text{d,app}}$ for Ca$^{\text{II}}$ to compare to $Mex$ LanM, which displays a conformational response with millimolar $K_{\text{d,app}}$. For $Xax$ LanM $K_{\text{d,app}} = 520.0$ nM and $n = 1.67$. 
This finding was surprising, as this $K_{d,\text{app}}$ is similar to typical EF hand proteins and distinct from *Mex* LanM, whereas the Ln affinity of this protein is much higher than most EF hand proteins.

This three EF hand protein seemingly only binds two equivalents of metal, where *Mex* LanM has four EF hands and binds three equivalents of metal tightly. Also, it seems from stoichiometric data that one site prefers Lns, where one has low Ln affinity and prefers Ca$^{ll}$. This protein undergoes a conformational change with high cooperativity which, based on stoichiometric and metal binding affinity data, suggest an oligomerization which is explored further.

2.3.6. **Oligomeric state determination**

On the Superdex Increase 75 column (SEC), Ca$^{ll}$ displayed a mostly monomeric state, not showing a shift into a higher order oligomer (**Figure 2.6A**). An EDTA sample (apo protein) runs at a higher apparent molecular weight, just as *Mex* LanM, due to its disordered state (**Figure 2.6A**). We found a seemingly mostly monomer state with La$^{lll}$-Gd$^{lll}$,
with a conformational difference in its metal bound state as apparent by the formation of a small shoulder in the trace, which could be a dimer or a distinct monomer conformational state (Figure 2.6B). However, with Dy\textsuperscript{III} and Yb\textsuperscript{III} there seems to be a shift to a monomer-oligomer equilibrium with two distinct peaks in the chromatogram (Figure 2.6C). Apparent molecular weights for each sample are shown in Table 2.3. Interestingly, upon metal analysis for some SEC fractions, Ca\textsuperscript{II} was bound as well as the Ln that was added to the protein. This further confirms the hypothesis previously discussed of a separate Ca\textsuperscript{II} preferred and Ln preferred site. Although SEC experiments such as these may be unreliable in determination of absolute molecular weights, we can conclude that \textit{Xax LanM} is undergoing a conformational change in the presence of metal, perhaps an oligomerization with the late Lns.

Table 2.3. Apparent molecular weights for each SEC peak ran with each metal/chelator.

<table>
<thead>
<tr>
<th></th>
<th>Apparent MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>~25 kDa</td>
</tr>
<tr>
<td>Ca\textsuperscript{II}</td>
<td>~12 KDa</td>
</tr>
<tr>
<td>La\textsuperscript{III}</td>
<td>~18 kDa</td>
</tr>
<tr>
<td>Nd\textsuperscript{III}</td>
<td>~19 kDa</td>
</tr>
<tr>
<td>Gd\textsuperscript{III}</td>
<td>~20 kDa</td>
</tr>
<tr>
<td>Dy\textsuperscript{III}</td>
<td>~19 kDa and ~32 kDa</td>
</tr>
<tr>
<td>Yb\textsuperscript{III}</td>
<td>~18 kDa and ~25 kDa</td>
</tr>
</tbody>
</table>
The complexity of the SEC results led us to pursue characterization by AUC and SEC-MALS. AUC can provide information about the interactions macromolecules have in solution\textsuperscript{15}. SEC-MALS can provide absolute molecular weight distribution data discerned from the diffraction patterns detected off the column\textsuperscript{36}. First, as a control, we examined apo and La\textsuperscript{III} bound Mex LanM which yielded data consistent with a monomer changing from a disordered to a more ordered state (Figure 2.7). With Xax LanM the AUC showed three species present, with two peaks present at a larger than expected molecular weight (perhaps due to the unfolded state), but one peak at a smaller molecular weight. In the La-bound form, the data were consistent with a

Figure 2.6. SEC traces for all metals run for oligomeric state test. Protein was 45 µM for each run. A) Trace with EDTA and Ca\textsuperscript{II}. B) Trace with La\textsuperscript{III}, Nd\textsuperscript{III}, and Gd\textsuperscript{III}. C) Trace with Dy\textsuperscript{III} and Yb\textsuperscript{III}.
monomer and a minor amount of dimer, one at ~11 kDa and a minor peak at ~22 kDa (Figure 2.8). These data were promising, but did not confirm our hypothesis. SEC-MALS analysis would be able to definitively provide molecular weight information for the various metallated states of Xax LanM. To this end, we sent a Ca$^{II}$ sample, a Ca$^{II}$/La$^{III}$ sample, and a Ca$^{II}$/Dy$^{III}$ sample, just as previously examined for stoichiometry (Figure 2.9).

Figure 2.7. AUC data for Mex LanM in the A) Apo state and B) La$^{III}$-bound state.
The SEC trace for Ca\textsuperscript{II} was a broad peak, with MALS data exhibiting an \~11 kDa protein, which suggests monomer. When La\textsuperscript{III} is added alongside Ca\textsuperscript{II} the SEC shows two peaks, as in previous runs, but the MALS data showing the same \~11 kDa protein, suggesting monomer once again. This confirms the earlier hypothesis that the SEC peaks represent two conformations of La\textsuperscript{III}-bound Xax LanM. For Dy\textsuperscript{III}-bound Xax LanM, there are three peaks present in the SEC trace, two of which are the same as the La\textsuperscript{III}-bound, monomer by scattering data. But, the third peak in the SEC trace is an apparent dimer from scattering data. Perhaps in the Ln series, when the metal radius is large enough, the protein dimerizes which is used by the cell as a sensor in order to discriminate which metal ions to utilize. This interesting dimerization could prove useful in future applications of separating early Lns from late Lns, as well as provide a starting point to investigating the \textit{in vivo} function of Xax LanM.

![Figure 2.8](image)

\textbf{Figure 2.8.} AUC data for Xax LanM in the A) Apo state and B) La\textsuperscript{III}-bound state.
2.3.7. Bioinformatics investigation for Xax LanM

Because of the interesting Ca\textsuperscript{II}/Ln dynamics of this protein, we carried out a BLAST search using Xax LanM as query, and looked at some of the common genera associated with this protein, and searched for association with putative MxaFI (Ca\textsuperscript{II}-dependent) and XoxF (Ln-dependent) like dehydrogenases. *X. axonopodis* does have an MDH that is likely Ln-dependent based on its alignment with *M. extorquens* XoxF, with an Asp residue at position 320. Through BLAST search, which provided 405 results, we further investigated 12 organisms from the aforementioned common genera for these MDHs as well as their EF hand characteristics. Interestingly, none of these organisms had MxaF present without XoxF present as well, suggesting all are capable of Ln-utilization in their MDH. One organism, *Arenimonas donghaensis* actually had neither MDH present, but was the only LanM to have a Pro present in
the EF hand, at the tenth position of EF hand 2. There are many instances of missing or additional carboxylates in these EF hands, which could potentially lead to unique metal binding characteristics, as observed in Xax LanM. These results are summarized below in Table 2.4, showing the unique properties that these organisms’ LanMs display.

