Surface Exposure of the Methionine Side Chains of Calmodulin in Solution

A NITROXIDE SPIN LABEL AND TWO-DIMENSIONAL NMR STUDY

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Binding of calcium to calmodulin (CaM) causes a conformational change in this ubiquitous calcium regulatory protein that allows the activation of many target proteins. Met residues make up a large portion of its hydrophobic target binding surfaces. In this work, we have studied the surface exposure of the Met residues in the apo- and calcium-bound states of CaM in solution. Complexes of calcium-CaM with synthetic peptides derived from the CaM-binding domains of myosin light chain kinase, constitutive nitric-oxide synthase, and CaM-dependent protein kinase I were also studied. The surface exposure was measured by NMR by studying the effects of the soluble nitroxide spin label, 4-hydroxyl-2,2,6,6-tetramethylpiperidinyl-1-oxy, on the line widths and relaxation rates of the Met methyl resonances in samples of biosynthetically [14C]-methyl-Met-labeled CaM. The Met residues move from an almost completely buried state in apo-CaM to an essentially fully exposed state in Ca2+-CaM. Binding of two Ca2+ to the C-terminal lobe of CaM causes full exposure of the C-terminal Met residues and a partial exposure of the N-terminal Met side chains. Binding of the three target peptides blocks the access of the nitroxide surface probe to nearly all Met residues, although the mode of binding is distinct for the three peptides studied. These data show that calcium binding to CaM controls the surface exposure of the Met residues, thereby providing the switch for target protein binding.

Calmodulin (CaM)1 (1) is a ubiquitous calcium regulatory protein that is found in all eukaryotic cells. This small acidic protein (16.7 kDa) can regulate at least 30 different target proteins in a calcium-dependent manner (1–6). Its target proteins include multiple components in muscle contraction such as myosin light chain kinases (MLCK), various protein kinases, protein phosphatase 2B (calcineurin), and nitric-oxide synthases. CaM responds to an increase in the intracellular Ca2+ concentration from 10−7 to 10−6 μM in vivo. CaM’s capacity to bind and activate a diverse range of target proteins originates in its structure. The x-ray structure of Ca2+-saturated CaM reveals a dumbbell-shaped molecule with each lobe containing two “helix-loop-helix” calcium binding motifs, giving a total of four Ca2+-binding sites (7, 8). The two lobes in Ca2+-CaM are connected by a 26-residue highly exposed α-helix in the crystal structures (7, 8). In contrast, structural and dynamic information derived by NMR spectroscopy as well as molecular dynamics simulations indicates that the region between amino acids 77 and 81 in Ca2+-CaM is flexible in solution (9, 10). This central linker region in Ca2+-CaM unravels further when a CaM-binding peptide from a target protein such as MLCK is bound (11, 12). High resolution x-ray and NMR structures of the complexes between Ca2+-CaM and the CaM-binding domain peptides of MLCK and CaM kinase II clearly indicate the different relative movement of the two lobes of CaM in the complexes as well as the bending and unaveling of the central linker region (11–13). This flexibility of the central linker region in Ca2+-CaM is one of the key reasons that allows CaM to activate so many different systems.

Another prominent feature that contributes to CaM’s flexibility are the two Met-rich hydrophobic surfaces on the N- and C-lobes of CaM, which become exposed upon calcium binding to CaM. The majority of the hydrophobic residues that make up these surface areas are buried inside the protein in apo-CaM (14, 15). In particular, all Met side chains involved in target binding are buried in apo-CaM, with the possible exceptions of Met124 and Met144, which appear to be partially exposed in the apo- and calcium-bound states (7, 8). The two lobes in Ca2+-CaM for target protein binding is obvious (11–13). The Met residues contribute 46% to the solvent-accessible surface area of these two exposed hydrophobic surfaces in calcium-CaM (16). In addition, almost all of the Met side chains in Ca2+-CaM appear to be involved in binding to the CaM-binding domain peptides from MLCK, CaM kinase II, caldesmon, constitutive nitric oxide synthase (cNOS), cyclic nucleotide phosphodiesterase, glycoprotein 41 of simian immunodeficiency virus, CaM-dependent protein kinase I (CaMKI), and plant glutamate decarboxylase (2, 11–13, 17–22). Site-directed mutagenesis of the Met residues of CaM further illustrates the importance of their side chains in the binding and activation of various target proteins (20, 23–25). Compared with other aliphatic hydrophobic amino acid side chains, the Met side chains possess a unique intrinsic flexibility and polarizability due to the presence of the sulfur atom as noted by Gellman (26). The flexible and malleable surfaces in Ca2+-CaM created by the

