Tryptophan Fluorescence Quenching by Methionine and Selenomethionine Residues of Calmodulin: Orientation of Peptide and Protein Binding†

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ABSTRACT: The two interaction surfaces of the dumbbell-shaped calcium-regulatory protein calmodulin (CaM) are rich in the amino acid Met. In this work we have used fluorescence spectroscopy to study the role of these Met residues in binding the single Trp residue that is found in many CaM-binding domain peptides. This approach is facilitated by the absence of Trp residues in CaM. In addition to the wild-type protein, we studied CaM containing the unnatural amino acid selenomethionine (SeMet), which was biosynthetically substituted for its nine Met residues. Furthermore, a CaM mutant protein in which all four Met residues in the C-terminal domain were mutated to Leu, and the N-terminal domain contained either Met or the unnatural SeMet, was studied. The Trp fluorescence quantum yield of many Trp-containing CaM-binding peptides increases upon binding to calcium-CaM. Moreover, the emission wavelength of the Trp fluorescence is blue-shifted from 353 to 325−333 nm. These parameters indicate movement of Trp from a solvent exposed to a hydrophobic environment. The fluorescence results obtained with these four CaM variants showed that Se is very effective at quenching Trp fluorescence in the calmodulin-bound peptides from myosin light chain kinase (MLCK) and CaM kinase I, while S is somewhat effective (Se > S > C). The quenching effect is markedly distance dependent, as it only influences the Trp residue of the bound peptide ($\leqslant$7 Å) but has little effect on the two Tyr residues in the C-terminal domain of CaM ($\geqslant$10 Å). Since the Trp fluorescence quenching is very dramatic, the protein containing Leu's in the C-terminal domain and SeMet's in the N-terminal domain allowed us to directly determine the orientation of the MLCK and CaM kinase I peptides bound to CaM; in both cases the Trp residue binds to the C-terminal domain of CaM. Our data indicate that SeMet quenching of Trp fluorescence could become a simple and useful tool for studies of protein folding, and protein−protein and protein−peptide interactions.

Calmodulin (CaM)[1] is a 16.7 kDa, ubiquitous protein that can regulate more than 30 different target proteins in eukaryotic cells (for reviews, see refs *1*−*6*). It is a dumbbell-shaped protein molecule with two Ca^{2+}-binding domains connected by a central linker region; it can bind a total of four Ca^{2+} ions. The structures of the calcium-saturated and apo forms of the protein have been reported (*7*−*10*). Each domain of CaM contains two helix−loop−helix Ca^{2+}-binding motifs that are connected to each other by a small β-sheet between the two Ca^{2+}-binding loops (*11*). Upon binding four Ca^{2+} ions, CaM exposes two Met-rich hydrophobic clefts that are important for the binding of target proteins (*3*, *7*, *10*, *12*−*14*). Because the binding of target proteins to CaM mainly involves hydrophobic interactions through amino acid side chains, the Met side chains of CaM have been the focus of much research (*2*, *3*, *5*, *12*). Methionine accounts for 6% of the total amino acids in CaM compared to only 1.5% in regular proteins (*12*, *15*). Moreover, all Met side chains in Ca^{2+}-CaM are exposed on the surface and ready for target protein binding (*7*), whereas they appear to be buried in the apo structure (*10*). The intrinsic flexibility of the methyl groups of the Met side chain and the polarizability of the sulfur atom may contribute significantly to the versatility of CaM, which allows it to activate more than 30 different target proteins in a sequence-nonspecific manner (*2*, *5*, *14*, *16*).

In complexes of CaM and synthetic peptides derived from the CaM-binding domains of skeletal and smooth muscle myosin light chain kinase (MLCK), the Met side chains of CaM contribute almost 50% of the binding surface (*12*, *17*, *18*). In both complexes, which were determined by high-resolution nuclear magnetic resonance (NMR) and X-ray crystallography, respectively, two large hydrophobic side chains (Trp, Phe, or Leu) dock the peptide into the two hydrophobic clefts of CaM. The side chain of Trp4 at the

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[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional; CaM, calmodulin; CT-CaM, calmodulin mutant with all four Met residues in the C-terminal domain of the protein mutated to Leu; HMQC, heteronuclear multiple quantum coherence; MLCK, myosin light chain kinase; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SeMet-CaM, calmodulin with all nine Met residues substituted by selenomethionine; SeMet-CT-CaM, CT-CaM with all five remaining N-terminal domain Met substituted by SeMet; WT, wild type.

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N-terminal end of the MLCK peptide makes extensive contacts with four Met residues (Met109, Met124, Met144, and Met145) in the C-terminal domain of CaM. A critical role for this Trp residue in the recognition event with CaM was indicated by site-specific mutagenesis studies of the intact MLCK protein (*19*, *20*). Alteration of this Trp to other amino acid residues, even other large hydrophobic side chain amino acids such as Leu, did not restore the normal activation properties of CaM on MLCK. Also, for other CaM-binding proteins, such as caldesmon, the Trp residues are pertinent for the interaction with CaM (*21*). Interactions between sulfur atoms of the Met residues in CaM and the Trp aromatic ring in the MLCK peptide (so called sulfur−aromatic interactions) may contribute significantly to the binding process. Weakly polar sulfur−aromatic interactions are one of many such weak bonds that can exist in proteins (*22*−*24*). Statistical analyses of protein structures has revealed that about 50% of Cys and Met sulfur atoms are located within 6 Å of an aromatic ring (*23*). A preferential orientation is favored, with the sulfur atom lying in the plane of the ring and pointing toward one of the aromatic ring protons, highlighting the weak polar interaction. Thermodynamic analysis of model peptides revealed favorable stabilizing energy of −0.2 to about −0.75 kcal/mol for the α-helical Phe-Met pair separated by three residues (*25*, *26*). Thus it seems that sulfur−aromatic interactions between CaM and the MLCK peptide may play an important role in the binding of the peptide and the overall stabilization of the complex (*17*, *18*).