**Table 2.4.** Summary of findings for BLAST search of Xax LanM. An ‘x’ represents a characteristic that the organism’s protein exhibits. These organisms represent the genera that appeared most in search.

<table>
<thead>
<tr>
<th>Organism</th>
<th>% id to Xax</th>
<th>mxaF</th>
<th>XoxF</th>
<th>neither</th>
<th>no proline</th>
<th>11th position 1st EF hand</th>
<th>11th position 2nd EF hand</th>
<th>9th position 3rd EF hand</th>
<th>9th position 3rd EF hand</th>
<th>12th position 2nd EF hand</th>
<th>10th position 1st EF hand</th>
<th>9th position 1st EF hand</th>
<th>N instead of D 3rd position 1st EF</th>
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<tbody>
<tr>
<td>Rhodanobacter denitrificans</td>
<td>48.42</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(A)</td>
<td>X (K)</td>
<td>X (D)</td>
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<td>X</td>
</tr>
<tr>
<td>Lysobacter enzymogenes</td>
<td>53.12</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(L)</td>
<td>X (S)</td>
<td>X (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>60.42</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(A)</td>
<td>X (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>X</td>
</tr>
<tr>
<td>Xanthomonas maltophilia</td>
<td>60.42</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(A)</td>
<td>X (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Arenimonas donghaensis</td>
<td>50</td>
<td>X</td>
<td></td>
<td>X</td>
<td>(A)</td>
<td>X (H)</td>
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<td>Luteibacter rhizomonicus DSM 16049</td>
<td>42.71</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(A)</td>
<td>X (N)</td>
<td>X (D)</td>
<td>X (D)</td>
<td>X (D)</td>
<td>X (D)</td>
<td></td>
<td>X (D)</td>
<td>X</td>
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<tr>
<td>Dyella jiangningensis</td>
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<td>X</td>
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<td>X (D)</td>
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<td></td>
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<td>X</td>
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<tr>
<td>Stenotrophomonas maltophilia</td>
<td>58.33</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(S)</td>
<td>X (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>X</td>
</tr>
<tr>
<td>Pseudomonas geniculata N1</td>
<td>58.33</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>(S)</td>
<td>X (D)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Xanthomonas oryzae pv.</td>
<td>94.79</td>
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<td></td>
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<td></td>
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<td></td>
<td>X</td>
</tr>
</tbody>
</table>

2.4. Conclusions and future directions

We have shown Xax LanM has a high affinity for Lns, in the femtomolar to picomolar regime, while maintaining a modest affinity for Ca\(^{II}\) in the nanomolar regime. This protein seems to preferentially bind Ca\(^{II}\) in one EF hand while binding a Ln in another. This protein displayed highly cooperative binding for Lns, suggesting an oligomeric state change. Through SEC, AUC, and SEC-MALS we have shown that Xax LanM oligomerizes in the presence of the late Lns, but
not in the presence of Ca$^{II}$ or the early Lns. In preliminary experiments, we are able to separate La$^{III}$ from Dy$^{III}$ but not Nd$^{III}$ from Dy$^{III}$. This protein has been relatively stable at room temperature as well as in the cold room, with storage capabilities without freezing for at least three weeks. The missing Pro residues at position two do not seem to disrupt the Ln binding capability of this protein. The extra carboxylate residues may explain the higher affinity for metal that $\text{Xax LanM}$ displays versus $\text{Mex LanM}$. Since the beginning of characterization of $\text{Xax LanM}$, we have made tremendous strides in understanding its metal binding capabilities and comparing them to the previously characterized $\text{Mex LanM}$.

Moving forward, there is still much work to be done in order to determine the full behavior of this protein. A crystal structure of $\text{Xax LanM}$ would be very useful in visualizing this oligomerization, leading to perhaps engineering of the protein’s EF hand residues and dimerization interface to optimize metal separations. Furthermore, since this protein does display high affinity for both Lns and Ca$^{II}$, it would be interesting to determine its in vivo application and its function in Ln or Ca utilization. Perhaps $\text{Xax LanM}$ is used as just a Ln sensor, discerning between early and late Lns, and just utilizing Ca$^{II}$ for structural reasons. Or, perhaps it is used for Ca$^{II}$ response in the cell. Finally, characterizing $\text{Xax LanM}$ at lower pHs, as $\text{Mex LanM}$, could be an interesting route to explore, seeing how the binding properties and separations potential are affected.
CHAPTER 3 INVESTIGATION OF EF HAND RESIDUES USING LAMP1 SENSOR

3.1. Introduction

The LanM-based fluorescent sensor, LaMP1 proved to be a valuable tool in the investigation of the metal binding of Mex LanM and gave insight into the in vivo function of this protein. LaMP1 variants are easily studied via FRET on a plate reader, leading to ease of characterization and rapid discernment if a variant is promising in its potential to industrial application. The groundwork laid out by the original characterization of this sensor, showed its promise in the study of LanM by these fluorescent means, as discussed previously. Mutation of the various EF hand residues allows us to form investigate how this protein exhibits such great Ln selectivity, and to potentially re-engineer that selectivity for specific Ln ions. Five mutant LanM variants were cloned into the LaMP1 sensor framework and investigated for their metal binding characteristics.

Two variants explored in-depth were N108D and 4P2A (formerly 4P4A, meaning the four Pro residues at the second position of each EF hand were changed to Ala), the latter of which was first explored in the original Mex LanM paper. N108D investigated the Asn residue in the first position of EF 4, as all three other EF hands have an Asp residue in that position instead. EF 4 displays weaker affinity for Lns than the other three EF hands, so it was hypothesized that this residue played a role in this weaker affinity. We created this mutant in hopes of generating LanM with four tight binding EF hands. 4P2A was created to explore the unique Pro residues at the second position of each EF hand, as explored in the original LanM paper. This mutant retained WT LanM’s full molar ellipticity change to the Lns and CaII, while responding to LaIII and NdIII at 5- to 10-fold lower concentrations and CaII at 300-fold lower concentrations via CD titrations. The substitution of Pro for Ala at position two of the EF hands suggests that these residues are
key in Nature’s framework for Ln-selectivity over Ca\textsuperscript{II}. The unique combination of Asn in EF 4 and Pro residues in the second position of each EF hand were thought to contribute to the overall Ln selectivity of Mex LanM.

Three additional variants were explored for their metal binding characteristics, but not as in depth as the previous two mutants. X\textsubscript{11}E was made to remove the positively charged Lys and Arg residues and add Glu residues at this position in the EF hands, hoping to engineer a LanM that exhibited tighter binding with the addition of more negatively charged metal binding residues. As a control, these positively charged residues were exchanged for an Ala at this position, yielding X\textsubscript{11}A. Finally, 4D\textsubscript{9}E was created to investigate whether a larger residue may improve binding or shift selectivity within the series. It was hoped that a larger residue could shift the WT LanM preference for the early Lns (larger) to the late Lns (smaller).

