Generation of a Calmodulin-Based EPR Calcium Indicator[†]

Junlong Shao, John Cieslak, and Adrian Gross*

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611

Received July 30, 2008; Revised Manuscript Received November 25, 2008

ABSTRACT: Calmodulin is a ubiquitous calcium dependent protein. In the presence of calcium, calmodulin adopts an altered conformation that leads to the generation of downstream cellular calcium signals. Here we describe the introduction of nitroxide EPR probes into calmodulin by means of site-directed spin labeling. These probes sense the calcium-dependent conformational change of calmodulin and, therefore, can serve as calcium indicators. In combination with a light-sensitive calcium chelator, spin-labeled calmodulin can be used to demonstrate calcium release by flash photolysis. These results provide an important step toward describing the molecular dynamics of calcium-induced conformational changes in proteins using EPR spectroscopy.

Cellular signals are generally propagated through sets of proteins that undergo structural changes and acquire altered function in response to an upstream signal. Many proteins in these signaling cascades can be viewed as molecular switches with two energetically favorable conformations, a functionally inactive conformation at rest and a functionally active conformation that is stabilized after the arrival of the signal. By determining the structure of the switch in both states, the nature of the conformational change induced by the upstream effector can be determined. However, it is of interest not only to understand the two end points of the structural transition but also to elucidate the pathway along which the inactive conformation transforms itself into an active conformation. One technique that has the potential to deliver such dynamic structural information is site-directed spin labeling in combination with EPR spectroscopy (*1*−*3*). In order to time resolve structural changes in a protein using EPR spectroscopy, a strategically placed spin label is monitored while a rapid perturbation of the sample conditions triggers the conformational change of the protein (*4*). The observed time-dependent data describe the relaxation of the protein structure in response to the perturbation at the location of the spin label.

Calcium is an important cellular signal and a suitable conformational trigger for the type of EPR studies described above. Calcium concentrations can be changed in an EPR[1] experiment by rapidly mixing two solutions with different calcium concentrations (*5*), or by flash photolysis of caged calcium (*6*). In the present study we have generated calcium sensitive calmodulin probes in order to test the feasibility of flash photolysis as a means of calcium release in EPR. Calmodulin was chosen as a model protein because it is well-behaved biochemically and undergoes a well-characterized structural change upon calcium binding (*7*). Indeed, calmodulin has previously been used as a calcium indicator for fluorescent applications (*8*). Calmodulin consists of two structurally homologous domains joined by a flexible linker. Each domain contains two helix-turn-helix motifs (EF-hands) that bind calcium ions with dissociation constants in the micromolar range (four calcium ions per calmodulin) (*9*). Based on the apo and holo structures of calmodulin (*10*), six spin-labeled mutants likely to report on the calcium-induced conformation change were generated. The most sensitive mutant was selected to serve as a calcium sensor to evaluate calcium release by flash photolysis inside the EPR cavity.

RESULTS

Six sites likely to report on the calcium-induced conformational change were selected based on the known apo and holo structures of calmodulin (Figure 1A). Two sites were selected in the N-terminal lobe, and four in the structurally homologous C-terminal lobe. The nitroxide spin probe R1 was introduced at each site by means of site-directed spin labeling (Figure 1B). The effect of calcium binding on the EPR line shape at each site was determined by acquiring room-temperature spectra at X-band in the presence and absence of calcium (Figure 2). Two mutants showed substantive line shape changes upon calcium binding: the line shape of I100R1 changed from highly mobile in the absence of calcium to immobile in its presence, whereas the reverse was observed for the line shape of M109R1. The line shape of I27R1, the homologous mutant to I100R1 in the N-terminal lobe, showed a similar transition from mobile to immobile as its counterpart, albeit with less dynamic range. The remaining three mutants (V35R1, L105R1, M145R1) showed only discrete line shape changes upon calcium binding and were not further studied.

[†] This work was supported by NIH Grant GM58568 to A.G.

* Correspondence should be addressed to this author. Tel:(312) 503-3375. Fax: (312) 503-5349. E-mail:a-gross@northwestern.edu.