In this work we have studied the sulfur−aromatic interactions in complexes of CaM with synthetic peptides derived from MLCK and CaM kinase I by fluorescence spectroscopy. The three-dimensional (3D) structure of the first complex has been reported (*17*, *18*); but for CaM kinase I only the uncomplexed structure is available (*27*). In addition to WT-CaM, we have utilized a CaM mutant, CT-CaM, in which the four Met in the C-terminal domain (Met109, Met124, Met144, and Met145) are all mutated to Leu (*28*). Furthermore, WT-CaM and CT-CaM containing the unnatural amino acid analog selenomethionine (SeMet) in place of Met were also produced (*29*). We have first characterized these proteins by gel electrophoresis, circular dichroism, enzyme activation studies, and NMR spectroscopy. Subsequently we systematically investigated the fluorescence quenching properties of the sulfur and selenium atoms in these proteins when complexed with the Trp-containing MLCK and CaM kinase I peptides. Our results demonstrate that Se is extremely effective at quenching the Trp fluorescence and that this property can be used for determining the orientation of peptide binding to CaM.

MATERIALS AND METHODS

Proteins. WT-CaM, CT-CaM, and SeMet-CaM were purified from *Escherichia Coli*, as described before using a strain harboring a plasmid with a synthetic gene that encodes the mammalian CaM sequence (*13*, *29*, *30*). The purified proteins were further applied to a Sephadex G-100 column (2 cm × 100 cm). Proteins were eluted by continued washing with 100 mM NH_4HCO_3, pH 8.0, and then these proteins were desalted by lyophilization. CT-CaM incorporated with SeMet (SeMet-CT-CaM) could also be prepared and purified by the same methods. However, some proteolytic fragments were found in this preparation; these were removed by reapplication to a phenyl-Sepharose column, essentially as we described for the separation of tryptic fragments of CaM (*31*). The phenyl-Sepharose column (volume 30 mL) was equilibrated with 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5, and the Ca^{2+}-saturated protein from the Sephadex G-100 column was applied to this column. The column was washed successively with 30 mL 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5; 60 mL 2 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5; and 100 mL H_2O, pH 7.5. The SeMet-CT-CaM was eluted by 2 mM EDTA, pH 7.5, and checked by SDS−PAGE to be devoid of fragments. The protein was lyophilized and desalted on a PD 10 column (Bio-Rad). All proteins were >98% pure as determined by SDS−PAGE and one-dimensional (1D) NMR. The concentration of all CaM samples was determined by ultraviolet spectroscopy, using $\epsilon_{276}^{1\%} = 1.8$; the concentration of some samples was confirmed by amino acid analysis.

The 22-residue MLCK peptide, KRRWKKNFIAVSAANRFKKISS, which encompasses residues 577−598 of the amino acid sequence of skeletal muscle MLCK (its CaM-binding domain, ref *32*), was synthesized by the Core Facility for Protein/DNA Chemistry, Queen's University, Kingston, Canada. The 22-residue CaM kinase I peptide, AKSKWKQAFNATAVVRHMRKLQ, which corresponds to residues 299−320 in rat brain CaM kinase I (its CaM-binding domain, ref *33*) was also synthesized at Queen's University. The purity of the peptide was >95% as judged by high-pressure liquid chromatography and mass spectrometry. The concentration of these peptides was determined spectrophotometrically by using the extinction coefficient for the sole Trp residue in the peptide ($\epsilon_{280} = 5500\ M^{-1}\ cm^{-1}$).

Electrophoresis. SDS−PAGE and nondenaturing urea−PAGE were performed as described before (*34*, *35*). Urea (4M) is added in the nondenaturing gel to prevent the formation of nonspecific interactions; this concentration of urea is insufficient to give rise to denaturation of calcium-CaM and its complexes.

NMR Spectroscopy. All NMR spectra were acquired on a Bruker AMX500 spectrometer with a broad-band 5 mm probe at 36 °C. Samples contained ∼1.5 mM protein (SeMet-CT-CaM was only ∼0.7 mM), 10 mM $CaCl_2$, and 100 mM KCl in 99.9% D_2O. The pH (pD) was 7.50 ± 0.05 without correction for the isotope effect. One-dimensional NMR spectra were collected with 8K complex points and 128 scans with low-power water presaturation. The spectra were processed on a Bruker X32 data station using UXNMR software with 1 Hz line broadening. The two dimensional (2D) ^1H, ^{13}C natural abundance heteronuclear multiple quantum coherence (HMQC) spectra were collected with 1024 × 512 data points in the phase-sensitive mode using the time proportional phase increment technique (TPPI) (*36*, *37*). The number of scans for each experiment was 128 (256 for SeMet-CT-CaM). After acquisition, the spectra were zero-filled once in both dimensions and processed with 72° shifted sine square window functions. All chemical shifts were referenced to 0 ppm for ^1H and ^{13}C using 5,5-dimethylsilapentanesulfonate (DSS).

Fluorescence Spectroscopy. Fluorescence spectra were acquired at room temperature on a Hitachi F-2000 spectrofluorimeter. All samples contained 10 mM Tris-HCl, pH 7.2, and 100 mM KCl. The CaM concentration was 15 μM for

MLCK peptide and 12 μM for CaM kinase I peptide; samples were measured in the presence of 1 mM $CaCl_2$ or 5 mM EDTA. The concentration of the MLCK peptide was 10 μM, and that of the CaM kinase I peptide concentration was 8 μM. Samples were equilibrated by allowing them to stand at room temperature for 3 h. Tryptophan residues were excited at 295 nm, and the emission spectra were recorded from 300 to 450 nm. Since CaM does not contain any tryptophan residues, the single peptide Trp residue of the bound peptide can be selectively excited. All spectra were obtained in triplicate with virtually identical results. The fluorescence contributions from buffer components were subtracted from the spectra of the peptides. For studies of the peptide−CaM variant complexes, the spectrum (excited at 295 nm) of a sample with an identical concentration of CaM was subtracted. In this fashion the relatively small (<10%, around 305 nm) contribution of the two Tyr residues of CaM to the Trp fluorescence spectra was eliminated. In the absence of the Trp-containing peptides, the Tyr fluorescence of CaM was excited at 278 nm and the Tyr emission spectra were recorded from 280 nm to 400 nm; the concentration of CaM was 25 μM in this experiment.

