Rational Design of a Calcium Sensing System Based on Induced Conformational Changes of Calmodulin

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In this paper, we demonstrate how molecular biosensors can be rationally designed through judicious genetic manipulation of proteins that undergo conformational changes upon binding to a targeted species. The Ca^{2+}-binding protein calmodulin was chosen as the protein of interest. A great deal of structural information on calmodulin and the calmodulin-Ca^{2+} complex has been obtained by X-ray[1] and NMR studies.[2] Two of the four Ca^{2+}-binding sites, III and IV, are located within the C-terminal domain, which is linked by a long solvent-exposed helix to the N-terminal domain containing two lower affinity Ca^{2+}-binding sites, I and II.[3] Calmodulin undergoes a conformational change in the presence of Ca^{2+}, and as a result, two hydrophobic pockets located at the C- and N-domains open up and allow for the interaction with calmodulin-binding proteins, peptides, and drugs such as trifluoropiperazine and phenothiazine.

To monitor the conformational changes induced by Ca^{2+}-binding, in the present study, genetically altered calmodulin was labeled with an environment-sensitive fluorophore at amino acid residues that are not directly involved in Ca^{2+} coordination. This was accomplished by employing site-directed mutagenesis and fluorescent labeling of a newly introduced single cysteine residue. Because naturally occurring calmodulins normally do not contain cysteine residues (except some plant calmodulins), introduction of a unique cysteine residue allows for labeling of the protein at a desired position. The plasmid pVUCH-1 that contains the gene of calmodulin[4] was used for genetic manipulation of the protein. Wild-type spinach calmodulin that has a single cysteine at position 26 was also used in this study. Single cysteine residues were introduced by the polymerase chain reaction (PCR) in the N-domain of calmodulin at position 38 (CaM38), at position 81 (CaM81) of the α-helix connecting the two domains, and in the C-domain at positions 101, 109, or 113 (CaM101, CaM109, CaM113, respectively). When the crystal structures of the calmodulin-trifluoropiperazine complex[5] and that of calmodulin[1] are compared, the regions of calmodulin that are involved in binding of hydrophobic drugs can be identified. In that respect, the residues at positions 38 and 81 are not involved in formation of hydrophobic pockets or in calcium coordination. However, residue 26 is positioned between adjacent Ca^{2+}-binding loops in the N-terminal domain of calmodulin.[1]

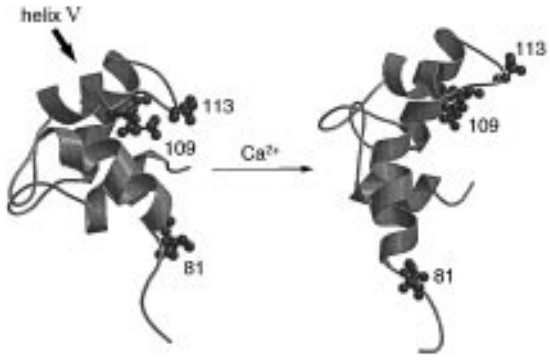

Figure 1. Ribbon diagram of a C-terminal fragment of calmodulin[7] in the absence and presence of Ca^{2+} (generated with the Rasmol 2.5,[8] Molscript 1.4,[8] and the Raster3D 2.0[8] software) with the unique mutation sites at positions 81, 109, and 113 shown in ball-and-stick.

Comparison of the structure of calmodulin and apocalmodulin suggests that upon calcium binding calmodulin undergoes a rearrangement so that hydrophobic residues that are buried in the apo-form are exposed to the solvent and become available for binding target molecules (Figure 1).[6] The structural change upon binding of Ca^{2+} results in the formation of a hydrophobic pocket on the surface of the C- and N-domains, which is absent in apocalmodulin. Since the largest conformational change occurs within the hydrophobic pocket, it seemed reasonable to expect that residues involved in the formation of this pocket would be subjected to significant changes in their microenvironment in the presence of Ca^{2+}. Therefore, by labeling a residue in a hydrophobic pocket, we should be able to sense conformational changes associated with binding of Ca^{2+} at very low levels of this ion.

The hydrophobic pocket in the C-domain of calmodulin is formed by the side chains of 14 residues (Phe92, Ile100, Leu105, Val108, Met109, Leu116, Val121, Met124, Ile125, Ala128, Val136, Phe141, Met144, and Met145).[1] One of them, methionine 109, is located in helix V and is on the surface of the hydrophobic pocket. This is an important consideration from the point of view that labeling can be very difficult if the amino acid of choice is sterically hindered. Further, structural analysis has shown that distances between hydrophobic residues in the C-domain (Ala88−Val108, Phe89−Val108, Phe89−Leu105, Leu105−Phe141, Leu105−Met144, and Val108−Phe141), some of which are in close vicinity to Met109 (i.e., Leu105 and Val108), increase significantly in the presence of Ca^{2+}.[6] Therefore, labeling with a fluorophore at site 109 should position the fluorophore within a microenvironment that undergoes significant changes in the presence of Ca^{2+} (Figure 1). In close vicinity to methionine 109 is glycine 113, except that this residue is not involved in hydrophobic interactions but rather resides on an exposed loop on calmodulin (Figure 1).[1] In order to compare the fluorescent response of two labeling sites located near each other but in different microenvironments, cysteine was introduced at position 113.