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-1-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OD, optical density; PMSF, phenylmethanesulphonylfluoride; R1, designation for spin labeled side chain; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi8014295 CCC: $40.75
Published on Web 12/30/2008

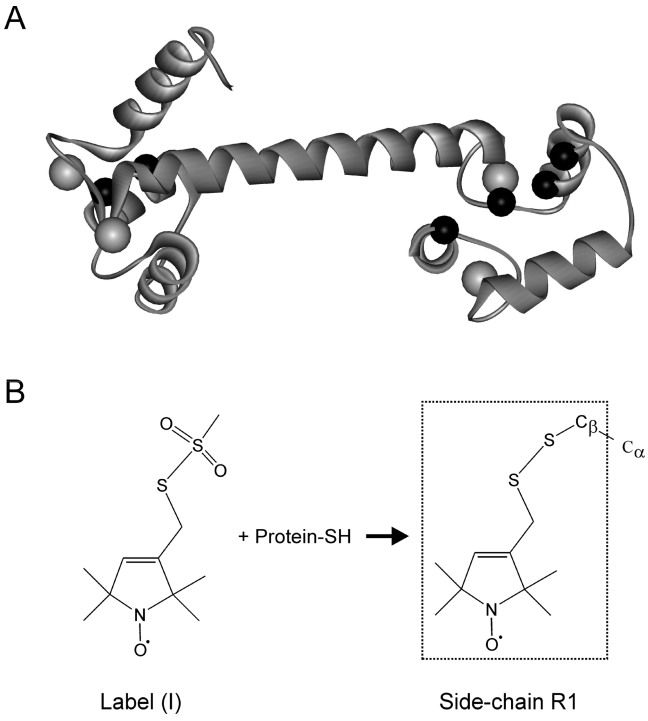

FIGURE 1: Site-directed spin labeling of calmodulin. (A) Labeled sites in calmodulin. The structure of calmodulin is shown as a gray ribbon, the labeled sites are black spheres, and calcium ions are gray spheres. (B) Reaction of the methanethiosulfonate reagent I with cysteine to generate the side chain designated R1.

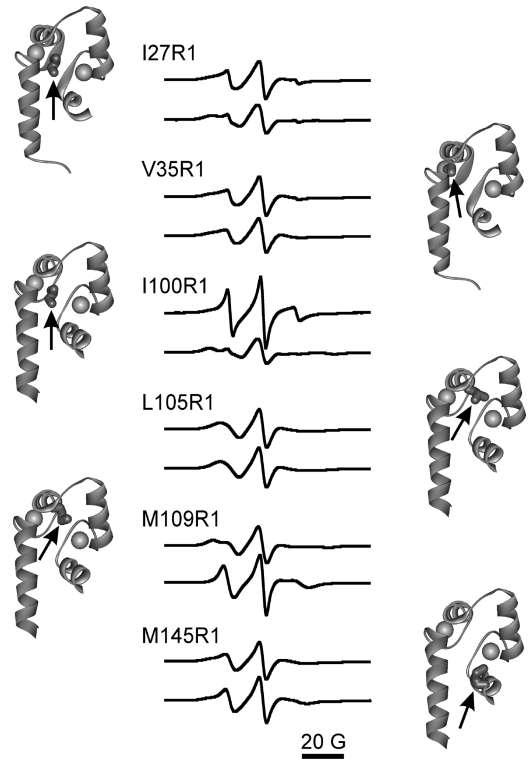

FIGURE 2: Calcium induced line shape changes of spin-labeled calmodulin. A single domain of calcium-bound calmodulin is depicted as a ribbon structure with the side chain of the mutated residue shown as CPK models (arrows). Two bound calcium ions are depicted as spheres. For each mutant, the calcium-free (top) and the calcium-bound (bottom) spectrum are shown (10 mM calcium). Significant calcium-induced spectral changes are observed for the I100R1 and M109R1 mutants.

The most sensitive mutant (I100R1) and its structural homologue in the N-terminal lobe (I27R1) were selected for further study (Figure 3). In a series of titration experiments a constant concentration of calmodulin (100 μM) was mixed with different concentrations of calcium (0−100 mM). Figures 3A,B show the observed line shapes for these two mutants as a function of increasing calcium concentration. As the calcium concentration is raised, a steady transition from the apo line shape to the holo line shape is observed in both mutants. Indeed, at each concentration of calcium, the line shape can be described as a linear combination of the apo and holo line shapes, indicating that calcium is binding to an increasing fraction of calmodulin with increasing concentration (data not shown). For further analysis of the structural transition, it is convenient to define a parameter based on the line shape. The ratio between two prominent line shape features (A_{im}/A_{p-p}, see inset of Figure 3C) is plotted as a function of total calcium in Figures 3C,D. These graphs demonstrate the conformational change of calmodulin as sampled locally by the two structurally homologous spin probes. It is immediately apparent that the two mutants react quite differently to increasing calcium. This difference is due to the different calcium affinities of the two sites. Figure 3E shows the conformational change as a function of the estimated free calcium concentration in solution. The data demonstrate that the local calcium affinity of the N-terminal site is about 1 order of magnitude higher than that of the C-terminal site.