Quenching data were collected by adding 20 μL aliquots of 10 M CsCl to a 1 mL sample. After mixing, the fluorescence emission at the respective maximum emission wavelength for the peptides or the CaM−peptide complexes were recorded. The emission intensities were subsequently corrected for the sample dilution.

RESULTS

Protein Purification, Characterization, and SeMet Incorporation. All four protein variants of CaM (WT-CaM, SeMet-CaM, CT-CaM, and SeMet-CT CaM) could be purified by calcium-dependent chromatography on phenyl-Sepharose as described before (*13*). This indicates that all of them expose similar hydrophobic clefts in the presence of Ca^{2+}, ready for binding to phenyl-Sepharose or target proteins. The extent of biosynthetic incorporation of SeMet into SeMet-CaM was about 85% (*29*). The incorporation of SeMet was even higher in CT-CaM, reaching close to 95% as judged by amino acid analysis (0.24 Met remaining in SeMet-CT-CaM, data not shown); the high level of SeMet incorporation was further confirmed by 2D ^1H, ^{13}C HMQC NMR spectra (see below). Optimal incorporation of SeMet into SeMet-CaM and SeMet-CT-CaM was necessary to ensure that our fluorescence data could be uniquely interpreted.

SDS−PAGE results obtained with the four CaM variants in the presence and absence of Ca^{2+} are shown in Figure 1A. All four calcium-saturated proteins display a calcium-dependent band shift relative to the apo form of these proteins. Although no detailed explanations have been offered for this phenomenon, it is generally agreed that it represents a tertiary structure difference between Ca^{2+}-CaM and apo-CaM (*28*, *38*). Therefore, these results suggest that all four proteins undergo a similar calcium-dependent structural change. We also conducted nondenaturing urea−PAGE electrophoresis experiments to study the interaction of these four proteins with the synthetic peptide derived from the skeletal muscle MLCK CaM-binding domain. Figure 1B shows urea−PAGE results obtained with CT-CaM and

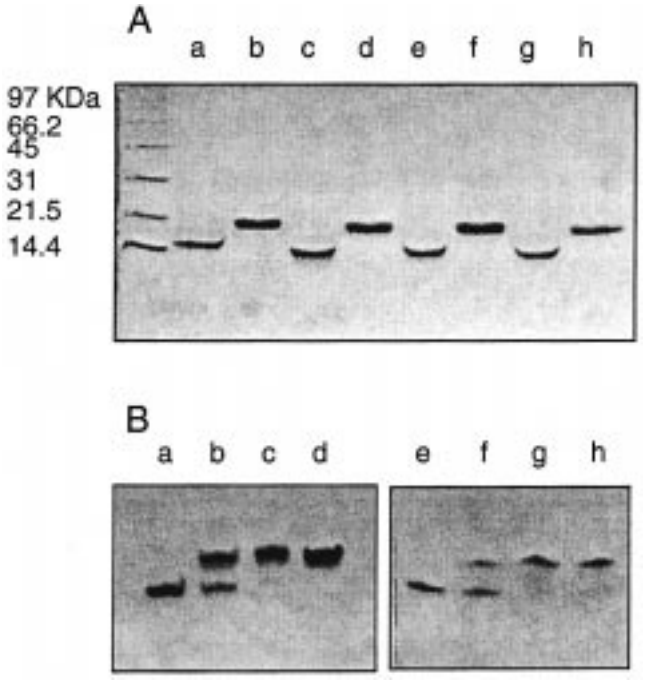

FIGURE 1: (A) SDS−PAGE for the four CaM variant proteins used in this study in the presence and absence of Ca^{2+}, WT-CaM (a, b), SeMet-CaM (c, d), CT-CaM (e, f), and SeMet-CT-CaM (g, h). (B) Nondenaturing urea−PAGE for CT-CaM and SeMet-CT-CaM complexed with the MLCK peptide in the presence of Ca^{2+}. The ratios for CT-CaM and the MLCK peptide were 1:0 (a), 1:0.5 (b), 1:1 (c), and 1:2 (d). The ratios for SeMet-CT-CaM and the MLCK peptide were 1:0 (e), 1:0.5 (f), 1:1 (g), and 1:2 (h). The electrophoresis was performed using the same conditions as described in ref *35*. The concentration of CT-CaM was 15 μM, and the concentration of SeMet-CT-CaM was 7.5 μM.

SeMet-CT-CaM. Typical band shifts are observed which indicate that a 1:1 molar ratio complex of protein and peptide is formed. This experiment revealed not only that all four CaM variants can form a 1:1 complex with the MLCK peptide but also that the binding affinities are in the nanomolar range (peptides with lower affinity for CaM generally do not give rise to a band shift in this assay). Additional band shift experiments also indicated the similarity of CT-CaM and SeMet-CT-CaM compared with WT-CaM and SeMet-CaM (data not shown), and thus these protein analogs are suitable for a comparison of their interactions with the MLCK peptide. We also noted that the interaction between these four CaM variants and the MLCK peptide were Ca^{2+}-dependent, since no band shifts were observed in urea−PAGE in the absence of Ca^{2+} (data not shown). Gel band shift assay results obtained with the CaM kinase I peptide were identical to those obtained with the MLCK peptide (data not shown). We also performed fluorescence titration experiments of the two peptides (2 μM) and the four CaM variants (protein concentrations between 0 and 4 μM; titrated from a 200 μM stock solution). These studies indicated that 1:1 complexes were formed and that the affinity of the peptide for the four proteins was similar and was in the nanomolar range (data not shown).

