VIP In-Cell NMR Spectroscopy Very Important Paper

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Direct Observation of Ca^{2+}-Induced Calmodulin Conformational Transitions in Intact *Xenopus laevis* Oocytes by ^{19}F NMR Spectroscopy**

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Abstract: *The Ca^{2+}-mediated conformational transition of the protein calmodulin (CaM) is essential to a variety of signal transduction pathways. Whether the transition in living cells is similar to that observed in buffer is not known. Here, we report the direct observation by ^{19}F NMR spectroscopy of the transition of the Ca^{2+}-free and -bound forms in Xenopus laevis oocytes at different Ca^{2+} levels. We find that the Ca^{2+}-bound CaM population increased greatly upon binding the target protein myosin light-chain kinase (MLCK) at the same Ca^{2+} level. Paramagnetic NMR spectroscopy was also exploited for the first time to obtain long-range structural constraints in cells. Our study shows that ^{19}F NMR spectroscopy can be used to obtain long-range structural constraints in living eukaryotic cells and paves the way for quantification of protein binding constants.*

Calmodulin (CaM), an intracellular Ca^{2+} sensor protein with two Ca^{2+} binding domains, is involved in many Ca^{2+}-mediated signaling pathways. In smooth muscle, Ca^{2+} binds CaM, thereby inducing a conformational change that enables binding to myosin light-chain kinase (MLCK). This binding activates the kinase, which is key to muscle contraction.[1] Studies in buffer show that Ca^{2+}-bound CaM (Ca-CaM) binds various cellular targets with dissociation constants (K_d) ranging from nM to μM (Figure 1).[2] CaM signaling may be regulated by these different affinities, but whether the conformational transitions, binding affinities, and structures in cells are similar to those in buffer is not known, because direct experimental verification in living cells is challenging.

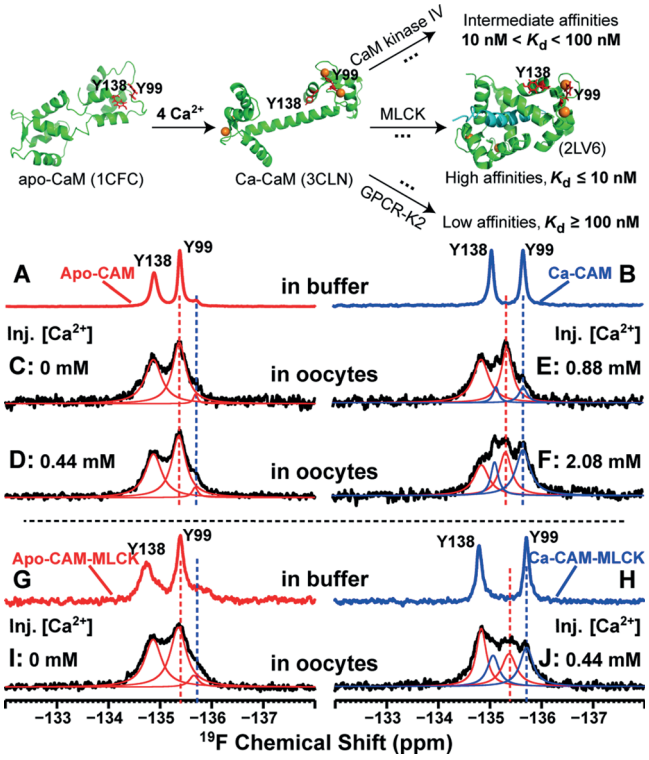

Figure 1. Conformational transitions of CaM in the presence of Ca^{2+} and cellular targets with different Ca^{2+} affinities (PDB_ID: 1CFC for apo-CaM, 3CLN for Ca-CaM, and 2LV6 for the CaM MLCK complex). The location of the ^{19}F labels at Y99 and Y138 are shown. One-dimensional ^{19}F NMR spectra of 3FY-labeled Ca^{2+}-free (apo-CaM, red; A) and Ca^{2+}-bound (Ca-CaM, blue; B) in buffer and in *Xenopus* oocytes injected with ≈20 nL of 5 mM apo-CaM in the presence of 0 (C), 22 (D), 44 (E), and 104 (F) mM Ca^{2+} per oocyte. The injected final intracellular Ca^{2+} concentrations are estimated to be 0, 0.44, 0.88, and 2.08 mM, as indicated. One-dimensional spectra of 3FY-labeled Ca^{2+}-free (apo-) CaM-MLCK (red) in buffer (G) and in oocytes (I), and Ca^{2+}-bound (Ca-) CaM-MLCK in buffer (H) and in oocytes (J). The red and blue dashed lines indicate chemical shifts from the buffer data. The peaks in the oocyte spectra were fitted according to the buffer data for apo-Cam, Ca-CaM, or CaM-MLCK.