The local nature of the structural transition reported by calmodulin spin labels extends to the level of the individual EF-hand. Figure 4 demonstrates how the I100R1 probe reacts to the destruction of the two calcium binding sites located within the same lobe as the probe itself (Figure 4A). The mutant D93A removes a coordinating carboxyl group of the calcium binding site located in the same EF-hand as the I100R1 probe, whereas the D129A mutant does the same for the remote EF-hand of the lobe. The effect of these two mutants on the calcium-dependent signal of I100R1 is very different (Figure 4B). The binding affinity was only slightly reduced by the D129A mutant (circles), suggesting little cooperativity between the two calcium binding sites, whereas the destruction of the local calcium binding site had a dramatic impact on the calcium-dependence of the spectrum (squares). The immobilization of the I100R1 side chain thus depends primarily on calcium binding to the site located in the same EF-hand as the probe. Indeed, a simple model with four independent calcium binding sites and strictly local immobilization fits the titration data of Figures 3C,D quite well (dotted lines). The data thus underscore the local nature of the conformational change reported by I100R1.

Having characterized the calcium-dependent properties of I100R1, we next used this mutant as a calcium sensor to demonstrate calcium release through photolysis of DM-nitrophen, a light-sensitive calcium chelator (Figure 5A, inset). In absence of light, DM-nitrophen binds calcium with high affinity (K_d ~5 nM) (*6*). In an initial test experiment, photolysis of DM-nitrophen was performed outside the EPR cavity, i.e. the sample was measured inside the cavity, illuminated with UV light outside the cavity, and then remeasured (Figure 5A). The concentrations of DM-nitrophen, calmodulin, and calcium were chosen so that the majority of calcium was bound to the chelator, thus generating a line shape of the mixture before illumination that was