Further supporting evidence for the suitability of these protein variants to study the interaction between CaM and the MLCK peptide has been obtained from enzymatic activation studies. Vogel and Zhang (*5*) showed that SeMet-CaM activates MLCK in a manner identical to that of WT-

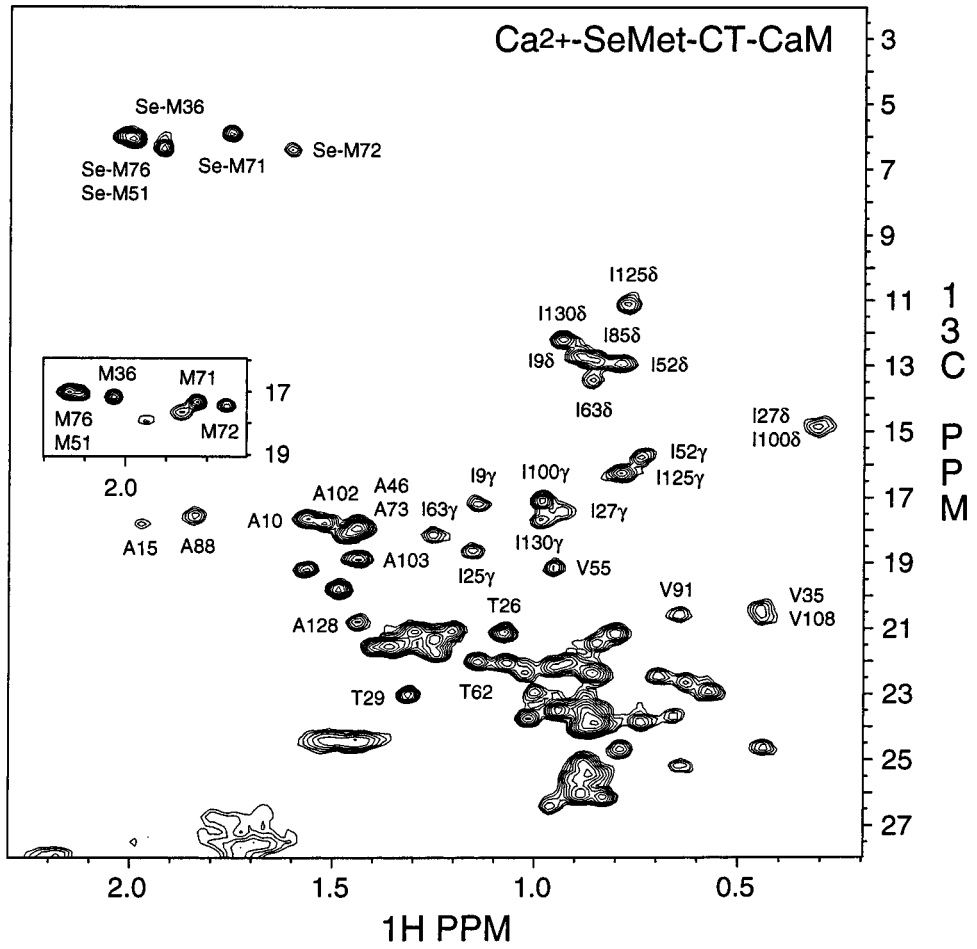

FIGURE 2: Methyl group region of the ^{1}H, ^{13}C natural abundance HMQC spectra of SeMet-CT-CaM in the presence of Ca^{2+}. The SeMet resonances were labeled as assigned (^{1}H, ^{13}C HMQC NMR spectrum for SeMet-CaM is shown in ref *29*). The inset is taken from the HMQC spectrum of CT-CaM, showing the presence of only five Met residues in this protein. Methyl resonances are labeled according to the assignments reported for WT-CaM (*52*). The minimal changes in the chemical shift of these resonances indicate the preservation of the tertiary structure in SeMet-CT-CaM.

CaM. This indicates that the substitution of SeMet for Met in CaM does not change the activity of CaM toward MLCK. Likewise the activation of cyclic nucleotide phosphodiesterase is identical for these two proteins (*29*). The mutant protein CT-CaM can also activate MLCK to only ~65%, but with a similar K_d (*53*), making it a relatively good mutant to study the interaction between CaM and the MLCK peptide. The outcomes of these enzyme assays are consistent with the gel band shift results.

We also recorded 1D ^{1}H NMR spectra for these four proteins in the presence of saturating Ca^{2+} concentrations. The proton resonances between 5.0 and about 5.5 ppm, which arise from the two small β-sheet regions in between the two Ca^{2+}-binding loops in each lobe of CaM, were similar (*39*). In addition, far-ultraviolet circular dichroism spectropolarimetry (not shown) revealed that these four proteins had an identical amount of α-helix. Evidence for the similarity in tertiary structure of the four protein variants is presented in Figure 2. This figure shows the methyl group region of a 2D ^{1}H, ^{13}C natural abundance HMQC NMR spectrum of SeMet-CT-CaM. We observed a very similar pattern in the spectra of the four variants, suggesting that the methyl groups in these proteins are in closely related environments. Because the two hydrophobic clefts in Ca^{2+}-CaM are dominated by these methyl groups (*7*), the four variants thus have a very similar hydrophobic surface available for target protein binding. In addition, it is directly apparent that the markedly upfield shifted resonances originating from the SeMet methyl groups are very strong. Integration of their intensity as well as those for the remaining Met methyl groups confirmed the level of incorporation of SeMet, as determined separately by amino acid analysis and electrospray mass spectrometry (~85% for SeMet-CaM and ~95% for SeMet-CT-CaM).

