

Chapter 7.

Conclusions

This work has been produced by adherence to a stepwise scientific strategy. First, mutants were designed based on the existing knowledge regarding parvalbumin amino acid positions that play an important role in Ca^{2+} -binding. Once these mutants were constructed via site-directed mutagenesis and the mutant protein was expressed, the next step was determining the metal ion-binding properties. In the case where the mutation produced interesting differences in binding properties, the subsequent endeavor was crystallization and structure determination of the mutant protein in complex with relevant cations. The crystal structure was then analyzed to identify the structural bases for the new metal ion-binding properties. Finally, the information gained from the biochemical and structural analyses of the mutant parvalbumin was used to design molecular dynamics (MD) simulations that might elucidate peripheral information that was not obtained from direct experimental observation. In this way, the parvalbumin PVEF-E101D triple mutant was identified as potentially informative and was therefore thoroughly analyzed.

The structures of the F102W and PVEF “parent” mutants were determined to elucidate whether the F102W and D51A substitutions, that were present in addition to E101D mutation in the PVEF-E101D triple mutant, structurally affected the EF site binding loop. The result was that the EF site in these parent mutants was observed to retain wild-type metal ion-binding behavior and wild-type EF-hand structure.

The PVEF-E101D mutant was originally designed by the James D. Potter laboratory because the residue at the last coordinating position of the loop, position 12, had been previously acknowledged as a central factor in the EF-hand metal ion selectivity process (Declercq et al., 1991; Strynadka and James, 1991; da Silva et al., 1995; Houdusse and Cohen, 1996). This residue was known to modulate the number of ligands available to the metal ion, through the change from bidentate Ca^{2+} coordination to monodentate Mg^{2+} coordination, and it to some extent defines the limits of the metal ion coordination sphere through its size and degree of flexibility.

The PVEF-E101D mutant disclosed the significant result that the binding affinity of the PVEF-E101D mutant protein for Ca^{2+} decreased 100-fold and the binding affinity for Mg^{2+} increased 10-fold. Furthermore, the mutant off-rate for Ca^{2+} was approximately 600-fold faster than the wild-type parvalbumin off-rate. The mutant crystal structure revealed that the substituted aspartate could not provide the preferred bidentate coordination of Ca^{2+} , thereby producing a sixfold, octahedral Ca^{2+} coordination geometry. Sixfold coordination is usually characteristic of Mg^{2+} in an EF-hand; EF-hands coordinate Ca^{2+} with a sevenfold, pentagonal bipyramid geometry. However, in the case of the PVEF-E101D parvalbumin mutant, the loop radius seemed to be too large for the aspartate to move in close enough to offer both oxygens to the Ca^{2+} ion. The plasticity of the binding loop was examined, and it was observed that loop flexibility is somewhat defined by the ability of the F helix to move in and out, toward and away from the metal ion, as circumstances warrant. It was clear in the PVEF-E101D/ Mg^{2+} crystal structure that the F helix was able to move in substantially,

on the order of 1 Å, in comparison to wild-type. However, the plasticity of the F helix was not sufficient to allow the aspartate to move in even farther for the bidentate coordination of Ca^{2+} . Therefore, the interplay between the nature (size and flexibility) of the last coordinating residue, and the plasticity of the binding loop imposes the limits on (1) the species of cations that are allowed to bind, and (2) which cation will coordinate with the lowest energy.

The final step in the analysis of the PVEF-E101D mutant was to perform molecular dynamics simulations designed to probe the conclusions derived from the examination of crystal structure. Specifically, MD simulations were used to further investigate the relationship between the last coordinating residue of the EF-hand binding loop and the overall plasticity and flexibility of the loop region.

The Alchemy simulation was crafted to investigate whether a classical MD model could correlate the sevenfold coordination of Ca^{2+} and the transition to the sixfold coordination of Mg^{2+} . In addition, we were interested in observing whether this process was reversible in our model and whether the model would predict that the glutamate at position 12, the last coordinating residue of the loop, would provide bidentate coordination of Ca^{2+} . The transition from Ca^{2+} to Mg^{2+} coordination was accomplished through beginning the simulation with van der Waals parameters representative of Ca^{2+} for the bound metal ions, then reducing this radius during the simulation, until the radius became representative of Mg^{2+} . Reversibility was determined by a final phase of the simulation where the van der Waals parameters were increased from Mg^{2+} values back to Ca^{2+} values. The glutamate at position 12 was