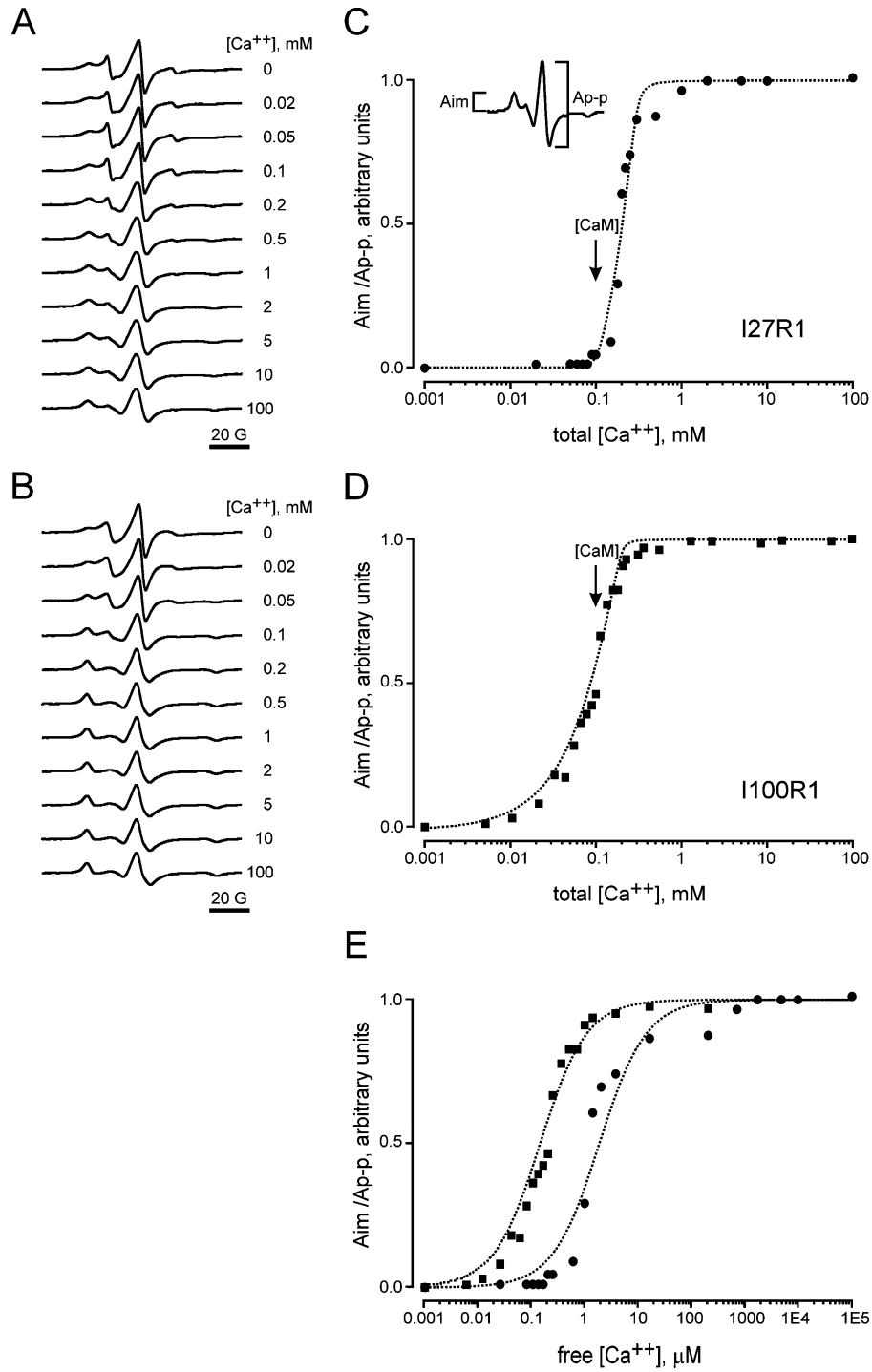

FIGURE 3: Titration of the I27R1 and I100R1 mutants with calcium. (A, B) Continuous wave EPR spectra of I27R1 (A) and I100R1 (B) in response to increasing concentrations of calcium. (C, D) Plot of the ratio of the line shape features A_{im} (immobilized) and A_{p-p} (peak-to-peak) (inset) as a function of increasing calcium concentrations for I27R1 (C) and I100R1 (D), respectively. The concentration of calmodulin (100 μM) is indicated by an arrow (CaM). The abscissa refers to total calcium. The data were fit (dotted lines) using a model with four different and independent calcium-binding sites and strictly local immobilization. (E) Structural change as a function of free calcium. The fit yields a local calcium affinity of ~0.13 μM and ~1.7 μM for sites I100R1 (squares) and I27R1 (circles), respectively.

essentially the same as in the complete absence of calcium (Figure 5A, left). After a few seconds of exposure to UV light (near 350 nm) outside the cavity, the line shape had immobilized substantially (Figure 5A, right), indicating successful photolysis of DM-nitrophen and binding of released calcium to calmodulin. In order to illuminate the sample directly inside the cavity, the light output of a xenon flash lamp was directed to the single light port of the EPR cavity via a light guide. The left trace in Figure 5B shows the EPR signal of a fresh mixture at a fixed magnetic field (at the most sensitive point in the spectrum, see asterisk) while a UV flash was administered to the cavity (arrow). After a short light-induced instability of the EPR signal, the trace settled on a lower level, indicating successful photolysis of DM-nitrophen inside the cavity. Photolysis is far from complete after a single flash (Figure 5B, right). This property can be used to improve the light-induced signal by averaging multiple traces. The light response is specific for calmodulin:

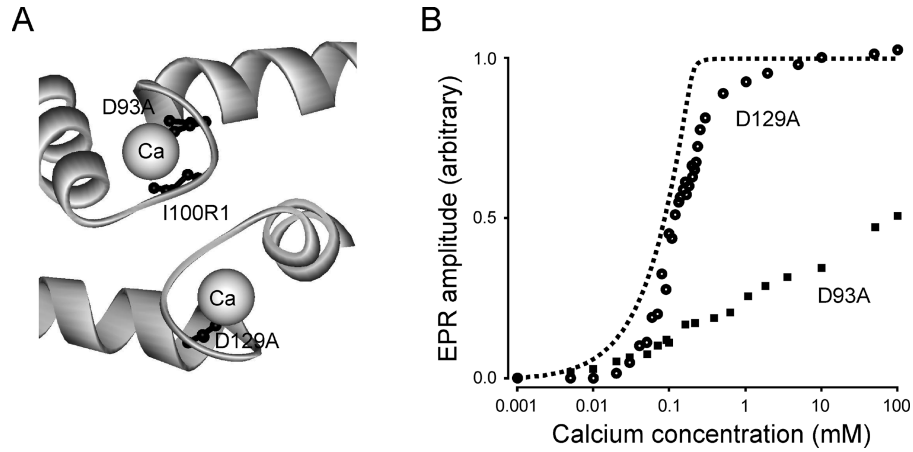

FIGURE 4: Spin-labeled calmodulin reports on local structural changes in proximity of the probe. (A) Close-up view of the two EF-hand domains in the C-terminal lobe of calmodulin (gray ribbons) with side chains draw in black and calcium ions shown as gray spheres. (B) Effect of removing the local calcium binding site (D93A, squares), and the remote calcium binding site (D129A, circles) on the spectral changes in I100R1 upon titration with calcium in comparison with the I100R1 wild type (dotted line, data of Figure 3D).

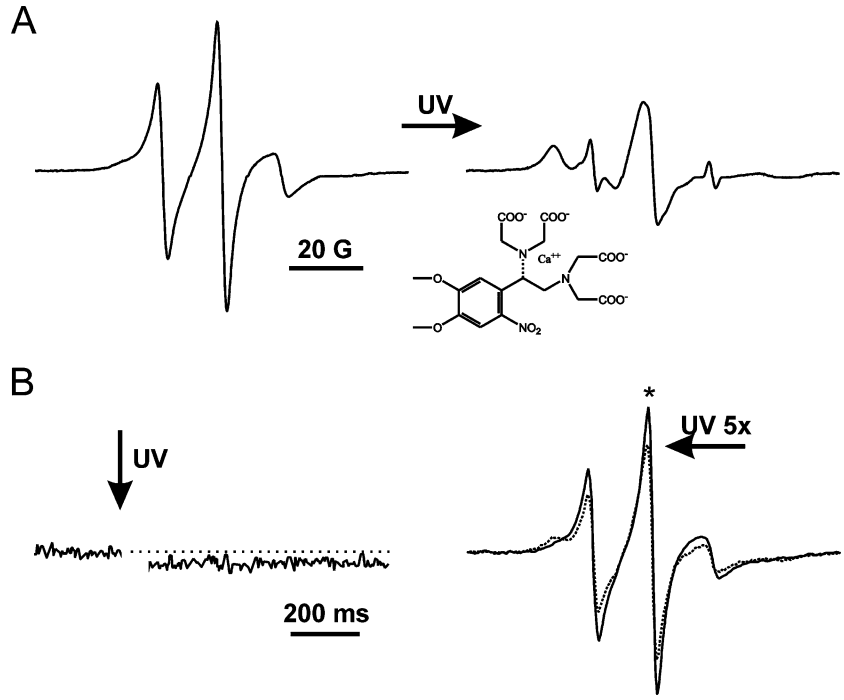

FIGURE 5: Flash photolysis of caged calcium using I100R1 as a calcium sensor. (A) Spectra of I100R1 before (left) and after (right) UV illumination outside of the EPR cavity. The chemical structure of DM-nitrophen is shown with the photocleavable bond indicated as a dotted line (inset). (B) (left) Time scan of I100R1 at a fixed magnetic field with a UV flash administered (arrow) within the EPR cavity. Average of 16 traces. The dotted line at the preflash level was drawn to support the eye. The signal is temporarily lost due to a light-induced instability in the EPR cavity. (right) Single scan spectra of the same sample before (solid line) and after (broken line) five UV flashes. Note the amplitude reduction of the center line (arrow) and the emergence of an immobilized component. The asterisk marks the position of the magnetic field for the time scan shown on the left.

a control sample (spin-labeled KcsA) showed only a transient light-induced instability, but no sustained signal change (data not shown). The data demonstrate that the calcium-induced structural transition of calmodulin was complete within 100 ms. Limiting the interaction between light and EPR cavity should help reduce or eliminate light-induced instabilities that prevent the transition from being observed. This could be achieved through the use of alternative cavities with a direct light path across the sample.