Fluorescence Spectroscopy. The interaction of the four CaM variants with two target peptides was studied by steady-state fluorescence of the sole Trp residue in the MLCK and CaM kinase I peptides in their respective complexes with CaM. The Trp maximum emission wavelength was at 353 nm for the free MLCK peptide, which indicates a full exposure of the Trp indole ring to an aqueous environment (Figure 3). Upon binding to calcium-CaM, the maximum wavelength of the Trp emission blue shifted to around 331 nm, which suggests that the Trp side chain was now in a solvent inaccessible hydrophobic environment (*40*, *41*). When EDTA was added to this sample to chelate the calcium, the original fluorescence spectrum of the unbound peptide was obtained (Figure 4). We obtained these characteristic changes in emission wavelength for all four CaM

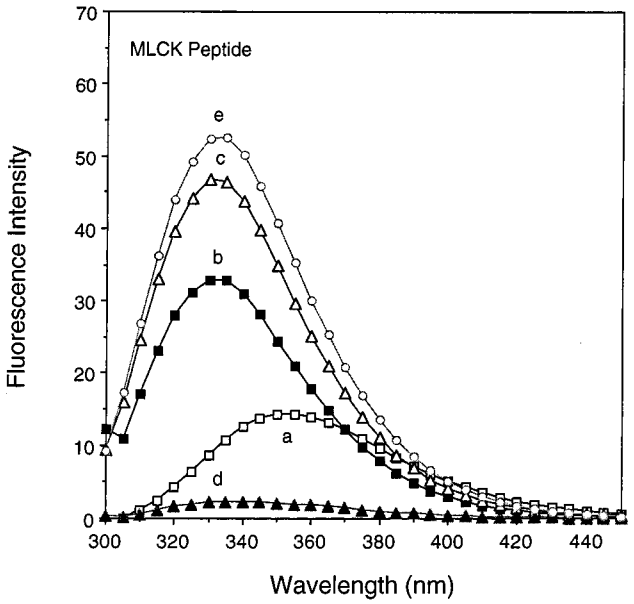

FIGURE 3: Steady-state Trp fluorescence of the MLCK peptide and CaM−MLCK peptide complexes in the presence of Ca^{2+}. (a) MLCK peptide, (b) WT-CaM−MLCK peptide complex, (c) CT-CaM−MLCK peptide complex, (d) SeMet-CaM−MLCK peptide complex, and (e) SeMet-CT-CaM−MLCK peptide complex. The protein and peptide concentrations were 15 and 10 μM, respectively.

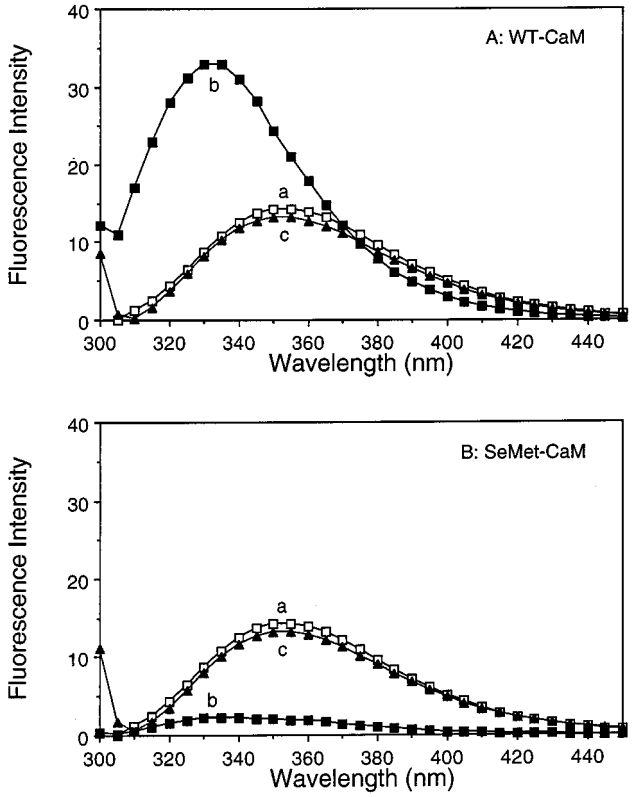

FIGURE 4: Tryptophan fluorescence spectra obtained for 10 μM MLCK peptide in the presence of WT-CaM (A) or SeMet-CaM (B). The spectra obtained in the absence of the protein (a), in the presence of 15 μM protein and 1 mM Ca^{2+} (b), and after addition of 5 mM EDTA to the complex (c) are shown.

variants complexed with the MLCK peptide (Figures 3 and 4, Table 1). However, our data indicate stronger fluorescence for the CT-CaM complex than WT-CaM and almost no fluorescence for the SeMet-CaM complex (Figures 3 and 4, Table 1). The Trp of the MLCK peptide acts as an anchoring

Table 1: Trp Fluorescence Parameters Observed for the CaM−MLCK and CaM−CaM Kinase I Peptide Complexes

	λ_{max} (nm)[a]	fluorescence intensity[b]
MLCK peptide	353	0.43
WT-CaM−MLCK	331	1.00
SeMet-CaM−MLCK	327	0.07
CT-CaM−MLCK	331	1.42
SeMet-CT-CaM−MLCK	333	1.60
CaM kinase I peptide	353	0.42
WT-CaM−kinase I	330	1.00
SeMet-CaM−kinase I	328	0.08
CT-CaM−kinase I	325	1.96
SeMet-CT-CaM−kinase I	326	2.14

[a] λ_{max}= the maximum emission wavelength. [b] Fluorescence intensity was measured as the height at λ_{max}, normalized to WT-CaM−MLCK or WT-CaM−kinase I as 1.00.

Table 2: Distances between the Trp Indole Ring of the MLCK Peptide and the Met Sulfur Atoms in the C-Lobe of CaM in the Ca^{2+}-CaM−MLCK Peptide Complex Structures (Å)[a]

PDB code	M109	M124	M144	M145
2BBM[b]	6.67	6.46	6.32	7.06
1CDL[c]	7.27	5.03	4.40	7.12

[a] The distance is expressed as the average distance from the Met sulfur atom to the nine heteroatoms on the Trp indole ring. The distances were measured by Insight II (Biosym, San Diego). Likewise, the solvent exposure of the Trp side chain in CaM kinase I (PDB code 1CKI) and in 2BBM were calculated from their respective structures as 80% and 4%, using the program MOLMOL. [b] 2BBM, Ikura et al. (*17*); 1CDL, Meador et al. (*18*). [c] We only present the distances in one of the four X-ray structures in the PDB coordinates. The distances in the other three structures are similar.