In-cell NMR spectroscopy can monitor protein conformational transitions in living cells at atomic resolution.[3] For example, ^{15}N-^{1}H HSQC spectra have been used to study the maturation of superoxide dismutase in live human cells.[3b] Nevertheless, it is known that most globular proteins yield low-quality (or even undetectable) in-cell ^{15}N-^{1}H HSQC spectra because the high cellular viscosity and ubiquitous weak interactions in cells (compared to buffer) lead to broad

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signals.[4] The ^{15}N-^{1}H HSQC spectrum of CaM in *Xenopus* oocytes displays broad, unresolved cross-peaks, which makes it impossible to distinguish conformational states (see Figure S1 in the Supporting Information). ^{19}F NMR spectroscopy has been used to show that CaM in *Escherichia coli* mainly exists in the Ca^{2+}-free apo form.[5] We turned to *Xenopus laevis* oocytes, a model eukaryotic cell system, to study the CaM conformational transition and CaM function because the Ca^{2+} level is difficult to manipulate in *E. coli* and because CaM is native to eukaryotes.

To monitor the conformational transition at different Ca^{2+} concentrations in oocytes, Y99 and Y138 were replaced with 3-fluorotyrosine (3FY). As shown in Figure 1 A,B, the ^{19}F NMR spectra of the Ca^{2+}-free (apo-CaM) and Ca^{2+}-bound (Ca-CaM) forms show distinct shifts in buffer, consistent with previous reports.[6] Resonance assignments were made by site-directed mutagenesis (see Figure S2 in the Supporting Information). We then injected approximately 20 nL of 5 mM apo-CaM along with increasing amounts of Ca^{2+}. The range of Ca^{2+} concentrations is 0 to 2 mM, assuming an oocyte volume of 1 μL. Spectra were then recorded as a function of the Ca^{2+} concentration (Figure 1 C–F). The resonances in cells are broader than those in dilute solution. The conformational state of CaM in cells was inferred by comparing the in-cell spectrum to that obtained in buffer. The in-cell signals were deconvoluted using the buffer data to yield the populations of apo- (red) and Ca-CaM (blue).

When apo-CaM was injected, the ^{19}F NMR spectrum (Figure 1 C) of the apo form is observed in cells, which suggests that the concentration of free Ca^{2+} is very low in oocytes, consistent with reports.[4a] The level of the apo form did not change over the course of 12 h. However, the Ca-bound form was observed after 18 h (see Figure S3 in the Supporting Information), which may be due to Ca^{2+} bursts from cell stress induced after such a long time in the NMR tube, as reported for HeLa cells.[3d]

To our surprise, when Ca^{2+}-bound CaM (final intracellular Ca^{2+} concentration 0.44 mM) was injected, the ^{19}F NMR spectrum (Figure 1 D) showed only the apo form, thus suggesting that Ca^{2+} was stripped from the protein after injection. We speculate that the Ca^{2+} loss may be due to competitive binding of some cell components. To address this possibility, we conducted competitive binding experiments in buffer between Ca-CaM and Mg^{2+} (the most abundant divalent cation in cells), membrane components, nucleic acids from *E. coli* and oocyte extracts (see Figure S4 in the Supporting Information). None of these conditions transformed Ca-CaM into apo-CaM. This result further indicates that Ca^{2+} is strictly regulated in living cells, possibly by Ca^{2+} pumps and exchangers.[7] We then increased the injected final intracellular Ca^{2+} concentration to 0.88 mM. The resulting spectrum (Figure 1 E) indicated the presence of Ca-CaM, with the peak fitting indicating a molar ratio of apo-CaM to Ca-CaM of approximately 6:1. When the injected intracellular Ca^{2+} concentration was increased to 2 mM, the spectrum (Figure 1 F) showed an increased population of Ca-CaM (molar ratio of apo-CaM to Ca-CaM of $\approx$ 1:1). These observations suggest that the protein can be saturated at high intracellular Ca^{2+} concentrations.

To test ideas about target activation, we injected the CaM-MLCK complex in the absence and presence of Ca^{2+} (Figure 1 I,J). The complex was constructed as described.[8] Briefly, 26 residues from the MLCK binding peptide were inserted into the C-terminus of CaM. This construct has a similar response to Ca^{2+} as CaM with an unattached MLCK peptide.[8] Figure 1 G–J shows the ^{19}F NMR spectra of apo- and Ca-CaM-MLCK in buffer and oocytes. Similar to what we observed for CaM, the chemical shift is sensitive to the apo and Ca form of the complex in buffer. Injected apo-CaM-MLCK remains in the apo form after injection (Figure 1 I). In contrast to what we observe with injected CaM (Figure 1 D), both apo- and Ca-CaM-MLCK are present at a final intracellular injected Ca^{2+} concentration of 0.44 mM (Figure 1 J, ca. 1:1 molar ratio of apo- to Ca-CaM-MLCK). In buffer, the Ca^{2+} K_d values for CaM and CaM-MLCK differ by about two orders of magnitude (7.2×10^{-6} M and 2.6×10^{-8} M, respectively).[8] Our observation in cells confirms the higher Ca^{2+} affinity of CaM upon binding to MLCK, one of the cellular target proteins. This observation means MLCK can be activated at lower Ca^{2+} concentrations in cells, thereby supporting the idea that target activity may be regulated in cells by differential affinity for CaM at different Ca^{2+} levels.