DISCUSSION

Among the strengths of site-directed spin labeling and EPR spectroscopy as a structural technique are the capabilities to detect conformational changes in proteins, and to follow their evolution in real time (*1*−*3, 11*). The latter capability requires that the conformational change occur inside the EPR spectrometer, i.e. the protein must be rapidly transitioned from one conformational state to another inside the cavity. This transition has been achieved through either rapid mixing of different solutions (*5*) or through the introduction of light (*4*). Whereas rapid mixing is suitable for virtually every system in which conformational changes can be elicited by altering sample conditions, light is a very selective trigger that is normally restricted to naturally light-sensitive proteins such as rhodopsin and bacteriorhodopsin (*4, 12*). However, flash photolysis of caged calcium (or of other caged

compounds) has the potential to substantially increase the number of systems amenable to triggering by light and to extend to these systems the substantive technical advantages of this mechanism, including low protein material requirement and fast response time (*13, 14*). The present study was designed to test flash photolysis as an EPR trigger by generating calcium sensors that can report on calcium release by photolysis of a commercially available calcium chelator under experimental conditions.

Toward this end, spin labels were introduced into both lobes of calmodulin at sites expected to report on the calcium induced conformational change. It was found that two structurally homologous sites (I27R1 and I100R1) reacted quite differently to increasing calcium concentrations (Figure 3). The C-terminal probe (I100R1) reports on a higher local calcium affinity than the N-terminal probe (I27R1), consistent with previous studies (*9, 15*). While it is beyond the scope of this study to describe calcium binding to calmodulin in detail, the differences in binding behavior are well described by a simple model of four independent calcium binding sites, different local calcium affinities (Figure 3E), and strictly local effects of calcium binding on the EPR line shape (dotted lines in Figures 3C,D). The primary importance of local structure on the EPR line shape is further illustrated by the strikingly different response to the destruction of two nearby calcium binding sites (Figure 4). This property of the technique is required to time-resolve the structural transition in a site-selective fashion.

The data in Figure 5 demonstrate the general feasibility of flash photolysis of caged calcium in EPR even though the conformational change of calmodulin could not be time-resolved. Nevertheless, the methods as reported here should be suitable for slow calcium-induced processes. Further improvements of the experimental conditions (i.e., resonator design, UV source, etc) should allow for an artifact-free observation of calcium-induced conformational transitions at high temporal resolution. In addition, the EPR probes designed here might prove useful for systems (e.g., light sensitive systems) where optical calcium methods cannot be applied.

METHODS

Protein Expression and Purification. Calmodulin was overexpressed and purified as described (*10*) with minor modifications. Briefly, the DNA sequence for *Xenopus* calmodulin was subcloned into the pQE60 expression vector (Qiagen) between *Nco*I and *Hin*dIII. Site-directed mutagenesis was performed with QuikChange (Stratagene) and verified by sequencing. The plasmids were transformed into Novablue cells (Novagen), grown in Luria broth and induced with IPTG at OD600 ~0.8 for three hours at 37 °C. Cells were harvested by centrifugation, resuspended in lysis buffer containing 40 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mg/L leupeptin/aprotinin, and disrupted using a French press. After centrifugation of the cell debris, the supernatant was loaded onto a DE52 ion-exchange column (Whatman). The column was washed with a buffer containing 20 mM Tris-HCl pH 7.5 and 1 mM $MgCl_2$, and bound calmodulin was eluted with a linear gradient of $(NH_4)_2SO_4$ (0 to 1.2 M) in the same buffer. The fractions containing calmodulin were identified using SDS−PAGE, pooled, and labeled with S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) methyl-methanesulfonothioate (Toronto Research) overnight at room temperature. Spin-labeled calmodulin was further purified using a Phenyl-Sepharose column (GE Healthcare) in the presence of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$, eluted by replacing $CaCl_2$ with equimolar EGTA, and dialyzed against a solution of 50 mM MOPS pH 7.0 and 500 mM NaCl. Samples were prepared for EPR by mixing appropriate amounts of the following stock solutions: 250 μM calmodulin (by UV absorbance), 700 mM MOPS pH 7.0, 5 mM $CaCl_2$, 60% w/v sucrose, and 1 mM DM-nitrophen (DMNP-EDTA, Invitrogen). No visible protein aggregation was observed in any sample.