residue for the C-lobe of CaM and is surrounded by four Met residues in this position in the complex (see Table 2; refs *17*, *18*). Therefore these different intensities likely reflect the different quenching properties associated with carbon (from Leu in CT-CaM), sulfur (from Met in WT-CaM), and selenium atoms (from SeMet in SeMet-CaM). Selenium apparently has the strongest quenching ability, leading to an almost complete loss of fluorescence intensity in the complex of SeMet-CaM and the MLCK peptide (Figure 3, Table 1). The sulfur atoms of Met also seem to possess some quenching ability; when we compare the fluorescence spectrum of the CT-CaM and the WT-CaM peptide complexes, we observe an increase of the fluorescence in the CT-CaM−MLCK peptide complexes. This suggests that the quenching of the Trp fluorescence by the sulfur atoms of Met in the WT-CaM−MLCK peptide complex is now removed (or at least reduced) in the complex with the mutant CT-CaM protein.

These findings also allow us to determine the orientation of the MLCK peptide binding to CaM. The enhancement of Trp fluorescence in the CT-CaM−MLCK peptide complex strongly suggests that the single Trp residue of the MLCK peptide binds to the C-lobe of CT-CaM, which has lost its four Met residues. Furthermore, a control experiment using SeMet-CT-CaM further substantiated this conclusion (Figure 3, Table 1); if the N-terminal Trp in the MLCK peptide binds to the N-lobe of SeMet-CT-CaM, the Se atoms in the N-lobe of CaM should quench its fluorescence. As expected, we observed an increase of fluorescence rather than quenching (Figure 3, Table 1). This outcome with SeMet-CT-CaM

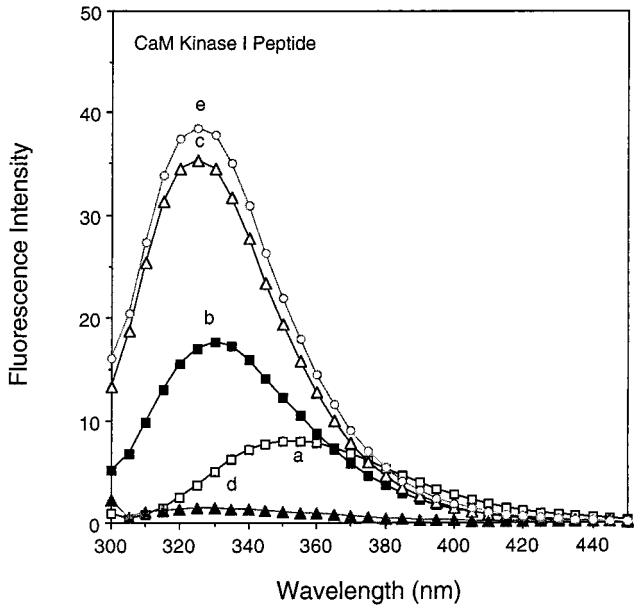

FIGURE 5: Steady-state Trp fluorescence of CaM kinase I peptide and CaM−kinase I peptide complexes in the presence of Ca^{2+}. (a) CaM kinase I peptide, (b) WT-CaM−kinase I peptide complex, (c) CT-CaM−kinase I peptide complex, (d) SeMet-CaM-kinase I peptide complex and (e) SeMet-CT-CaM−kinase I peptide complex. The protein and peptide concentrations were 12 and 8 μM, respectively.

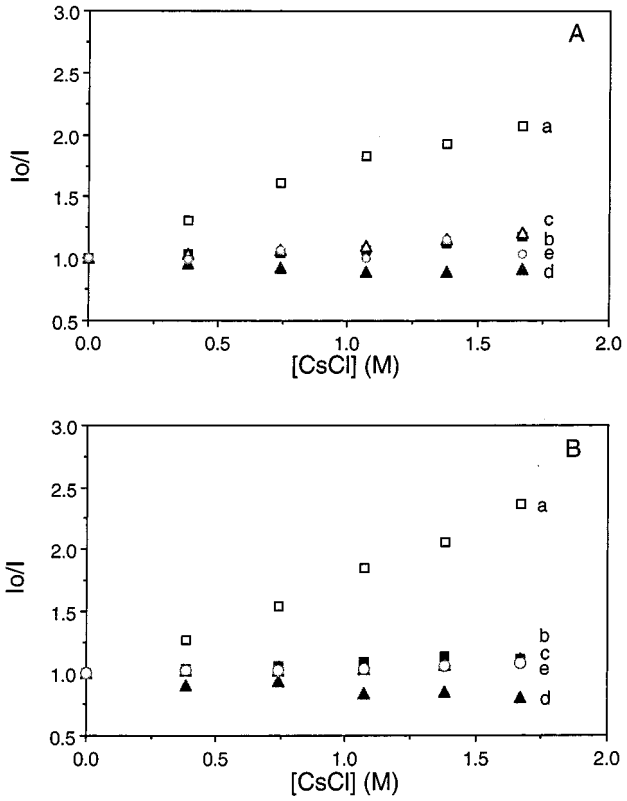

FIGURE 6: (A) Stern−Volmer plots of the CsCl quenching experiments for the MLCK peptide or the CaM−MLCK peptide complexes in the presence of Ca^{2+}. (a) MLCK peptide, (b) WT-CaM−MLCK peptide complex, (c) CT-CaM−MLCK peptide complex, (d) SeMet-CaM−MLCK peptide complex, and (e) SeMet-CT-CaM−MLCK peptide complex. (B) Stern−Volmer plots of the CsCl quenching experiments for the CaM kinase I peptide or the CaM−kinase I peptide complexes in the presence of Ca^{2+}. (a) CaM kinase I peptide, (b) WT-CaM−kinase I peptide complex, (c) CT-CaM−kinase I peptide complex, (d) SeMet-CaM−kinase I peptide complex, and (e) SeMet-CT-CaM−kinase I peptide complex. I_0 is the fluorescence in the absence of CsCl, and I is the fluorescence at each titration point after correction of the dilution.