As described previously, LaMP1 has not only been useful in reporting on the metal binding of Mex LanM, but has also provided key insight into the in vivo use of Lns in bacteria. Whereas bacteria are the only organisms that have been demonstrated to utilize Lns in specific roles, plants have been seen to incorporate Lns into chlorophyll, as well as display enriched growth in soils supplemented with La\textsuperscript{III}, up to about 100 $\mu$M\textsuperscript{37,38}. These metals have been seen to be taken up by plants through ICP-MS studies of digested material, but the specifics of localization, trafficking, and function of Lns in plants has yet to be determined\textsuperscript{39–41}.

Plants seem to accumulate Lns in proportion to their concentration in the soil, suggesting that their uptake may occur nonspecifically\textsuperscript{42,43}. The proportion of Ln accumulated within the cells versus outside the cell is not known, leaving open the questions of uptake and use within the plant. One theory is that plants utilize Lns available to them through Ln-utilizing bacteria located in the phyllosphere. These Ln-utilizing bacteria may be relevant to the aforementioned positive effects that Lns have on plants. More work must be done in order to fully understand the mechanism of Ln utilization in plants, in which the contents of this chapter aim to address a
starting point for these studies. Through modification of a Ca\textsuperscript{II} sensor from plants, Yellow Cameleon 3.6 (YC3.6)\textsuperscript{44}, we cloned Mex LanM in place of the Ca\textsuperscript{II} binding protein and introduced this sensor into \textit{Arabidopsis thaliana}. Through preliminary characterization, we have seen some promise in the ability of this FRET sensor answered questions about Ln usage in plants.

### 3.2 Experimental

All primers used in this chapter are listed in below.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD-f</td>
<td>5'-ATGCCATAGCATTTTTTATCC-3'</td>
</tr>
<tr>
<td>pBAD-r</td>
<td>5'-GATTTAATCTGTATCAGG-3'</td>
</tr>
<tr>
<td>ECFP-mid</td>
<td>5'-CAACCACCTACCTGAGC-3'</td>
</tr>
<tr>
<td>ECFP-LanM-f</td>
<td>5'-TTCTGTGACGCACGCCCCGCATGCCCTCGAGTACGTACCGCCTGCCCAAGTTGACGCGCC-3'</td>
</tr>
<tr>
<td>cpV-LanM-r</td>
<td>5'-CACGCCCTTTTGACCTGCTGAGCAGCAGCAGCAGC-3'</td>
</tr>
<tr>
<td>ECFP-mid-plant</td>
<td>5'-ATCGAGGACGGCAGC-3'</td>
</tr>
<tr>
<td>cpV-plant-r</td>
<td>5'-CGGCTCCCGGTACCAAGATGAC-3'</td>
</tr>
</tbody>
</table>

#### 3.2.1. Cloning for \textit{in vitro} work

All LaMP1 variants were created by double digestion of the gBlock gene fragment of the variant of interest (IDT) with SacI and SphI for 1 h at 37\textdegree C. pBAD-LaMP1 was also digested using the same restriction enzymes and, following gel electrophoresis, the fragment containing the vector and ECFP/citrine was excised and purified using the Zymoclean Gel DNA Recovery Kit. The digested gBlock was ligated into the gel purification product at a 3:1 insert:vector ratio using T4 DNA ligase according to the manufacturer’s protocol. After transformation, colonies
were screened using PCR (OneTaq Quick-Load) with pBAD-f and pBAD-r and confirmed by sequencing with ECFP-mid (Genewiz).

3.2.2. *Expression of LaMP1 variants*

Chemically competent *E. coli* 10beta cells were transformed with each construct and plated on LB-agar plates containing 100 µg/mL ampicillin (Amp) and grown at 37°C for ~12 h. A single colony was used to inoculate 100 mL LB (100 µg/mL Amp in all media for growth) and grown for ~16 h with shaking at 200 rpm at 37°C. After shaking, 40 mL of starter culture were used to inoculate 2 L of LB and grown with shaking at 200 rpm at 37°C. At OD$_{600}$ ~0.5, the culture was placed at 4°C for ~30 min, after which L-arabinose was added to a final concentration of 500 µM and the flask incubated at 23°C with shaking at 200 rpm for ~16 h. After incubation, the cells were pelleted by centrifugation at 7000 x g for 7 min at 4°C, yielding ~3 g of cell paste per liter.

3.2.3. *Purification of LaMP1 variants*

All purifications were done at 4°C. Cell paste was resuspended in 5 mL/g of Buffer F [50 mM sodium phosphate, 10 mM imidazole, 5% glycerol, pH 7.0], 0.40 mM PMSF, 2U/mL DNAse, and complete mini protease inhibitor Tablets (1 per 10 mL). Cells were lysed on ice with sonication for 20 s on and 40 s off for 20 min at 50% amplitude. Debris was pelleted by centrifugation at 40,000 x g for 30 min at 4°C. The supernatant was decanted and loaded to a 1.5 x 4.5 cm (8.0 mL) Ni-NTA column pre-equilibrated with Buffer F. The column was washed with 15 CV of Buffer F followed by elution with Buffer F + 250 mM imidazole. Eluted protein was concentrated to ~2 mL using an Amicon Ultra 10-kDa MWCO centrifugal filter device. Protein was buffer exchanged into Buffer E using a 0.5 x 5.0 cm (10 mL) G50 column. Yields were determined using UV/Vis with $\varepsilon_{515\text{nm}} = 77,000 \text{ M}^{-1}\text{cm}^{-1}$ and listed below.
### 3.2.4. Preparation of buffered Ln$^{III}$-EDDS solutions

The procedure for preparing buffered Ln$^{III}$-EDDS solutions is described in detail in (ref). EDDS was obtained as a ~1 M solution in water (Sigma). A solution of Buffer E and 10 mM EDDS was prepared and 300 µL were added to a 1 mm cuvette for CD titration. To determine how much Ln$^{III}$ was needed to create a final buffered solution, ~100 mM Ln$^{III}$ was titrated into the cuvette in 1.5-4 µL increments until there was no longer a change in CD signal at 222 nm. The volume of stock needed to reach the equivalence point was calculated from the intersection of the linear regression line fitted to the first half of the titration and the average ellipticity from the first three points after constant signal. The final solution (20 mL) was prepared with 10 mL 2 x Buffer E, 200 µL ~1 M EDDS, and the appropriate amount of ~1 M Ln$^{III}$ stock as determined by the titration followed by pH adjustment to 7.2.