EPR Measurements. Continuous wave EPR spectra were recorded at room temperature on an X-band EMX spectrometer fitted with an ER4119HS resonator (Bruker Biospin). Sucrose was added (30%) to the labeled protein in Figures 3 and 4 to decrease molecular tumbling and isolate the spectral properties of the R1 side chain (*16*). The spectral changes are selective for calcium: magnesium at a concentration of 10 mM has no significant effect on the line shape of I100R1 (data not shown). The data in Figure 3 were globally fit to a model with four independent calcium sites with each mutant reporting on one site. The free calcium concentration was calculated from the model assuming no nonspecific calcium binding to calmodulin. Photolysis experiments outside the cavity were performed with a standard UV illuminator box (FisherBiotech) as the light source. For time-dependent flash experiments, a xenon flash lamp (SP-20, Rapp OptoElectronic) with a custom-made waveguide was used to direct the light into the single light port of the cavity.

ACKNOWLEDGMENT

We thank Michael Lenaeus for critically reading the manuscript.

REFERENCES

1. Hubbell, W. L., Mchaourab, H. S., Altenbach, C., and Lietzow, M. A. (1996) Watching proteins move using site-directed spin labeling. *Structure 4*, 779–783.
2. Hubbell, W. L., Cafiso, D. S., and Altenbach, C. (2000) Identifying conformational changes with site-directed spin labeling. *Nat. Struct. Biol. 7*, 735–739.
3. Columbus, L., and Hubbell, W. L. (2002) A new spin on protein dynamics. *Trends Biochem. Sci. 27*, 288–295.
4. Farahbakhsh, Z. T., Hideg, K., and Hubbell, W. L. (1993) Photoactivated conformational changes in rhodopsin: a time-resolved spin label study. *Science 262*, 1416–1419.
5. Hubbell, W. L., Froncisz, W., and Hyde, J. S. (1987) Continous and stopped flow EPR spectrometer based on a loop gap resonator. *Rev. Sci. Instrum. 58*, 1879–1886.
6. Kaplan, J. H., and Ellis-Davies, G. C. (1988) Photolabile chelators for the rapid photorelease of divalent cations. *Proc. Natl. Acad. Sci. U.S.A. 85*, 6571–6575.
7. Chin, D., and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol. 10*, 322–328.
8. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature 388*, 882–887.
9. Linse, S., Helmersson, A., and Forsen, S. (1991) Calcium binding to calmodulin and its globular domains. *J. Biol. Chem. 266*, 8050–8054.

10. Chou, J. J., Li, S., Klee, C. B., and Bax, A. (2001) Solution structure of Ca(2+)-calmodulin reveals flexible hand-like properties of its domains. *Nat. Struct. Biol. 8*, 990–997.
11. Hubbell, W. L., Gross, A., Langen, R., and Lietzow, M. A. (1998) Recent advances in site-directed spin labeling of proteins. *Curr. Opin. Struct. Biol. 8*, 649–656.
12. Steinhoff, H. J., Mollaaghababa, R., Altenbach, C., Hideg, K., Krebs, M., Khorana, H. G., and Hubbell, W. L. (1994) Time-resolved detection of structural changes during the photocycle of spin-labeled bacteriorhodopsin. *Science 266*, 105–107.
13. McCray, J. A., Fidler-Lim, N., Ellis-Davies, G. C., and Kaplan, J. H. (1992) Rate of release of Ca^{2+} following laser photolysis of the DM-nitrophen-Ca2+ complex. *Biochemistry 31*, 8856–8861.
14. Ellis-Davies, G. C., Kaplan, J. H., and Barsotti, R. J. (1996) Laser photolysis of caged calcium: rates of calcium release by nitrophenyl-EGTA and DM-nitrophen. *Biophys. J. 70*, 1006–1016.
15. Haiech, J., Klee, C. B., and Demaille, J. G. (1981) Effects of cations on affinity of calmodulin for calcium: ordered binding of calcium ions allows the specific activation of calmodulin-stimulated enzymes. *Biochemistry 20*, 3890–3897.
16. Mchaourab, H. S., Lietzow, M. A., Hideg, K., and Hubbell, W. L. (1996) Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry 35*, 7692–7704.

BI8014295