provides an important control to determine the correct orientation of the MLCK peptide, because it excludes the possibility that the N-terminal Trp of the peptide interacts with the N-lobe of CaM. In principle, the method described here for determining the orientation of the MLCK peptide should be applicable to other CaM-binding peptides that contain a Trp residue. Thus, in order to establish whether similar fluorescence data could be obtained for other CaM-binding peptides, we studied the complex of CaM with the CaM kinase I peptide. CaM kinase I is also activated by Ca^{2+}-CaM (*33*), and the crystal structure of the uncomplexed enzyme has been reported (*27*). This structure reveals that its CaM-binding domain lies on the ATP binding loop of the kinase domain; this region contains the single Trp303 residue which sticks out into solution in the crystal structure. Our fluorescence data obtained with a synthetic peptide encompassing the CaM-binding domain of CaM kinase I are depicted in Figure 5 (see also Table 1). The peptide in aqueous solution has a fluorescence maximum around 353 nm, which shifts to 330 nm upon binding to WT-CaM; this is accompanied by approximate 2-fold increase in the relative fluorescence intensity. As observed with the MLCK peptide, binding of the CaM kinase I peptide to SeMet-CaM almost abolishes the Trp fluorescence, while further increased intensity is observed with CT-CaM. Also the results obtained with SeMet-CT-CaM are consistent, as the increased fluorescence obtained demonstrates that the Trp residue of the CaM kinase I peptide is bound to the C-terminal domain of CaM in a manner which closely resembles that of the MLCK peptide. This outcome is also in agreement with our NMR studies of nitroxide spin-labeled CaM kinase I peptides complexed with CaM (Yuan and Vogel, unpublished results).

In order to exclude the possibility that the Trp residue of the two peptides studied binds in a manner different from that of the four variants of CaM used here, we have studied the Trp exposure in the two CaM−peptide complexes by fluorescence quenching experiments with CsCl. O'Neil et al. (*42*) have earlier measured the exposure of Trp residues on CaM-bound peptides by quenching experiments with acrylamide. The results of our quenching experiments with CsCl are fully consistent with their data (Figure 6). We have found that the quenching results obtained for CaM−peptide complexes with acrylamide and CsCl are similar (data not shown). The peptides in aqueous solution have a highly exposed Trp residue, and upon binding to any of these four proteins in the presence of Ca^{2+}, the accessibility of this Trp is dramatically decreased (Figure 6).

Finally, we have studied the distance dependence of the fluorescence quenching effects observed with the CaM variants. Such information can contribute to an understanding regarding the mechanism that governs effects of Se and S on the fluorescence intensity. For this we studied the intrinsic Tyr fluorescence of CaM; this protein contains two Tyr residues in the C-lobe of apo- and Ca^{2+}-CaM. They are at a relatively larger distance than the Trp from the Met sulfur atoms in the X-ray and NMR structures in the various forms of CaM (see Table 3). We find that the introduction

Table 3: Distances between the Tyr/Phe 99 and Tyr 138 Benzene Ring and the Met Sulfur Atoms of the C-Lobe of CaM in the Apo-CaM, Ca^{2+}-CaM, and Ca^{2+}-CaM−MLCK Peptide Complex Structure (Å)[a]

	Tyr 99				Tyr 138			
PDB code	M109	M124	M144	M145	M109	M124	M144	M145
apo-CaM								
1DMO[b]	16.21	14.89	12.56	14.64	10.57	14.33	12.57	11.82
ICFD	15.02	18.16	15.37	16.77	12.41	14.44	13.06	11.65
Ca^{2+}-CaM								
3CLN	18.64	17.87	16.55	15.68	17.13	18.07	13.26	9.09
1CLL	19.90	18.09	16.63	16.83	17.52	18.12	13.29	9.93
Ca^{2+}-CaM−MLCK								
2BBM	18.07[c]	18.85[c]	16.96[c]	15.82[c]	18.55	19.65	12.96	9.57
1CDL[d]	18.75	18.32	16.29	16.94	17.12	17.42	11.86	10.28

[a] The distance is expressed as the average distance from the Met sulfur atom to the six carbon atoms on the Tyr/Phe benzene ring. The distances were measured by Insight II (Biosym). [b] 1DMO, Zhang et al. (*10*); 1CFD, Kuboniwa et al. (*9*); 3CLN, Babu et al. (*7*); 1CLL, Chattopadhyaya et al. (*8*); 2BBM, Ikura et al. (*17*); 1CDL, Meador et al. (*18*). [c] Phe 99. [d] We only present the distances in one of the four X-ray structures in the PDB coordinates. The distances in the other three structures are similar.

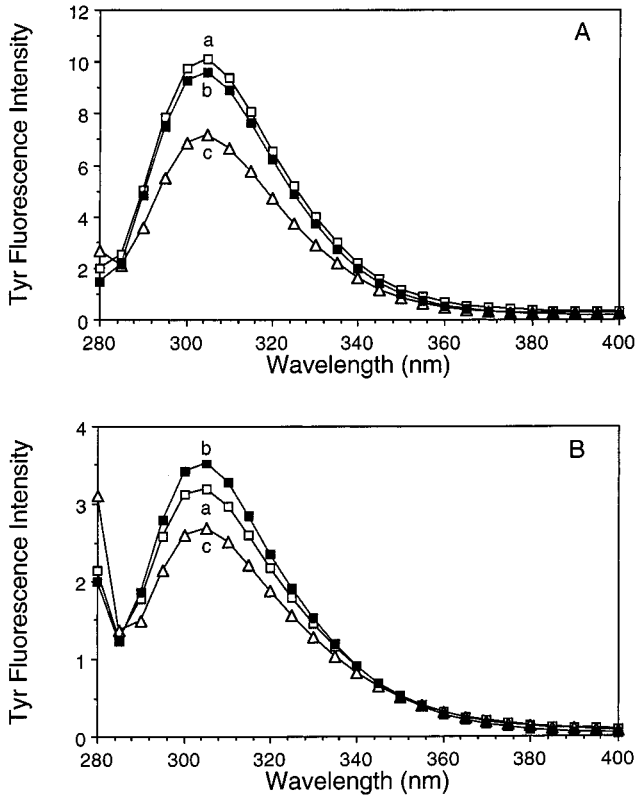

FIGURE 7: (A) Tyrosine fluorescence of three CaM variants in the presence of Ca^{2+}. (a) WT-CaM, (b) CT-CaM, (c) SeMet-CaM. (B) Tyrosine fluorescence of three CaM variants in the absence of Ca^{2+}. (a) WT-CaM, (b) CT-CaM, (c) SeMet-CaM. The protein concentration used was 25 μM.

of Se or C atoms into CaM only has relatively small effects on the Tyr fluorescence intensity (Figure 7).