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1 The abbreviations used are: CaM, calmodulin; CaMKI, calmodulin-dependent protein kinase I; cNOS, constitutive nitric-oxide synthase; cTnC, cardiac muscle troponin C; HMGC, heteronuclear multiple quantum coherence; MLCK, myosin light chain kinase(s); R1, spin-lattice relaxation rate; T1, spin-lattice relaxation time; TEMPO, 4-hydroxyl-2,2,6,6-tetramethylpiperidinyl-1-oxy.
Met side chains provide a highly adjustable interaction area that can accommodate the binding of a wide range of target proteins (1, 2, 4).

4-Hydroxyl-2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPOL; also called HyTEMPO) is a nonperturbing nitroxide-soluble spin label that can be used for mapping surface-exposed hydrophobic side chains in proteins. It has been used for the characterization of different protein folding intermediates, identification of the critical residues involved in ligand-binding, and simplification of 1H NMR spectra for assignment purposes (Refs. 27–33; for a review, see Ref. 34). The idea behind the TEMPOL method is that NMR resonances for residues that are (or become) exposed on the surface of proteins will be dramatically broadened by the paramagnetic nitroxide in the surrounding medium (34). In contrast, the relaxation rate of buried residues will not be influenced by this probe. As such, the relative surface exposure of various groups in a protein can be gauged. The method works well for studying hydrophobic areas on protein surfaces (27, 34). The combination of high sensitivity proton-detected 1H, 13C heteronuclear multiple quantum coherence (HMQC) NMR spectra and the availability of the residue-specific assignments for the nine Met methyl groups in a number of physiologically relevant states of CaM provided us with a unique opportunity to investigate the Met methyl group exposure in solution for different states of CaM. Here we have studied apo-CaM, Ca2+-CaM, Ca2+-C4-CaM, the Ca2+-C4-CaM-MLCK peptide complex, and complexes of Ca2+-CaM with cNOS and CaMKI CaM-binding peptides. In addition, we have also studied the Met methyl groups in the separated N- and C-lobes of CaM (TR1C and TR2C) in the presence and absence of two Ca2+ ions using the same experimental approach. Our results reveal that changes in the surface exposure of the Met side chains play a key role in allowing CaM to act as a versatile calcium regulatory protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—TEMPOL was purchased from Sigma. A synthetic mammalian CaM gene was expressed in *Escherichia coli* (35, 36). The purification of wild-type CaM and [methyl-13C]Met-labeled CaM was accomplished as described before (17, 36). The N-lobe of CaM, TR1C (residues 1–75), was cloned using the DNA polymerase chain reaction; the expression and purification of TR2C followed the same protocol as for wild-type CaM (17, 36). The C-lobe of CaM, TR2C (residues 78–148), was generated by limited tryptic digestion of intact CaM in the presence of saturating levels of calcium (37). Although the purified TR2C fragment may contain three different fragments of slightly different length (38), the vast majority was the fragment 78–148, as determined by electrospray mass spectrometry (data not shown). Two-dimensional 1H, 13C HMQC NMR spectra of the [methyl-13C]Met-labeled TR2C fragment (see below) showed only about 20% intensity for the Met 76 resonance compared with the other Met resonances (see Fig. 4C), in agreement with the outcome of the protein mass spectrometry. The absence of trace calcium ions in apo-form preparations of CaM and fragments after Chelex 100 chromatography was confirmed by one- and two-dimensional NMR spectroscopy. The MLCK peptide, cNOS peptide, and CaMKI peptide were commercially synthesized by the Core Facility for Protein/DNA Chemistry at Queen’s University (Kingston, Ontario, Canada). The preparation of CaM-peptide complexes for NMR analysis has been described before (19, 39). The concentration of CaM was calculated using an extinction coefficient of ε76 = 1.8. The concentrations of the TR1C and TR2C fragments were determined by using molar extinction coefficients of ε276 = 1073 M⁻¹cm⁻¹ and ε266 = 2666 M⁻¹cm⁻¹, respectively, which were confirmed by quantitative amino acid analysis.