### 3.2.5. FRET titrations for LaMP1 variants in vitro

The procedure for preparing and analyzing samples is described in detail. Solutions were mixed at various free metal concentrations to 100 µL total using EDDS- and EGTA-buffered solutions made as described above. LaMP1 and all variants were diluted to 500 nM. Following 15-minute incubation, the measurements were taken in a Greiner Cellstar 96-well half-area μClear plate with a 433 nm excitation, 450-550 nm emission, and a gain of 80. FRET ratios were

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Yield (mg/L culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4P$_2$A</td>
<td>~25</td>
</tr>
<tr>
<td>N108D</td>
<td>~30</td>
</tr>
<tr>
<td>4D$_3$E</td>
<td>~10</td>
</tr>
<tr>
<td>X$_{11}$E</td>
<td>~15</td>
</tr>
<tr>
<td>X$_{11}$E</td>
<td>~10</td>
</tr>
</tbody>
</table>
determined from the intensities of the fluorescence emission peak of ECFP (475 nm) and citrine (529 nm).

3.2.6. Cloning of LanM in place of Ca\textsuperscript{II} binding protein in YC3.6

The Gateway entry vector pENTR11 containing YC3.6 was acquired from Gabriele Monshausen. YC3.6 was double digested with SphI and MscI for 1 h at 37°C and, following gel electrophoresis, the fragment containing the vector and ECFP/cpVenus was excised and purified using the Zymoclean Gel DNA Recovery Kit. LanM was amplified using standard PCR protocol with ECFP-LanM-f and YFP-LanM-r and a Gibson assembly was performed at 50°C for 4 hours. The assembly reaction was transformed and was screened using colony PCR with primers ECFP-mid-plant and cpV-plant-r (OneTaq Quick-Load) and confirmed by sequencing with ECFP-mid-plant. After sequencing was confirmed, the new construct, named YCLanM, was transferred to the Connolly Lab for further investigation by Brendon Juengst.

The YCLanM gene was transferred from the Gateway Entry Vector pENTR11 to the Gateway destination vector pEarleyGate100, with the Invitrogen Gateway LR Clonase Kit II using manufacturer’s protocol. The new YCLanM/pEarleyGate100 vector was introduced into Arabidopsis thaliana using the established floral dip protocol\textsuperscript{45}, using Agrobacterium strain GV3101. T\textsubscript{1} seeds were selected on MS plates supplemented with 25 mg/L BASTA and 100 mg/L Timentin (to remove remaining Agrobacterium), with surviving plants being transferred to soil. The full sequence of YCLanM is below with ECFP in green, LanM in blue, YFP in red, and restriction sites underlined.

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAGGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTC
TTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTC
TTCAAGGACGAGCGCACCTTGACCTGGGGCGTGCAGCTCGCC

\textsuperscript{45}
WT Arabidopsis (genotypes Columbia-0 or Nossen) were grown in a Percivial Growth Chamber with 16 hours day/8 hours night at 22°C, 100-150µM/m²/s light intensity. Seeds were sterilized by shaking in 70% Ethanol for two minutes, decanting, then shaking in 50% bleach and 0.1% Tween 20 for 10 minutes. Seeds were then washed with sterile water and plated in a laminar flow hood. Seeds were then vernalized for two days at 4°C before moving to growth chamber.

Half-strength Murashige and Skoog plates supplemented with 1 mM MES, 0.5% sucrose, 0.7% agar and pH 5.8 were used for transgenic selection and experiments. Plants were grown with Sunshine Mix 4 potting soil supplemented with 3 Tablespoons Osmocote per 2 gallons soil.

3.2.8. ICP-MS of plant samples

Plants were grown in the presence and absence of LaIII and the shoots were dried for ICP analysis. Plant samples (ranging from 0.51 – 1.2 mg) were incubated for ~16 h with 100 µL of analytical grade HNO3 and then heated at 95°C for two hours. These digested samples were then
diluted to 7 mL with 2% analytical grade HNO₃ and sent for ICP analysis to evaluate their La³⁺ content.

3.2.9. Confocal imaging

For observation on the confocal, plants were grown vertically on MS plates for 6 days. Then were transferred to a chamber with a microscope slide 24 hours before observation. The plants were placed on the slide, then a thin layer of 0.7% agar was placed over the roots, before placing the chamber vertically in a magenta box with a moist paper towel at the bottom. For root observations, plants were exposed to La³⁺ immediately before first observation on the microscope. For leaf observation, plants were grown for two weeks in soil, in either 0mg/kg LaCl₃ soil, or in 100mg/kg LaCl₃ soil.

Images were acquired with the Zeiss LSM 510 Meta confocal microscope. Roots were imaged with a 40X, 1.2 aperture, C-Apochromat objective, and leaf tissue with a 10X, 0.3 aperture, Plan-Neofluar objective. The sensor was excited with the 458 nm line of an argon laser. Images were collected with an HFT 458 primary dichroic mirror, and two emission filters: one from 480-510nm for CFP florescence, and the other from 525-535nm for YFP florescence. Bright field images were acquired with the microscope’s transmission detector. CFP/YFP ratio calculations were measured using imagej.

3.3. Results and discussion

3.3.1. In vitro characterization of LaMP1 variants

N108D exhibited unexpected results; this alteration did not only create a higher affinity EF 4 as expected, instead it changed the cooperativity between the EF hands, showing two separate binding events. The affinity for EF 4 could, in fact, have increased, but this change also
interferes with this coupling of this EF hand with EF 2. We believe that these two phases, as seen in Figure 3.1, can be assigned to first EF 2/3 binding followed by EF 1/4. Unpublished data from our group, where Trp residues were mutated into each EF hand also suggest this order of binding, as the second EF hand mutation leads to no conformational change detected by CD, leading us to think that this is the first binding event. The first binding event, which is cooperative, displays relatively the same affinity across the Ln series similar to that of WT Mex LanM, with the second binding event, also cooperative, getting tighter across the series. Although this modification did not create the higher affinity that was hoped for, it does help provide a little more detail to the mechanism of this protein, as well as provide a starting point for possible modifications.

Although EF hand 4 starts with an Asn residue and does not have a high of an affinity for Lns, it does provide key structural integrity for the cooperativity of this protein, as evident by the mutation causing this distinct two-phase binding. The $K_{\text{d,app}}$ data as well as Hill coefficients for all metals tested is summarized in Table 3.1.
4P₂A gave us the first clue into how to perhaps increase Mex LanM’s selectivity within the Ln series. By mutating these four Pro residues, we observed a splitting of binding into two phases once again, albeit a bit more subtle than N108D, for just the late Lns and not the early Lns, as seen in Figure 3.2. This early response to the later Lns seems to be non-cooperative while the lower affinity response is cooperative. The most likely explanation for this observation is first EF1 binding non-cooperatively because of its lack of adjacent metal-binding partner, followed by EF2/3 binding to account for the cooperative response. We also observe the affinity for Lns increase across the series, the direct opposite of the observation made in wild type Mex LanM, as seen in Table 3.1. The 240 fold difference in $K_{d,app}$ for between Ho$^{III}$ and La$^{III}$ gives great promise in this variant’s ability to separate metals within the Ln series from one another. These data
provide an insight into how the EF hands of Mex LanM are binding metal, and allow for further investigation into some of these unique Pro residues in the second position.