Plasmids containing the mutated genes were transformed[9] in *Escherichia coli*. After expression, the proteins were purified on a phenothiazine-affinity column.[10] It should be pointed out that although CaM109 contained a mutation of a residue

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Table 1. Effect of the Labeling Site on the Fluorescence Response of the Different Mutants Labeled with MDCC in the Presence of 3×10^{-6} M Ca^{2+}

protein	labeling site	increase in fluorescence (%)
spinach CaM	26	15
CaM 38	38	15
CaM 81	81	16
CaM 109	109	96
CaM 113	113	28

involved in hydrophobic interaction with phenothiazine,[1] this did not affect its ability to bind to the phenothiazine column in the presence of a Ca^{2+}-containing buffer and subsequently to be eluted with an EGTA-containing buffer. The elution of the mutants was monitored at 280 nm, and the purity of the eluted calmodulin mutants was confirmed by SDS-PAGE.

For selective labeling of thiol groups, we have used a thiol-reactive fluorescent label, *N*-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC).[11] This fluorophore was chosen because the quantum yield of coumarins is dependent on the local environment and, thus, is a good label to probe changes in protein conformation.[12] The purified mutants and spinach calmodulin were labeled with MDCC, and the fluorescence response of these labeled calmodulins was recorded in the absence or presence of 3×10^{-6} M Ca^{2+} (Table 1). The Ca^{2+} concentration was controlled by EGTA at pH 8.0, and the free Ca^{2+} concentrations were calculated by using the program Chelator.[13]

Practically, the same enhancement in fluorescence was observed for spinach calmodulin, CaM38, and CaM81 (Table 1). These data suggest that the environment of the fluorophore does not change significantly upon Ca^{2+}-binding for these three calmodulins. A previous study with spinach calmodulin labeled with 2-(4-maleimidoanilino)napthalene-6-sulfonic acid also showed a small (5%) increase in fluorescence upon Ca^{2+}-binding.[14] We were unable to obtain any data with CaM101 because of very low coupling efficiency of the fluorophore to this calmodulin mutant. It seems that the thiol group at position 101 is not as susceptible to labeling as at position 26 probably due to its location within a Ca^{2+}-binding site. With regard to the MDCC-labeled CaM109 and CaM113, the percent enhancement upon Ca^{2+} binding was much higher (Table 1). This suggests a much more dramatic change in the environment around the fluorescent probe compared to the other MDCC-labeled calmodulin mutants. As it was discussed earlier, this can be related to the position of the labeling site. The fluorescent label at site 109 is much more sensitive to the conformational change induced by Ca^{2+}-binding than the one at position 113, the reason being the location of residue 109 inside the hydrophobic pocket of calmodulin (Figure 1). It is interesting that a difference in position of only four amino acids can have such a significant effect on the fluorescence response and, therefore, on the sensitivity of the system for Ca^{2+}.

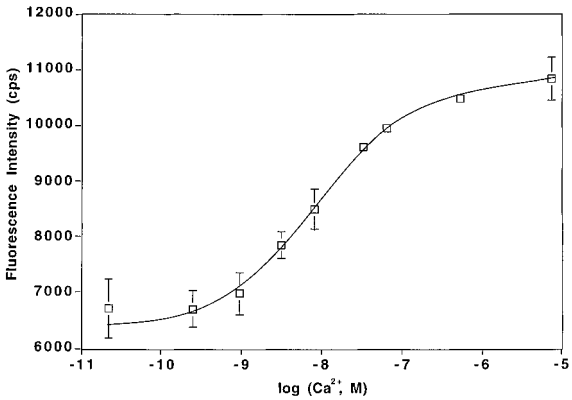

Figure 2. Calibration curve for Ca^{2+} obtained using the CaM109 mutant.

Since the MDCC-labeled CaM109 has shown the largest increase in fluorescence, this labeled protein was used to obtain a calibration curve for Ca^{2+} (Figure 2). The detection limit (determined as the concentration of Ca^{2+} at S/N = 2) was 2×10^{-9} M. It should be noted that calmodulin labeled with fluorescein isothiocyanate (FITC) undergoes a 23% increase in fluorescence signal upon Ca^{2+}-binding[15] and gives a detection limit of 5×10^{-8} M.[16] This is because FITC labels lysine residues on calmodulin. Therefore, given that calmodulin has nine lysines, this fluorophore is distributed throughout the calmodulin molecule. As our studies have indicated, the enhancement in fluorescence is highly dependent on the calmodulin structure because of differences in the Ca^{2+}-induced conformational changes in the various regions of the protein. Thus, in order to obtain larger changes in fluorescence signal in the presence of Ca^{2+}, site-specific labeling is required as demonstrated herein. Another advantage of site-specific labeling is related to the fluorescence background signals. With the single-site labeling, a lower fluorescence background and higher percent change in fluorescence is observed, whereas multiple nonspecific labeling, such as for FITC−calmodulin, is manifested by a smaller percent increase in signal over a larger background. This contributes to the ability of the site-directed MDCC-labeled calmodulin to detect lower levels of Ca^{2+} compared to that of FITC-labeled calmodulin.

In conclusion, rational design of molecular sensing systems by site-directed mutagenesis and site-specific fluorescent labeling can be a powerful tool in the development of very sensitive biosensors. Given the abundance of proteins that undergo structural conformations upon binding to specific ligands,[17] molecular biosensors for other important biomolecules could be designed on the basis of this strategy.[18]

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Supporting Information Available: Experimental materials and methods (8 pages). See any current masthead page for ordering and Internet access instructions.

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