**EPR**—EPR spectra were acquired at ambient temperature (22 °C) using a Bruker ER-200D-SRC spectrometer located at the Department of Chemistry, University of Alberta (Edmonton, Canada). The parameters used were as follows: modulation amplitude, 1 G; microwave power, 18 dB; microwave frequency, 9.770 GHz; time constant, 0.2 s. EPR spectra were acquired for 0.08 mM TEMPOL in the absence or presence of 1/6, 1, or 6 eq of Ca2+-CaM. The solution contained 100 mM KCl, pH 7.50 ± 0.05, which was similar to the conditions used for the NMR experiments except that H2O was used as the solvent instead of D2O. EPR spectra of TEMPOL were also obtained in the absence of apo-CaM and the Ca2+-CaM-MLCK peptide complex.

**NMR Spectroscopy**—All NMR samples contained 1 mM [methyl-13C]Met isotopically labeled protein in 100 mM KCl, 99.9% D2O. The pD was determined as 7.50 ± 0.05 using a glass electrode; no corrections for the isotope effects were made. All NMR spectra were acquired on a Bruker AMX-500 spectrometer at 298 K. One-dimensional 1H NMR spectra were obtained with a sweep width of 6024 Hz and 128 scans. A 1-Hz line broadening was applied before the Fourier transformation. A concentrated stock solution of TEMPOL in D2O was prepared, and 1-μl amounts of the spin label were tritiated into the protein sample. Two-dimensional 1H, 13C HMQC spectra were acquired as described before.
Quadrature detection in the F1 dimension was obtained using the time-proportional phase increment method. The sweep width was 6 ppm in the 1H and 13C dimensions, with the 1H carrier set at 500.1383 MHz and the 13C carrier at 125.7613 MHz. The size of the HMQC spectra gave a 1024^3 128 real data matrix with eight scans for each experiment. Spin-lattice relaxation ($T_1$) measurements were also performed as described before (39), with a 1024 × 128 real data matrix and 32 scans for each experiment. The different relaxation delays used in the $T_1$ experiments were 10, 50, 150, 300, 600, 900, 1500, and 2500 ms.

NMR spectra were processed on a Bruker X32 data station using Bruker UXNMR software. Two-dimensional NMR spectra were zero-filled to 1024 × 256, and a 72° sine square window function was applied before Fourier transformation. $T_1$ spectra were integrated using an integration subroutine in UXNMR, and the integration data were fitted to a single exponential decay curve to give the $T_1$ values (39). All 1H chemical shifts are referenced to HDO as 4.78 ppm, which is further referenced to DSS (2, 2-dimethyl-2-silapentane-5-sulfonate) as 0 ppm. All 13C chemical shifts are indirectly referenced to DSS using a converting ratio $^{13}C/^{1}H = 0.251449530$ as suggested by Wishart et al. (40).

RESULTS

EPR Studies of Potential CaM-TEMPOL Interactions—We first acquired EPR spectra of TEMPOL in the absence and presence of CaM to assess whether any specific interactions between TEMPOL and CaM existed. Such interactions have been reported for other proteins (e.g., Ref. 31), and they complicate the interpretation of subsequent NMR experiments, where TEMPOL is used as a general relaxation enhancement reagent. The formation of complexes between the TEMPOL nitroxide spin label and the protein should lead to a broadening of the EPR nitroxide signal, accompanied by a commensurate decrease in the signal intensities (31). As can be seen in Fig. 1, no changes were detected in the EPR spectra of TEMPOL in the absence or presence of 6 eq of CaM. We also did not observe any changes in the EPR spectra recorded with TEMPOL/CaM at a ratio of 6, TEMPOL/apo-CaM at a ratio of 1/6 or 6, and the TEMPOL/CaM-MLCK-peptide complex at a ratio of 1/6 or 6 (data not shown). Therefore, the outcome of these EPR studies indicates that there are no specific interactions between TEMPOL and CaM; consequently, the soluble spin label is suitable to probe the exposed hydrophobic surfaces and the exposure of Met methyl groups in CaM (29, 32). Furthermore, in our NMR studies of CaM in the presence of TEMPOL, we also did not observe any chemical shift changes for the Met methyl resonances, which is also consistent with the ab-
sence of specific interactions between the spin label and the protein (see below). Other relaxation enhancement reagents, such as lanthanide compounds, may give rise to specific interactions with proteins and chemical shift changes in the protein NMR spectra (41). Thus, choosing TEMPOL as a reagent to probe the exposed hydrophobic surface in CaM seems appropriate.