4D₉E, which was created in order to attempt at a shift in selectivity to the late Lns, resulted in a variant that was not as useful as first hoped. This protein only exhibited a 3-4-fold change in FRET ratio upon metal binding, whereas the original sensor, as well as the N108D and 4P2A display an approximate 8-fold change. Plots of these titrations are shown in Figure 3.3 and data in Table 3.1, which show this smaller fold change overall, most likely due to not reaching the full conformational response in the concentration range evaluated using EDDS buffered solution (reaching low nanomolar). As a result, this variant was not explored in detail.

X₁₁A and X₁₁E, created to explore the removal of positively charged residues, both showed a bit more promise than the 4D₉E variant. Both X₁₁A and X₁₁E displayed affinities similar to wild type, but, again did not reach the same maximum FRET (4-5-fold response) that was expected, albeit higher than 4D₉E. Also, for binding to LaIII and NdIII, both of these variants displayed a very distinctive two-phase response, whereas all other metals tested were fitted to one phase. Once again, this result most likely reflects the concentration range for some of these
metals, as Nd$^{III}$ does get up to even a 7-fold response for X$_{11}$E, suggesting that its full conformational change has been reached in these metal titrations. Since these curves yielded reasonable data that were able to be fit for $K_{d,app}$ values, analysis was complete and summarized in Figure 3.4 and 3.5 and data in Table 3.1. This shows small promise in perhaps using these variants to separate metal within the series, but, thus far, 4P$_{2}$A seems to be the most promising.

**Figure 3.4.** Determination of $K_{d,app}$s for Ln$^{III}$-X$_{11}$A as determined by fluorescence plate reader assay using 500 nM X$_{11}$A and EDDS-buffered solutions. Plots are fitted to Hill equation with one or two sets of interacting sites. A) Titration with La$^{III}$. B) Titration with Nd$^{III}$. C) Titration with Sm$^{III}$. D) Titration with Ho$^{III}$. 
Figure 3.5. Determination of $K_{d, app}$ for Ln$^{III}$-X$_{11}$E as determined by fluorescence plate reader assay using 500 nM X$_{11}$E and EDDS-buffered solutions. Plots are fitted to Hill equation with one or two sets of interacting sites. A) Titration with La$^{III}$. B) Titration with Nd$^{III}$. C) Titration with Sm$^{III}$. D) Titration with Ho$^{III}$. 
Table 3.1. Summary data for all LaMP mutants created with $K_{d,\text{app}}$, $n$ value, and fold change.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Metal</th>
<th>$K_{d,\text{app}}$ (pM)</th>
<th>$n$</th>
<th>Fold Change ($R/R_0$)</th>
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<td>4.7</td>
<td>2.6</td>
<td>4.4</td>
</tr>
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</table>
3.3.2. **Characterization of Lns in plants**

Brendon Juengst of the Connolly lab spearheaded the completion of these experiments, as acknowledged previously. For preliminary experiments, plant roots were exposed to 1, 10, or 100 µM La<sup>III</sup> over a course of 4.5 hours on a microscope slide with agar media on top of the plant sample (Figure 3.6). The La<sup>III</sup> solutions, in water, were added into the area between the microscope slide and the agar. The plant samples that were exposed to 1 µM as well at 10 µM displayed an increase in FRET signal over time, whereas the 100 µM sample actually displayed a decrease in FRET signal. Higher concentrations have been seen to be toxic to plants, as stated previously, which could explain the decrease in FRET for the 100 µM sample. The slow response time may suggest an endocytosis mechanism, as discussed in some previous work. Positive and

![Figure 3.6. FRET ratio data for initial plant experiments where roots were exposed to 0, 10, and 100 µM La<sup>III</sup> on slide topped with media. Time points were taken at 0, 5 minutes, and 4.5 hours.](image-url)
negative controls were completed where YC3.6 and YCLanM were exposed to indole-3-acetic acid (IAA), which is a hormone that induces Ca\textsuperscript{II} release in plants. As expected, the YC3.6 construct responded to this with an increased FRET signal, while the YCLanM samples did not (Figure 3.7). These experiments suggested the ability of YCLanM to sense La\textsuperscript{III} in plants.

However, we note that there were spots to change experimental design such as changing of buffers used in the agar cushion as there was boric acid as well as monopotassium phosphate present, which could induce La\textsuperscript{III} precipitation.

Furthermore, we could also change La\textsuperscript{III} concentrations, which has been seen to have a different effect on many different types of plants (such as promoting or hindering growth). Finally, we could also use a difference in time points, and observe different parts of the plant as the uptake mechanism and utilization is unknown, we could be currently targeting the wrong time course and portions of the plant. These preliminary findings motivate future experiments in the plant system changing some of these variables.

**Figure 3.7.** Control experiments where plant roots were exposed to IAA to induce Ca\textsuperscript{II} release for A) YC3.6 plants and B) YCLanM plants.
To ensure these plants were, in fact, uptaking La$^{III}$, we grew wild type *Arabidopsis* with and without La$^{III}$ supplementation (100 mg/kg soil or ~20µM) and sent digested samples for ICP-MS analysis. On average, the La$^{III}$ supplemented plants had double the amount of La$^{III}$ present per mg of dry cell weight than the non-supplements plants after 14 days (*Table 3.2*), meaning these samples did effectively uptake the metal. With this confirmation, we

**Figure 3.8.** Imaging of plant leaf samples in La$^{III}$ treated and untreated samples. A) CFP fluorescence of leaf. B) YFP fluorescence of leaf. C) Brightfield image of leaf. D) Bar graph for average YFP/CFP ratio of ten samples.
moved forward with further characterization, looking at YCLanM FRET signal in the leaves of plants grown in a La\textsuperscript{III}-supplemented or non-supplemented environment. While there was FRET in the leaves (Figure 3.8A, B, C), unfortunately there was no significant increase in FRET ratio between the two conditions (Figure 3.8D). This could be for a multitude of reasons, including the microbiome of these growths not being right for Ln-uptake, as discussed previously. Nonetheless, these experiments have been just the beginning of exploring Ln\textsuperscript{III} uptake and utilization in plants, and further optimization of this sensor and how it works could provide useful insight into this system.

### 3.4. Conclusions and future directions

Overall, LaMP1 has been a useful and convenient tool for the evaluation of the various interesting EF residues of \textit{Mex} LanM. Through mutation of the fourth EF hand in N108D, we observed a shift in cooperativity rather than just a strengthening of the affinity of EF 4. For 4P\textsubscript{2}A, we have created a shift in selectivity within the series, as well as observed a bifurcation of the Ln binding. 4D\textsubscript{9}E was not characterized in detail due to its conformational change not achieving maximum FRET for the few metals tested. Finally, X\textsubscript{11}A and X\textsubscript{11}E showed a smaller fold change for most metals, but again shifted into two phase binding for the Lns. All these data show promise in the field of Ln separations, and could hopefully glean insight into how this protein can be used for industrial purposes through engineering of \textit{Mex} LanM.