It has been reported that some proteins adopt a more compact structure in crowded conditions that mimic the intracellular environment.[4e,9] To address whether the structure of Ca-CaM-MLCK in living cells is the same as it is in buffer, we used pseudocontact shifts (PCSs) to derive structural information about Ca-CaM-MLCK in *Xenopus* oocytes. In buffer, PCSs are a well-established tool for obtaining long-range structural constraints.[10] PCSs provide information about the distance and orientation between an observed nucleus and a paramagnetic center. The CaM variant N60D has higher affinity for lanthanide ions at the second Ca^{2+} binding site, so we used the paramagnetic lanthanide ion Tb^{3+} to induce PCSs, which were measured by calculating the difference in the shift between the paramagnetic N60D $(CaTb)_N(Ca_2)_C$CaM and the diamagnetic N60D $(Ca_2)_N(Ca_2)_C$CaM.[11] We used the 3FY-labeled N60D variant to obtain ^{19}F PCSs because the ^{15}N-^{1}H HSQC spectrum of Ca-CaM-MLCK is poorly resolved in oocytes (see Figure S5 in the Supporting Information). As shown in Figure 2 A,B, the ^{19}F NMR spectra of the 3FY-labeled wild-type and N60D variant are the same, which suggests that the mutation has a minimal structural effect. Two more resonances appear in the presence of 0.7 equivalents of Tb^{3+}, and the population of this species, $(CaTb)_N(Ca2)_C$CaM, is approximately equal to the population of $(Ca2)_N(Ca2)_C$CaM. We calculated a PCS of 0.32 ppm for Y138, and 0.13 ppm for Y99 (Figure 2 C). Inspection of the crystal structure reveals that the labeled position in Y138 is much closer (29 Å) to the Tb^{3+} ion than is Y99 (40 Å). Thus, the PCS for Y138 should be larger than that for Y99, consistent with our observation. When additional Tb^{3+} ions were added (Figure 2 D), the concentration of $(CaTb)_N(Ca2)_C$CaM increased, thus confirming the assignment of the diamagnetic and paramagnetic forms. We then injected $(CaTb)_N(Ca2)_C$CaM-MLCK into oocytes and observed a PCS almost equal to that observed in buffer (Figure 2 E,F). As a consequence of the limited

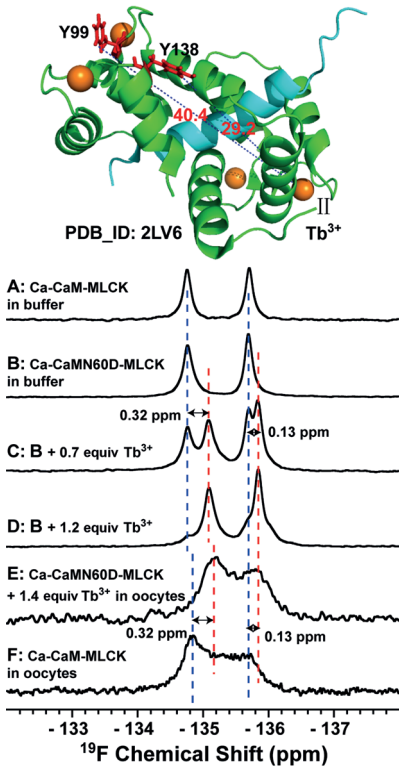

Figure 2. One-dimensional ^{19}F NMR spectra of Ca-CaM-MLCK (A), Ca-CaMN60D-MLCK (B), and Ca-CaMN60D-MLCK in the presence of 0.7 equiv of Tb^{3+} (C), 1.2 equiv of Tb^{3+} (D) in buffer, or in the presence of 1.4 equiv of Tb^{3+} in *Xenopus* oocytes (E, a molar ratio of Ca^{2+} to CaMN60D-MLCK of 5:1), and diamagnetic Ca-CaM-MLCK in oocytes (F). The Ca-CaMN60D-MLCK model is shown at the top (PDB_ID: 2LV6). The distances between the ^{19}F nuclei and the paramagnetic center (II, Tb^{3+}) are indicated.

number of PCS values obtained here, it is difficult to draw a conclusion about cellular effects on the whole protein structure. Nevertheless, our ^{19}F PCS data show that it should be possible to obtain long-range structural constraints by including more ^{19}F labeling sites.

In summary, we have, for the first time, directly observed the CaM conformational transition in intact *Xenopus* oocytes by using ^{19}F NMR spectroscopy. Under physiological conditions, most CaM is in the apo from, and Ca-CaM only appears at high Ca^{2+} levels. The affinity of Ca^{2+} for CaM is enhanced by MLCK in cells, which suggests that MLCK can be actived at lower Ca^{2+} concentration, thereby resulting in further signal transduction. Our observations support the idea that activation of the signaling pathway is regulated by the relative binding affinity of CaM targets at different Ca^{2+} concentrations. Our data also show that in-cell ^{19}F PCSs can yield long-range structural constraints. We believe the ^{19}F method demonstrated here with CaM can be applied to other cellular signal transduction systems in living cells.

Keywords: calmodulin · fluorine · NMR spectroscopy · protein conformation · signal transduction

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