Ca\textsuperscript{2+}\textsubscript{4}-CaM, Ca\textsuperscript{2+}\textsubscript{2-TR1C}, and Ca\textsuperscript{2+}-TR2C—Three regions of the one-dimensional 1H NMR spectra of Ca\textsuperscript{2+}\textsubscript{4}-CaM, which were recorded in the absence and presence of 6 eq of TEMPOL, are shown in Fig. 2 A. Several resonances for aromatic side chains were significantly broadened upon the addition of TEMPOL. These observations are in accordance with the x-ray structure of Ca\textsuperscript{2+}\textsubscript{4}-CaM, in which four Phe residues are found in the exposed hydrophobic patches (7, 8). The broadened upfield-shifted methyl proton resonances are also consistent with this notion. Val\textsuperscript{35}, Val\textsuperscript{108}, Ile\textsuperscript{27}, and Ile\textsuperscript{100} are all part of the two exposed hydrophobic surface areas in the x-ray structure of Ca\textsuperscript{2+}\textsubscript{4}-CaM (7, 8). In contrast, the downfield-shifted \( \alpha \)-proton resonances between 5.0 and 5.5 ppm are well protected from TEMPOL; these resonances correspond to the two buried \( \beta \)-sheets between the two Ca\textsuperscript{2+}-binding loops in each lobe of Ca\textsuperscript{2+}\textsubscript{4}-CaM in the x-ray structure (7, 8).

Relaxation enhancement effects caused by TEMPOL on the resonances in NMR spectra can be assessed quantitatively by measuring the enhancement of relaxation rates in the presence of TEMPOL (28, 30, 32, 34). Because all resonances (except for Met\textsuperscript{76}) in 1H, 13C HMQC spectra of [methyl-\textsubscript{13}C]Met-labeled Ca\textsuperscript{2+}-CaM disappeared in the presence of 1 eq of TEMPOL (data not shown), we decided to use only 0.4 eq of TEMPOL to study the relaxation effects in Ca\textsuperscript{2+}-CaM. Severe line broadening of the resonances of all of the Met methyl groups with the exception of Met\textsuperscript{76} can be seen in Fig. 3 A. The four N-terminal Met residues (Met\textsuperscript{36}, Met\textsuperscript{51}, Met\textsuperscript{71}, and Met\textsuperscript{72}) and the four C-terminal Met residues (Met\textsuperscript{109}, Met\textsuperscript{124}, Met\textsuperscript{144}, and Met\textsuperscript{145}) showed an average 53 and 66% increase, respectively, in their 13C spin-lattice relaxation rates \( R_1 \) (Table I); this result is in agreement with the qualitative picture obtained by looking at the line broadening (Fig. 3A). As a control, we also measured the increase of the \( R_1 \) relaxation rate for the isolated [methyl-\textsubscript{13}C]Met amino acid, and in the presence of 0.4 eq of TEMPOL the ratio of \( R_1 (0.4 \text{ eq})/R_1 (0 \text{ eq}) = 2.07 \). Thus, the results obtained with the protein indicate that the Met methyl groups are almost fully exposed on the surface of Ca\textsuperscript{2+}-CaM with the exception of Met\textsuperscript{76} (see below).