<table>
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<tr>
<th>Condition</th>
<th>La\textsuperscript{III} present (mg/gDCW)</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>1</td>
<td>0.0716</td>
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<tr>
<td>2</td>
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<tr>
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<td>5</td>
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There is still much work to be done in understanding Ln selectivity within the series. *Mex* LanM could be engineered further in order to learn a bit more about its future hope into industrial separations. Furthermore, characterization of 4P$_2$A and N108D for structural information would give key insight in how to move forward in some of this engineering. Also, as displayed in Chapter 2 and later in Chapter 4, examination of LanM homologs, both in their natural form as well as in this sensor framework, could provide more insights using biomolecules to separate Lns from each other.

YCLanM has shown some promise in the characterization of Lns in plants, exhibiting a FRET response in initial experiments when treated with La$_{III}$. However, subsequent trials showed similar FRET ratios for La$_{III}$ treated and untreated plants. There is much future work to be done on these systems, as only preliminary experiments have worked in showing Ln uptake. Moving forward, these experiments must be optimized to show maximum FRET response which could potentially include changing of plant growth conditions, imaging points, and time scales of metal exposure. Once this sensor is optimized and working, it can then be used for more specific organelle targeting in order to show localization within the plant cell. These results could lead to a multitude of discoveries and tests in order to elucidate the mechanism of Ln uptake and utilization in the cell.
CHAPTER 4 MISCELLANEOUS PROTOCOLS AND RESULTS

4.1. Introduction

This chapter describes several miscellaneous projects related to characterization of protein involved in lanthanide utilization. First, untagged PqqT C192A was expressed and purified for the sake of crystallography in order to understand PQQ recognition by this protein. This protein has been characterized from *M. extorquens* and has been seen to be a periplasmic binding protein for PQQ. It has been shown to bind one equivalent of PQQ and been implicated as the first protein used in cellular trafficking of this cofactor\(^4^7\). Unfortunately, crystallization has not yet been successful.

Furthermore, LanM homologs from two additional organisms were explored for their metal binding characteristics. First, *Hansschlegelia* sp. Dub (*Hans*) LanM was found by BLAST search and exhibited 31% sequence identity with Mex LanM, although its EF hand sequences were unusual (Figure 4.1). *Hans* was of particular interest because only one EF hand had a Pro residue at position two, unlike all four EF hands in Mex LanM. In addition, *Hans* LanM has three EF hands with an Asn residue at position one, and only one Asp. Asn residues at the first position often abrogate Ca\(^{II}\) binding. Given that Mex LanM’s EF 4 starts with an Asn and is the weakest binding site, it was unclear what affect this would have on lanthanide binding.

<table>
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<th>DPDSDDGTVSLAE</th>
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<td>DPNDGDGLDLKE</td>
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<td>NPNDNDGTIDARE</td>
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<td>DPNDGDGLDAKE</td>
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*Figure 4.1*. EF Hand sequences for *Mex* LanM, *Hans* LanM, and Rhal LanM.
Second, an organism from *Beijerinckia* (named Rhal LanM) was found to grow in pH 5 conditions. These methylotrophs were grown in media at pH 5 and in the presence of Lns, just as *M. extorquens*, but at an even lower pH\textsuperscript{48}. It was thought that perhaps this LanM would perform better at lower pH than even *Mex* LanM (as discussed previously) because of the organism’s natural growth conditions. Rhal LanM has 56% sequence identity with *Mex* LanM, with three of its four EF hands containing Pro residues at position two (Figure 4.1). Also, its first EF hand is missing a carboxylate at the ninth position. The possible library of LanM proteins is vast, and the goal of these studies is to explore the diversity of the Ln coordination in Nature.

Finally, in collaboration with the Giedroc group at Indiana University, we aimed to discover new Ln-binding proteins. The only such proteins currently known in *M. extorquens* are XoxF1, ExaF, LanM, Lanb, and XoxF2, but we hypothesize additional Ln-binding proteins likely exist. We have followed metalloproteomic methods reported by the Robinson group\textsuperscript{49,50}, running lysate from *Mex* using SEC followed by injection into ICP-MS to detect La\textsuperscript{III} containing fractions.

### 4.2 Experimental

All primers used in this chapter are listed in below.

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<th>Sequence</th>
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#### 4.2.1 Untagged PqqT C192A

#### 4.2.1.1 Cloning

PCR was performed using PqqT-f and PqqT-r with an annealing temperature of 68°C. The purified PCR product was digested with NdeI and EcoRI-HF for 1 h. pET-24a was digested with the same restriction enzymes and, following gel electrophoresis, the vector fragment was excised and purified using the Zymoclean Gel DNA Recovery Kit. The digested PCR product
was ligated into the digested vector at 3:1 insert:vector using T4 DNA ligase according to the manufacturer’s protocol. Colonies were screened by PCR (OneTaq Quick-Load) and confirmed by sequencing using T7P and T7T (Genewiz).

4.2.1.2. Expression

Chemically competent *E. coli* BL21 (DE3) cells were transformed with the pET-24a-untagged PqqT C192A and plated on LB-agar plates with 50 µg/mL kanamycin and grown at 37°C. A single colony was used to inoculate 100 mL of LB (50 µg/mL Kan) for ~16 h at 37°C with shaking at 200 rpm. After overnight shaking, 40 mL of starter culture was used to inoculate 2 L of LB (50 µg/mL Kan) supplemented with 0.5 mM MgCl\(_2\) and 0.1 mM CaCl\(_2\). At OD\(_{600}\) ~0.6, IPTG was added to a final concentration of 0.2 mM and the temperature was decreased to 18°C for ~18 h. The cells were pelleted by centrifugation for 7 min at 7000 x g at 4°C yielding ~2.5 g of cell paste per L of culture.

4.2.1.3. Purification

The cell paste was resuspended in 5 mL/g of cell paste in Buffer A, 2 U/mL DNAse, 0.25 mM PMSF, and complete mini protease inhibitor Tablets (1 per 10 mL). Cells were lysed on ice using sonication with pulse sequence of 20 s on/40 s off with 50% amplitude and debris was pelleted by centrifugation at 40,000 x g for 20 min at 4°C. The suspension was decanted and applied to a 2.5 cm x 3 cm (15 mL) Q-sepharose column pre-equilibrated with 10 CV of Buffer A. The column was washed with 2 CV of Buffer A and eluted with a 40 x 50 mL gradient from 10 mM KCl to 300 mM KCl. The eluted protein was concentrated to 2 mL using an Amicon Ultra 10-kDa MWCO centrifugal filter device. Higher and lower molecular weight contaminants were removed and buffer was exchanged into Buffer E by SEC on a HiLoad 160/600 Superdex 75 pg column. After column equilibration, protein was loaded using 2 mL capillary loop which washed with 3 mL Buffer B and the column eluted with 1.2 CV Buffer E at 0.75 mL/min with 1.5 mL fractions collected (in peak fractionation mode). Untagged PqqT C192A containing fractions
were collected and concentrated using an Amicon Ultra 10-kDa MWCO centrifugal filter device to ~2 mL (yield of ~5 mg/g cell paste) and stored in -80°C freezer.