accurately and reversibly predicted to be the source of bidentate ligation of Ca^{2+} in our simulations. This outcome not only verified that the model was appropriate for performing additional simulations, it provided strong evidence that protein structure to some extent imposes this role in selectivity on the residue at position 12. This result was construed from the fact that the electronic properties of glutamate and aspartate are described by identical electrostatic charge distributions in the model system. Additionally, the CD binding site contains an additional glutamate, the coordinating residue at position 9, that could offer bidentate ligation of Ca^{2+} , if the length and flexibility of a glutamate residue were all that was required. It is likely that the residue at position 12 is assigned the bidentate to monodentate transition because (1), the glutamate oxygens are in a favorable position with respect to the other coordinating oxygens in the binding loop for bidentate coordination and (2), the last coordinating residue of the loop is positioned at the outermost edge of the loop, with only water molecules available for hydrogen bonding, and thus, the sidechain carboxylate oxygens are very available to the bound metal ion. In contrast, the coordinating sidechains at loop positions 1, 3, 5 and 9 are in a more crowded region, protected within the binding loop where they can easily make intramolecular hydrogen bonds with nearby residues.

An additional and unexpected piece of information that came from the Alchemy simulation was the implication that serine may not be a good ligand for Mg^{2+} . Our model displays a trend for the serine at position 5 of the CD binding site to move away from the Mg^{2+} ion during MD simulations. It is possible that this result is an artifact of the imprecise charge distribution representation for serine in the MD simulation, and

further investigation would be required to infer definitively that serine coordinates Ca^{2+} well, but Mg^{2+} poorly. However, in the existing EF-hand/ Mg^{2+} structures that contain serine in the binding site, serine never directly coordinates the Mg^{2+} ion. For that reason, further research may be warranted regarding whether EF-hand proteins might exploit serine to discriminate between Mg^{2+} and Ca^{2+} .

A second simulation, the Aspartate simulation, produced results that correlated well with the experimental result that an E101D substitution at EF loop position 12 resulted in monodentate Ca^{2+} coordination. The simulation was intended not only to observe whether our model would predict binding geometry in agreement with the crystal structure, but also to scrutinize the F helix movement that would be required for the aspartate in our model to be able to bind cations. The F helix was able to move in to the binding cavity to allow one aspartate oxygen to bind the Ca^{2+} ion, but the aspartate was unable to achieve a favorable orientation for bidentate Ca^{2+} coordination. It is our postulate that the tug-of-war between the attraction of the carboxylate sidechain oxygens for Ca^{2+} , and their requirement to drag the F helix along with them as they move in towards the metal ion, to a large degree defines the range of coordination spheres that can be achieved in a particular EF-hand site.

In summary, our findings showed that the range of coordination spheres that can be attained by a specific EF-hand binding site were delimited by (1), the length and flexibility of the ligating sidechains, particularly the sidechain at position 12 of the loop, and (2), the plasticity of the binding loop, which was dominantly influenced by the pliability of the F helix. Additionally, the glutamate at position 12, the last

coordinating residue of the binding loop, was chosen in MD simulations as the bidentate Ca^{2+} ligand over other carboxylate sidechains present in the loop, in spite of the fact that the electronic properties of glutamate and aspartate are described by identical electrostatic charge distributions in the model. The bidentate to monodentate transition is assigned to the last coordinating loop residue, position 12, by at least two factors. First, the glutamate oxygens are in a favorable position with respect to the other coordinating oxygens in the binding loop for bidentate coordination in that there are no nearby steric hindrances that would prevent both position 12 sidechain oxygens from binding Ca^{2+} . Second, the position 12 residue is positioned at the outermost edge of the loop, with only water molecules available for hydrogen bonding. This arrangement leaves both of the position 12 sidechain carboxylate oxygens very available to the bound metal ion. As a final point, observation of the CD binding site behavior in MD simulations predicted that serine may be a poor coordinator of Mg^{2+} , and suggested that EF-hands may use this property to help confer a distinction between Mg^{2+} - and Ca^{2+} -binding parameters in certain binding sites. The EF-hand binding site is extraordinary in its ability to discriminate between two small cations, similar in charge, size and electronic configuration. This study has shown that these binding sites exploit subtle, but crucial, differences in the properties of all the pertinent components involved in metal ion binding. These properties include those dictated by structural configuration, properties of the amino acids involved in metal ion coordination, and properties of the metal ions themselves.