We also studied the individual isolated lobes of Ca\textsuperscript{2+}-CaM using the TEMPOL broadening experiment. Fig. 3, B and C, shows the 1H, 13C HMQC spectra obtained for Ca\textsuperscript{2+}\textsubscript{2}-TR1C and Ca\textsuperscript{2+}\textsubscript{2}-TR2C. The assignments for Ca\textsuperscript{2+}\textsubscript{2}-TR1C and Ca\textsuperscript{2+}\textsubscript{2}-TR2C were obtained by comparison with the assignments for Ca\textsuperscript{2+}-CaM in A. Again, significant broadening effects are observed in the two isolated lobes of the bilobal CaM.
Ca\textsuperscript{2+} TR\textsubscript{2C}, respectively, in the absence and presence of 0.4 eq of TEMPO. By utilizing the similarities between the NMR spectra of these two protein fragments and intact CaM in the Ca\textsuperscript{2+} form as well as the apo-form, the assignment for the Met methyl resonances could be readily made (Figs. 3 and 4). Table I includes the results obtained for the R\textsubscript{1} relaxation rate measurements for Ca\textsuperscript{2+} TR\textsubscript{C} and Ca\textsuperscript{2+} TR\textsubscript{2C}. Clearly, we obtained a very similar result for the two isolated fragments Ca\textsuperscript{2+} TR\textsubscript{C} and Ca\textsuperscript{2+} TR\textsubscript{2C}, when compared with intact Ca\textsuperscript{2+} CaM (Fig. 3, Table I). These data imply that Ca\textsuperscript{2+} TR\textsubscript{C} and Ca\textsuperscript{2+} TR\textsubscript{2C} have a very similar exposure for the Met methyl groups as in Ca\textsuperscript{2+} CaM. The broadening effect is slightly less dramatic, but this possibly reflects the decrease of the correlation time of TR\textsubscript{C} and TR\textsubscript{2C} compared with intact CaM. A higher TEMPOL concentration is generally needed to achieve similar broadening effects for small molecules (34). Be that as it may, the \textsuperscript{13}C R\textsubscript{1} measurements definitively show a comparable increase in the spin-lattice relaxation rates in the presence of 0.4 eq of TEMPO (Table I). These results suggest that Met side chains are almost fully exposed on the protein surface in Ca\textsuperscript{2+} TR\textsubscript{C} and Ca\textsuperscript{2+} TR\textsubscript{2C}.

Apo-CaM, Apo-TR\textsubscript{C}, and Apo-TR\textsubscript{2C}—Next, we acquired the one-dimensional \textsuperscript{1}H NMR spectra of apo-CaM in the absence and presence of 6 eq of TEMPO (Fig. 2B). In contrast to the results obtained with Ca\textsuperscript{2+} CaM (Fig. 2A), we observed almost no line broadening effects in the aromatic region and upfield-shifted methyl group region of the proton NMR spectra upon the addition of TEMPO (Fig. 2B). This is consistent with the burial of the majority of these side chains inside the protein as seen in the apo-CaM structure (14, 15).

Because of the lower response to TEMPO in the NMR spectra of apo-CaM, two-dimensional \textsuperscript{1}H,\textsuperscript{13}C HMQC spectra of apo-CaM were acquired in the absence and presence of 4 eq of TEMPO rather than the 0.4 eq we had used for the Ca\textsuperscript{2+} saturated protein. Under these conditions, we observed a relative lack of broadening for the Met methyl groups in the N-lobe of apo-CaM. These appear to be completely buried inside the protein (Fig. 4A). This notion is confirmed by the R\textsubscript{1} relaxation rate enhancement data (Table II). Interestingly, the Met methyl groups in the C-lobe of apo-CaM seem more exposed than those in the N-lobe, as evidenced by the broader resonances for the C-lobe of apo-CaM (Fig. 4A), as well as a more significant relaxation rate enhancement (average 69% compared with 3%, Table II). This may be related to a semi-open conformation adopted by the C-lobe of apo-CaM, as suggested by a computer modeling study (42). It should be noted that because of the different amounts of TEMPO added, the quantitative results presented in Tables I and II cannot be compared as a direct measure of surface exposure. However, if a 10-fold increase in the TEMPOL concentration is required to achieve the same relaxation rate enhancement, the residues are obviously less exposed (34).

Apo-TR\textsubscript{C} and apo-TR\textsubscript{2C} again showed similar surface exposure properties for their Met methyl groups compared with their counterparts in intact apo-CaM (Fig. 4 and Table II). Similarly, we find that the Met methyl groups are fully buried in apo-TR\textsubscript{C} and only partially buried in apo-TR\textsubscript{2C}. The spin-lattice relaxation rate enhancement in apo-TR\textsubscript{C} on average is 73% compared with 69% in apo-CaM. These data suggest that the apo-TR\textsubscript{C} alone may also adopt a semi-open conformation as suggested for the C-lobe in apo-CaM (42).