4.2.2. Hansschlegelia sp. Dub LanM

G-block for Hans LanM is as follows with the His-tag in blue, and restriction sites underlined:

AATA CATATG CATCATCACCATCATCAGCAAGTGGCGGATGCTTTGAAGGCCTTAACCAAGACAATGACGATTCGCTGGAAATTGCAGAGGTAATCCACGCAGGCGCAACTACGTTGACAGAAAGGATTTGGCTAGAGCTAATAAGACGGGAACTGAGTGGACGAATGGCTGAAGATCCTGCGTACTAGATTTAAAAGAGCCGATGCTAATAAGGATGGCAAATT AACGGCTGCGAGGGTTAGGATTCCAAAGCGGGGCAAGGGGTATTGGTCATGATCATGAAATAAGAATTCTATT

4.2.2.1. Cloning

N-terminally His-tagged Hans gblock was acquired (IDT) and double digested using NdeI and EcoRI for 1 h at 37°C. pET-24a was digested in the same manner following gel electrophoresis, the vector fragment was excised and purified using the Zymoclean Gel DNA Recovery Kit. The digested gblock was ligated into the digested vector at 3:1 insert:vector using T4 DNA ligase according to the manufacturers protocol. Transformation was screened using colony PCR (OneTaq Quick-Load) and confirmed by sequencing using T7P and T7T (Genewiz).

4.2.2.2. Expression of Hans LanM

Hans LanM was transformed into chemically competent E. coli BL21 (DE3) cells and plated on LB-agar plates with 50 µg/mL kanamycin (Kan) and grown at 37°C for ~10 hours. A single colony was used to inoculate 100 mL of LB (50 µg/mL Kan) for ~16 hours at 37°C with shaking at 200 rpm. After overnight shaking, 40 mL of starter culture was used to inoculate 2 L of LB (50 µg/mL Kan) and grown at 37°C with shaking at 200 rpm. At OD_{600} ~0.6, IPTG was added to a final concentration of 0.2 mM and shaken for an additional 3 hours at 37°C. The cells were pelleted by centrifugation for 7 min at 7000 x g at 4°C yielding ~1.5 g of cell paste per L of culture.
4.2.2.3. **Purification**

All purifications were done at 4°C. Cell paste was resuspended in 5 mL/g of Buffer F [50 mM sodium phosphate, 10 mM imidazole, 5% glycerol, pH 7.0], 0.40 mM PMSF, 2U/mL DNAse, and complete mini protease inhibitor Tablets (1 per 10 mL). Cells were lysed on ice with sonication for 20 s on and 40 s off for 20 min. Debris was pelleted with centrifugation at 40,000 x g for 30 min at 4°C. Supernatant was decanted and loaded to a 1.5 x 4.5 cm (8.0 mL) Ni-NTA column pre-equilibrated with Buffer F. The column was washed with 15 CV of Buffer F followed by elution with Buffer F + 250 mM imidazole. Eluted protein was concentrated to ~2 mL with an Amicon Ultra 3-kDa MWCO centrifugal filter device. High and low molecular weight contaminants were removed and buffer was exchanged into Buffer E by size-exclusion chromatography on a HiLoad 160/600 Superdex 75 pg column. After column equilibration, protein was loaded using 2 mL capillary loop which washed with 3 mL Buffer B and the column eluted with 1.2 CV Buffer E at 0.75 mL/min with 1.5 mL fractions collected (in peak fractionation mode). Hans LanM containing fractions were collected and concentrated using an Amicon Ultra 10-kDa MWCO centrifugal filter device to ~2 mL, quantified using $\varepsilon_{280} = 11000 \text{ M}^{-1} \text{cm}^{-1}$ (yield of ~8 mg/g cell paste) and stored in -80°C freezer.

4.2.2.4. **Stoichiometry and metal binding**

Hans was assayed for its stoichiometry with La$^{III}$ and metal affinity for La$^{III}$, Nd$^{III}$, and Gd$^{III}$ as described in Chapter 2 with Xax LanM protein.

4.2.3. **Beijerinckia LanM (Rhal LanM)**

The sequence for Rhal LanM is as follows:

ATGGCACAATGGACATGAAGCCATCGACCACGAGATGATGGGACTTTCTCTTGC
TGAGGCTCAAAGACCAGACGGCCCAGAAAATTTGCGGCTATGGACCTGACCATGAGGGA
CAATTGATCTTTAAAAAGAAGCTAAGGGAAGATGGCTAAAGCGAAGTTCAAAAAGACGGAC
GCAGATACGACGGGACCCGTAGACAAGGGTACACTCTCTGCTTTGTTTTGAATCTGCAATT
CAAGGCCGACATGCCGGGAAGCAGGGGACCCCTGAGCGACAGCTAAGAGTAGAATTAGAAAC
CAGCAGAACAAATATTCTTTCCCTTATTTAGTTA
4.2.3.1 Expression

Rhal LanM was acquired from Twist Bioscience (pET-29b-Xax) and transformed into chemically competent *E. coli* BL21 (DE3) cells which were plated on LB-agar plates with 50 µg/mL kanamycin (Kan) and grown at 37°C for ~10 hours. A single colony was used to inoculate 100 mL of LB (50 µg/mL Kan) for ~16 hours at 37°C with shaking at 200 rpm. After overnight shaking, 40 mL of starter culture was used to inoculate 2 L of LB (50 µg/mL Kan) and grown at 37°C with shaking at 200 rpm. At OD$_{600}$ ~0.6, IPTG was added to a final concentration of 0.2 mM and cells were grown for an additional 3 hours at 37 °C. The cells were pelleted by centrifugation for 7 min at 7000 x g at 4 °C yielding ~1.5 g of cell paste per L of culture.