DISCUSSION

In this work we have studied the steady-state fluorescence characteristics of two CaM-binding domain peptides derived from skMLCK (*32*) and CaM kinase I (*33*). We note that when both peptides bind to WT-CaM, a characteristic blue-shift and increase in fluorescence intensity is observed (Figure 3 and 5). This indicates that the Trp is moved from a solvent exposed to a buried hydrophobic position (*40*, *41*, *43*), and this effect has been observed earlier for Trp residues in other peptides that became buried upon binding to CaM (*42*, *44*). We also studied the interaction of these two peptides with three CaM variants: CT-CaM, SeMet-CaM, and SeMet-CT-CaM. These proteins contain four C-terminal Leu instead of Met and/or ∼90% Se instead of S atoms. These proteins gave rise to a similar wavelength shift of the Trp fluorescence (see Table 1). The fluorescence quenching experiments (Figure 6) also indicate that the Trp residue of the MLCK and CaM kinase I peptides becomes almost fully buried upon binding to these four CaM variants; similar quenching properties are observed in all instances. This interpretation of the fluorescence data is consistent with the known structures of the CaM−MLCK peptide complexes (*17*, *18*).

For the four variants of CaM used in this study we have studied their Ca^{2+}-dependent gel band shift, their peptide-bound urea gel band shift (Figure 1), their enzyme activation properties, their β-sheets, α-helices, and methyl groups (Figure 2), as well as the burial of the peptide Trp groups (Figure 6) to demonstrate that these CaM variants interact with CaM-binding peptides in a manner that closely resembles that of the wild-type protein. All these data indicate the high similarity in the structure and activation properties of these four CaM proteins. Consequently, we are comfortable in interpreting the dramatic changes in relative fluorescence intensity that are observed in Figures 3 and 5 by changes in the quenching properties of these proteins compared to that of the wild-type protein. Since the only differences in these four proteins are in their Met residues, these residues likely directly dominate the observed quenching effects. We find that SeMet is an extremely efficient quencher of Trp fluorescence. In addition, Leu (in place of Met) gives rise to an increased fluorescence, suggesting that the S atoms of Met also can give rise to the quenching of Trp fluorescence, albeit less efficiently than Se. The effect appears to be much stronger at a short distance (Figure 7), suggesting that dynamic (collisional) or static quenching rather than energy transfer mechanisms are responsible for these effects (*43*). Since both quenching mechanisms require molecular contacts between the quencher and the fluorophore (*43*), such effects are always short-range. It is well known that many groups in proteins can give rise to fluorescence quenching; especially electron-rich entities such as disulfides have been demonstrated to be effective (*41*, *43*, *45*−*48*). Both Se and S atoms carry a relatively large electron cloud and

are more polarizable than C atoms. These two physical properties should contribute to the efficiency of the quenching mechanism; the larger polarizability of Se would also explain why it is more efficient than S at quenching. Further studies, involving fluorescence lifetime measurements (*43*), should shed more light on the exact physical mechanism of the Se quenching effect; such experiments are currently underway. Be that as it may, the quenching effects reported here are substantial and allowed us to confirm the orientation of the MLCK peptide with respect to CaM; it binds with its N-terminal end to the C-terminal domain of CaM, and this orientation is consistent with other structural studies (*17*, *18*). Also the CaM kinase I peptide, for which the binding orientation has not been reported before, binds in the same orientation. Thus this approach provides a simple fluorescence method for determining the binding orientation of other Trp-containing CaM-binding peptides. Since many CaM-binding domains contain a single Trp residue (*3*, *5*), this experiment is a useful tool for further studies with CaM. Furthermore, because of the relatively short range at which this quenching effect acts, it should also be possible to perform similar studies on the interaction of other proteins and peptides. Moreover, it might be possible to study interactions between Trp and Met/SeMet residues that arise during the protein folding process through stopped flow fluorescence experiments; for example, quenching of the Trp fluorescence by a Met residue has been used to study the initial folding of apomyoglobin (*49*).

In closing, we would like to briefly discuss the potential importance of the interaction between the Trp residue in a target protein and calcium-CaM. In some target proteins, e.g., calcineurin and adenylate cyclase (*50*, *51*), the CaM-binding domain appears to be flexible and unstructured, and the structure of the calmodulin-binding domain could not be defined in the crystal structure of these two proteins. On the other hand, the CaM-binding and autoinhibitory domain of CaM kinase I has a very-well defined fold, and it appears to form an almost integral part of the protein (*27*). The crystallographic *B* factors indicate the absence of flexibility in this region of the protein. Therefore, the almost fully solvent exposed indole ring of Trp303 of CaM kinase I is the only obvious recognition site for binding CaM (*27*). When CaM is bound, this Trp side chain becomes completely buried (this study), and this first step might trigger further changes in this region of CaM kinase I, which allows the remainder of the domain to bind to CaM as well, hence removing the CaM binding autoinhibitory domain and activating the protein kinase. Thus, the free energy provided by the complete burial of the large indole ring of Trp into the C-terminal domain of CaM appears to be an important step in the activation of several CaM-activated enzymes. This would explain why, in protein engineering experiments with several mutant target proteins of CaM, the Trp residue cannot be replaced with smaller hydrophobic amino acids (*19*−*21*).

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