Ca\textsuperscript{2+} CaM—In order to obtain information about the side chain exposure in the N- and C-lobes of half-saturated CaM, experiments were performed with only 2 eq of Ca\textsuperscript{2+} added to intact apo-CaM. Since there is approximately a 10-fold difference in the calcium-binding constants of the C- and N-terminal domains, only the two sites in the C-lobe of CaM will be fully occupied under these conditions (38). When two Ca\textsuperscript{2+} ions are added to apo-CaM, the resonances for the methyl groups of Met\textsuperscript{108}, Met\textsuperscript{124}, Met\textsuperscript{144}, and Met\textsuperscript{145} undergo slow exchange on the \textsuperscript{13}C NMR time scale, and a set of new resonances for the Ca\textsuperscript{2+} form appeared (Fig. 5). However, the methyl resonances of N-terminal Met\textsuperscript{26}, Met\textsuperscript{51}, Met\textsuperscript{71}, and Met\textsuperscript{72} residues moved to new positions (Fig. 5), although these effects were much less dramatic than when the N-terminal half is directly binding Ca\textsuperscript{2+}. The four Met methyl groups in the C-lobe of Ca\textsuperscript{2+} CaM are all fully exposed; upon the addition of 0.4 eq of TEMPOL, similar effects as in Ca\textsuperscript{2+} CaM were observed for the C-terminal lobe resonances (data not shown). The addition of 1 eq of TEMPOL completely broadened these C-lobe resonances. The four Met methyl groups in the N-lobe of Ca\textsuperscript{2+} CaM become partially exposed, as indicated by the observed relaxation enhancement in the presence of 2 eq of TEMPO (Table III). The N-lobe of Ca\textsuperscript{2+} CaM may maintain a similar structure as in apo-CaM. However, our data clearly show an increase in the relaxation rates of the Met\textsuperscript{26}, Met\textsuperscript{51}, Met\textsuperscript{71}, and Met\textsuperscript{72} methyl groups. Perhaps a change in dynamics takes place in the N-lobe upon the binding of two Ca\textsuperscript{2+} ions to the C-lobe of apo-CaM; i.e., the N-lobe of Ca\textsuperscript{2+} CaM may be more flexible than in apo-CaM or apo-TR\textsubscript{C}. This change may be transmitted through the central linker region, after the binding of two Ca\textsuperscript{2+} to the C-lobe of apo-CaM (see below). We also observed some heterogeneity in the Met\textsuperscript{76} resonance in the half-saturated CaM (Fig. 5). Additional experiments were also performed with an equimolar mixture of TR\textsubscript{C} and TR\textsubscript{2C} in the presence of 2 eq of Ca\textsuperscript{2+}. In this case, the spectra closely

### Table I

| R\textsubscript{1} methyl group | N average | C average |
|-------------------------------|-----------|-----------|
| Ca\textsuperscript{2+} CaM     |           |           |
| R\textsubscript{1} (0 eq)\textsuperscript{a} | 0.48      | 0.51      |
| R\textsubscript{1} (0.4 eq)     | 0.74      | 0.70      |
| R\textsubscript{1} (0.4 eq)/R\textsubscript{1} (0 eq) | 1.54      | 1.49      |
| Ca\textsuperscript{2+} TR\textsubscript{C} |           |           |
| R\textsubscript{1} (0 eq)     | 0.51      | 0.45      |
| R\textsubscript{1} (0.4 eq)     | 0.58      | 0.65      |
| R\textsubscript{1} (0.4 eq)/R\textsubscript{1} (0 eq) | 1.14      | 1.44      |
| Ca\textsuperscript{2+} TR\textsubscript{2C} |           |           |
| R\textsubscript{1} (0 eq)     | 0.48      | 0.53      |
| R\textsubscript{1} (0.4 eq)     | 1.00      | 0.94      |
| R\textsubscript{1} (0.4 eq)/R\textsubscript{1} (0 eq) | 2.08      | 1.77      |

\textsuperscript{a} Assignments taken from Siivari et al. (39).

\textsuperscript{b} Average values compared in this study are underlined.
resembled those obtained for Ca\(^{2+}\)-CaM. While Met resonances of TR\(_2\)C were fully broadened upon the addition of 1 eq of TEMPOL, the surface exposure of the Met residues in TR\(_1\)C did not change (the average ratio of \(R_1(2)/R_1(0)\) was 1.12 (Table III), compared with 1.67 in Ca\(^{2+}\)-CaM (see Table III). This result also makes the possibility unlikely that the increased

FIG. 4. Two-dimensional \(^1\)H, \(^{13}\)C HMQC NMR spectra of apo-CaM, apo-TR\(_1\)C, and apo-TR\(_2\)C in the absence and presence of 4 eq of TEMPOL. The concentration of TEMPOL used is 10-fold higher than that used in Fig. 3. A. Apo-CaM. The assignments were taken from Siivari et al. (39). Selective broadening effects for the Met methyl resonances from the C-lobe of apo-CaM can be seen. The Met\(^{145}\) resonance can only be observed at lower contour level. B and C, apo-TR\(_1\)C and apo-TR\(_2\)C, respectively. The assignments were obtained by comparing with the assignments of apo-CaM in A. Note that no broadening effects are observed for apo-TR\(_1\)C, while they do occur for apo-TR\(_2\)C.