4.2.3.2 Purification

The cell paste was resuspended in 5 mL/g of cell paste in Buffer A, 2 U/mL DNAse, 0.25 mM PMSF, and complete mini protease inhibitor Tablets (1 per 10 mL). Cells were lysed on ice using sonication with pulse sequence of 20 s on/40 s off with 50% amplitude and debris was pelleted by centrifugation at 40,000 x g for 20 min at 4°C. The supernatant was applied to a 2.5 cm x 3 cm (15 mL) Q-sepharose column pre-equilibrated with 10 CV of Buffer A. The column was washed with 2 CV of Buffer A and eluted with 10 CV of 250 mM KCl in 1 CV fractions. The eluted protein was concentrated to 2 mL using Amicon Ultra 3-kDa MWCO centrifugal filter device. Higher and lower molecular weight contaminants were removed and buffer was exchanged into Buffer E by SEC on a HiLoad 160/600 Superdex 75 pg column. After column equilibration, protein was loaded using 2 mL capillary loop which washed with 3 mL Buffer B and the column eluted with 1.2 CV Buffer E at 0.75 mL/min with 1.5 mL fractions collected (in peak fractionation mode). Rhal LanM containing fractions were collected and concentrated as above to ~2 mL, quantified using $\varepsilon_{280} = 1490$ M$^{-1}$ cm$^{-1}$ (~ 10 mg/g cell paste) and stored in -80°C freezer.
4.2.3.3. Stoichiometry and metal binding

Rhal was assayed for its stoichiometry with La\textsuperscript{III} and metal affinity La\textsuperscript{III} (pH 7.2 and 6), Nd\textsuperscript{III}, Gd\textsuperscript{III}, and Ho\textsuperscript{III} as described in Chapter 2 with XαL LanM protein.

4.2.4. Discovery of new Ln-binding proteins via SEC-ICP-MS

Cell pellets of *M. extorquens* AM1 were grown in 100 mL, 250 mL and 500 mL volumes according to established protocols with 1 µM La\textsuperscript{III} and sent to Matthew Jordan at Indiana University for further study\textsuperscript{26}. The 250 mL cell pellet (~0.3 g) was resuspended in 2 mL of Buffer G [25 mM HEPES, 50 mM NaCl, pH 7] and lysed by homogenizer with glass beads. Lysate was centrifuged at 15,000 rpm for 10 mins at 4°C and supernatant was decanted and the centrifugation was repeated. Lysate was loaded on SP-FF or Q-sepharose column with 14 injections of 100 µL and flow through was collected. A gradient was applied from 50 mM NaCl to 1 M NaCl and 900 µL fractions were collected and 100 µL of each fraction were loaded onto the SEC and directly injected into the ICP-MS after the column with \textsuperscript{139}La being detected. After cell growths, all work was done by Matthew Jordan of the Giedroc group at Indiana University.

4.3 Results and discussion

Untagged PqqT expressed, purified, and assayed for its PQQ binding via spectrophotometric titration and ITC as defined in the literature for tagged PqqT\textsuperscript{47}. Untagged PqqT exhibited a $K_a$ for PQQ of $(7.13 \pm 1.84) \times 10^6$ M$^{-1}$ and an $n = 1.08 \pm 0.01$, consistent with
the tagged version. Removal of the tag did not affect PQQ binding to the protein (Figure 4.2) and untagged PqqT was sent for crystallography which has been unsuccessful to date.

_Hans_ LanM was characterized for its stoichiometry and select metal affinities via CD at pH 7.2. _Hans_ LanM bound 4 equivalents of La$^{III}$ according to its conformational change monitored by CD. _Hans_ LanM also displayed picomolar affinity for Lns, as seen in Figure 4.3 with the few $K_{d,app}$’s acquired, although data is preliminary and proper titration curves have not been made. Gd$^{III}$ showed a modest two phase response, whereas La$^{III}$ and Nd$^{III}$ did not. It is interesting with the Asn residues in the first position and the one Pro residue that this protein retains a picomolar affinity for Lns. Further studies are necessary to characterize this protein’s metal binding and its potential to industrial applications.

**Figure 4.2.** A) Spectrophotometric titration of PQQ into PqqT. B) ITC for binding of PQQ to PqqT.
Rhal LanM has not been characterized very extensively, as very few Lns have been tested for affinity. However, this LanM was interesting in the fact that it only bound 2 equivalents of La\textsuperscript{III} as seen in CD titrations as well as XO titrations (Figure 4.4). Rhal LanM exhibits a modest two phase response to La\textsuperscript{III} while Nd\textsuperscript{III}, Gd\textsuperscript{III}, and Ho\textsuperscript{III} exhibit a one phase response (Figure 4.5). Titrations with La\textsuperscript{III} carried out at pH 5 displayed the same two phase response with affinities in the nanomolar range, opposed to Mex LanM which maintains an ~100 pM affinity. The difference in response between Rhal LanM and Mex LanM could be due to the EF hand features, where Rhal is missing one Pro residue as well as a carboxylate residue in EF 1, as discussed previously. These features must be explored in more detail in order to fully characterize this protein’s Ln utilization.

\textbf{Figure 4.3.} Ln-EGTA buffered metal titration for Hans LanM with $K_{d,app}$s of 20 pM for La\textsuperscript{III}, 6 pM for Nd\textsuperscript{III}, and 2 and 0.07 pM for Gd\textsuperscript{III}. 

Figure 4.4. Stoichiometric binding of La$^{III}$ to Rhal LanM. A) CD ellipticity change at 222 nm upon addition of 1 equivalent aliquots of metal. B) XO titration with 13 µM protein. Signal change occurs at 28 µM in this titration.
Figure 4.5. Ln-EGTA titrations for various metals with Rhal LanM at pH 7.2 unless otherwise specified. A) Titration with La\textsuperscript{III} to give a $K_{d,\text{app}}$ = 8.6 and 120 pM and n = 2.8 and 2.0. B) Titration with Nd\textsuperscript{III} to give a $K_{d,\text{app}}$ = 7.1 pM and an n = 3.9. C) Titration with Gd\textsuperscript{III} to give a $K_{d,\text{app}}$ = 1.0 pM and an n = 6.9. D) Titration with Ho\textsuperscript{III} to give a $K_{d,\text{app}}$ = 0.74 pM and an n = 7.4. E) Titration with La\textsuperscript{III} at pH 5 to give a $K_{d,\text{app}}$ in the nanomolar regime (data could not be fit in this preliminary state).
First experiments in the discovery of more La-binding proteins were promising, as shown in Figure 4.6, there are at least five peaks in the ICP-MS analysis, suggesting there are other La-binding proteins present that are not known. Currently, in *M. extorquens* only XoxF1, ExaF, LanM, Lanb, and XoxF2 to bind La$^{III}$, as previously mentioned, but we hypothesize there are many more, necessitating this type of analysis. Based on SEC standardizations, although the range of standards run is not broad, suggest that peak 1 or 2 could be XoxF, and peak 3 may be LanM. All five fractions were sent for MS analysis, but the protein samples were too dilute to get proper signal and identification of protein. More *M. extorquens* lysate that has been run over a Q-sepharose column has been sent to Indiana University for analysis, as previous studies had been done on small 100 µL injections, but the data has yet to be acquired. Those results will hopefully lead to higher concentration fractions off the ICP-MS for further analysis and discovery of new La-binding proteins. The discovery of additional Ln-binding proteins, could lead to a better understanding of Ln usage in biology.

![Figure 4.6. Map of ICP-MS data against Q and SEC fractions for La$^{III}$ grown *M. extorquens*.](image-url)
References


49. Waldron, K. J.; Rutherford, J. C.; Ford, D.; Robinson, N. J. Metalloproteins and metal