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**The Role of Low Frequency Collective Modes  
in Biological Function: Ligand Binding and  
Cooperativity in Calcium-Binding Proteins**

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# **The Role of Low Frequency Collective Modes in Biological Function: Ligand Binding and Cooperativity in Calcium-Binding Proteins**

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## **Abstract**

We have studied the protein dynamics that have been widely discussed as important for direct control of protein function. We used a combination of NMR relaxation, x-ray scattering and molecular dynamics (MD) simulation and to probe the dynamic fluctuations in the  $\text{Ca}^{2+}$ -binding protein calmodulin (CaM). The experimental data provided critical tests for the MD simulations. We also completed spectroscopic studies of a structurally and functional homologous protein, calcineurin to investigate  $\text{Ca}^{2+}$  binding and cooperativity between different  $\text{Ca}^{2+}$  binding sites in that protein. Our project was aimed at obtaining a fundamental understanding of the relationship between protein structural dynamics and function.

## **Background and Research Objectives**

The focus of this proposal was to study the low-frequency collective modes in proteins that have been widely discussed as potential means for direct control of biochemical processes. While it has been shown that protein dynamics are required for protein function, there is scant experimental data to establish a clear link between the specific details of the dynamics and control of a specific function. We used a combination of isotope labeling with NMR relaxation to probe the dynamic fluctuations within an individual domain of the calcium-binding protein calmodulin. Cellular functions are regulated via the actions of a number of "messengers" of which the divalent calcium ion is perhaps the simplest.  $\text{Ca}^{2+}$  exerts its control by binding to a variety of proteins, whose  $\text{Ca}^{2+}$ -binding domains share a common structural motif – the EF hand.

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Calmodulin is a model for this large class of proteins that have been much studied in order to gain insights into how these proteins that use a common structural scaffold while varying the specific side-chains in order to tune the binding properties for specific functions in the cell [1]. The molecular dynamics simulations, x-ray scattering and NMR experiments we completed were aimed at obtaining quantitative information on the dynamic fluctuations widely speculated to be important in target enzyme-binding and calcium-binding in calmodulin, in order to evaluate how the dynamic fluctuations might be influencing function. We also used optical spectroscopy (specifically Fourier Transform Infra-Red spectroscopy) to study another  $\text{Ca}^{2+}$ -binding protein calcineurin which is structurally and functionally homologous to calmodulin [2,3].

### **Importance to LANL's Science and Technology Base and National R&D Needs**

This project addresses fundamental science underpinnings that are key to a broad range of biotechnology applications that depend upon engineering proteins for modified function, e.g. in bioremediation, designer enzymes for industrial applications. The project supports the Laboratory's Bioscience and Biotechnology core competency and our missions in the environment and threat reduction. Our combined experimental and computational approach also supports Laboratory goals in experimental validation of modeling and simulation.

### **Scientific Approach and Accomplishments**

Calmodulin (CaM) and the regulatory component of calcineurin (CnB) represent a large class of  $\text{Ca}^{2+}$ -binding proteins that are ubiquitous in eukaryotic systems and regulate the functions of a large number of activities. These proteins are structurally homologous. The crystal structure of CaM has been solved [4], showing two globular  $\text{Ca}^{2+}$ -binding lobes connected by a central helix that functions as a “flexible tether” [5,6]. The concept of the flexible tether function obtained its strongest support from structural studies of CaM complexed with isolated peptides containing the core CaM-binding sequences in smooth [7] or skeletal [8,9,10,11] muscle myosin light chain kinase, CaM-dependent protein kinase II [12], and CaM-dependent protein kinase kinase [13]. CaM undergoes a dramatic conformational collapse upon binding the peptides. Flexibility in the central helix allows the two lobes of CaM to come together, enfolding the helically-configured target peptides with the hydrophobic clefts in each CaM lobe apposing hydrophobic surfaces in the target peptides.

A current dogma is that target enzyme binding is facilitated by a  $\text{Ca}^{2+}$ -induced conformational change in CaM that results in the exposure of a hydrophobic cleft in each globular lobe of CaM. Interactions between this cleft and hydrophobic residues on the target are important in determining the high affinities for the CaM-target enzyme interactions. There is less structural data available for calcineurin, but there is a crystal structure of the multisubunit enzyme [2,3] that reveals the same general fold for CnB as for CaM.

*Studies of the internal dynamics of calmodulin:* CaM(1-77) has two EF hand type,  $\text{Ca}^{2+}$  binding sites. An EF hand consists of a helix-loop-helix motif, and it is the loop that forms the  $\text{Ca}^{2+}$ -binding site. The arrangement of the EF-hand pairs in CaM(1-77) is typical of these proteins with the two  $\text{Ca}^{2+}$ -binding loops forming the base of a cup-shaped domains. The sides of the cup are formed by the four helices, and it is the inner surface of that cup-shape that forms the hydrophobic cleft.

The major differences observed in the molecular dynamics simulations for the apo- and  $\text{Ca}^{2+}$ -bound CaM(1-77) structures was that  $\text{Ca}^{2+}$  binding stabilized the calcium binding loop regions of the structure making them more rigid, and mobilized collective the motions of the helices that form the hydrophobic cleft and give rise to the open and closed states.

Molecular dynamics studies of  $\text{Ca}^{2+}$ -loaded CaM(1-77) specifically show that the solvent exposed hydrophobic cleft seen in the crystal structure of CaM(1-77) actually transitions from an exposed hydrophobic cleft (open state) to a buried hydrophobic cleft (closed state) occurs over a time scale of nanoseconds. As a consequence of the burying, the radius of gyration,  $R_g$ , of the protein is reduced by 1.5 Å. Based on this prediction, x-ray scattering experiments were conducted on this domain over a range of concentrations. Models built from the scattering data show that the  $R_g$  value and general shape is consistent with the simulation studies of CaM(1-77) (see Figure 1). Based on these observations we postulate a model in which the conformations of CaM(1-77) fluctuates between two different states that expose and bury this hydrophobic cleft. In aqueous solution the closed state dominates the population, while in the presence of peptides, the open state dominates. This inherent flexibility of CaM(1-77) may be the key to the versatility in recognizing peptides of CaM.

Our model for CaM(1-77) dynamics conflicts with the currently accepted hypothesis based on observations in the crystal structure, where upon  $\text{Ca}^{2+}$  binding, the hydrophobic cleft is exposed to solvent. We postulate that crystal packing forces stabilize the protein conformations toward the open configuration. In comparing the dynamics of the apo- and  $\text{Ca}^{2+}$ -loaded CaM(1-77) it is tempting to speculate that the key effect of  $\text{Ca}^{2+}$ -binding is to stabilize the  $\text{Ca}^{2+}$ -binding loops.

This stabilization could provide a stable pivot point for the large amplitude motions of the helices that open and close the hydrophobic cleft in order to facilitate target protein binding. Thus it would be the dynamics of the structure regulating the interactions with target proteins rather than a simple conformational change.

NMR relaxation data have been acquired on the N-terminal domain of CaM, with and without  $\text{Ca}^{2+}$ , using strategic isotope labeling ( $^2\text{H}$  and  $^{13}\text{C}$ ). An initial round of dynamical analysis has been carried out with the  $\text{Ca}^{2+}$ -free data. The magnitude of the rotational motion for internal mobility in the ps to ns timeframe has been estimated for the resolved  $^{13}\text{C}$  and  $^{15}\text{N}$  nuclei. The feasibility of extracting both fast limit and generalized order parameters has been demonstrated. These parameters indicate the magnitude of motion faster than approximately 10 ps and faster than molecular tumbling ( $\sim 4$  ns). In marked contrast to earlier studies, this study provided relaxation data for multiple main chain and side chain atoms of a given amino acid residue. These more extensive data allow for dynamical interpretations in terms of correlated conformational fluctuations thus yielding estimates for the conformational entropy of individual protein side chains. Full analysis of the data will continue beyond the duration of this project, and will provide further experimental data to evaluate against the molecular dynamics results.

*Studies of cooperativity between  $\text{Ca}^{2+}$ -binding sites:* We used site directed mutagenesis, flow dialysis, and Fourier Transform InfraRed (FTIR) spectroscopy to study  $\text{Ca}^{2+}$ -binding to the regulatory component of calcineurin (CnB). Single Glu-Gln (E  $\rightarrow$  Q) mutations were used to inactivate each of the four  $\text{Ca}^{2+}$  binding sites of CnB in turn, generating mutants designated as Q1, Q2, Q3, and Q4 with the number indicating which  $\text{Ca}^{2+}$  site is inactivated. The  $\text{Ca}^{2+}$ -binding studies and the FTIR data confirm that each mutant can be fully decalcified, and that mutations in site 1, 3, and 4 leave three titratable  $\text{Ca}^{2+}$  binding sites that are mostly filled ( $> 90\%$ ) with three equivalents of  $\text{Ca}^{2+}$  added. The wild type protein also only has three titratable sites, but this is because it has one very high affinity site that cannot be decalcified even with extensive dialysis against chelating agents. Changes in the FTIR spectra are observed in the region assigned to the carboxylate stretching frequencies of the glutamate and aspartate residues involved in  $\text{Ca}^{2+}$  ligation, as well as in the amide I' region that provides information on the protein's secondary structure. The FTIR data and the binding data show that there is communication between the  $\text{Ca}^{2+}$  binding sites such that mutation of one site can affect binding at the remaining three sites.

Further, this communication includes communication between the N- and C-terminal domains of CnB. There are significant differences in the spectral changes upon  $\text{Ca}^{2+}$  binding between the wild type, Q1, Q2, and Q3 mutants. However, the  $\text{Ca}^{2+}$  induced spectral changes for the wild type and Q4 mutant are most similar, suggesting that the same three  $\text{Ca}^{2+}$  binding sites are being titrated. The FTIR data thus suggest that site 4 is the very high affinity site.

## Publications

1. Gallagher, S. C., Wang, X., Gao, Z-H., Dyer, R. B., Trehella, J., and Klee, C. B. "Ca<sup>2+</sup> Binding to Calcineurin B Studied Using Site Specific Mutagenesis and Fourier Transform Infrared Spectroscopy, to be submitted to *Biochemistry*.
2. Vigil, D., Gallagher, S. C., Trehella, J., and Garcia, A. E. "A Dynamic of the Hydrophobic Cleft in the N-Terminal Domain of Calmodulin," to be submitted to *Biophysical Journal*.
3. LeMaster, D. M. "NMR Relaxation Order Parameter Analysis of the Dynamics of Protein Sidechains," *J. Amer. Chem. Soc.* 131, 1726-1742 (1999).

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the Calmodulin-Binding Domain of Myosin Light Chain Kinase," *Biochemistry* **28**, 6757-6764 (1989).

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Figure 1: Three views of the crystal structure of  $\text{Ca}^{2+}$ -loaded nCaM (gray ribbon), the final simulation structure of the  $\text{Ca}^{2+}$ -loaded nCaM (black ribbon), and the best-fit model of the  $\text{Ca}^{2+}$ -loaded nCaM calculated from solution small-angle x-ray scattering data (gray crosses). It is easily seen that the final simulation structure is very close in overall size and shape to that seen by the scattering. The crystal structure, on the other hand, is much more elongated than the others.