TABLE II

|           | Met\(^{36}\) | Met\(^{51}\) | Met\(^{71}\) | Met\(^{72}\) | Met\(^{109}\) | Met\(^{144}\) | Met\(^{145}\) | N average | C average |
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-----------|-----------|
| Apo-CaM   |             |             |             |             |             |             |             |           |           |
| \(R_1\) (0 eq)\(^a\) | 0.56        | 0.63        | 0.75        | 0.63        | 0.56        | 0.56        | 0.61        | 0.60      | 0.64      |
| \(R_1\) (4 eq) | 0.55        | 0.70        | 0.68        | 0.71        | 1.15        | 1.00        | 0.91        | 0.94      | 0.96      |
| \(R_1\) (4 eq)/\(R_1\) (0 eq) | 0.98        | 1.11        | 1.08        | 1.08        | 1.27        | 1.78        | 1.14        | 1.49      | 1.39      |
| Apo-TR\(_1\)C |             |             |             |             |             |             |             |           |           |
| \(R_1\) (0 eq) | 0.58        | 0.54        | 0.65        | 0.61        |             |             |             | 0.60      |           |
| \(R_1\) (4 eq) | 0.75        | 0.76        | 0.77        | 0.82        |             |             |             | 0.78      |           |
| \(R_1\) (4 eq)/\(R_1\) (0 eq) | 1.29        | 1.41        | 1.18        | 1.34        |             |             |             | 1.29      |           |
| Apo-TR\(_2\)C |             |             |             |             |             |             |             |           |           |
| \(R_1\) (0 eq) | 0.67        | 0.55        | 0.57        | 0.64        |             |             |             | 0.61      |           |
| \(R_1\) (4 eq) | 1.06        | 1.01        | 1.03        | 1.14        |             |             |             | 1.06      |           |
| \(R_1\) (4 eq)/\(R_1\) (0 eq) | 1.58        | 1.84        | 1.81        | 1.78        |             |             |             | 1.73      |           |

\(^a\) Assignments taken from Siivari et al. (39).
\(^b\) Average values compared in this study are underlined.
exposure in the N-lobe of Ca\(^{2+}\)-CaM arises from transient binding of Ca\(^{2+}\) to this domain.\(^2\)

Ca\(^{2+}\)-CaM-Target Peptide Complexes—When the TEMPOL relaxation enhancement approach was applied to several Ca\(^{2+}\)-CaM-peptide complexes, significant differences were observed when compared with Ca\(^{2+}\)-apo-CaM. The most striking result is that the majority of the Met resonances are now buried. Almost all Met methyl groups in the N-lobe and C-lobe of Ca\(^{2+}\)-CaM have become inaccessible to TEMPOL (4 eq), as would be expected if they are involved in the complex interface (see Fig. 6 and Table IV). Interestingly, from the broadening in the spectra and the \(R_1\) relaxation enhancement data, we noted some small differences between the three complexes (Fig. 6 and Table IV). For example, the methyl groups of Met\(^{72}\) and Met\(^{145}\) are partially exposed in the Ca\(^{2+}\)-CaM-MLCK peptide complex, with Met\(^{72}\) being more exposed than Met\(^{145}\) (Table IV). However, the MLCK peptide still partially covers the Met\(^{72}\) methyl group, because we still can measure its \(R_1\) rate in the presence of 2 eq of TEMPOL, while the same resonance in the Ca\(^{2+}\)-apo-CaM is unobservable after the addition of 1 eq of TEMPOL (Table IV). Different results were obtained for the Ca\(^{2+}\)-CaM-cNOS peptide and the Ca\(^{2+}\)-CaMKI peptide complexes. Upon cNOS peptide binding, Met\(^{124}\) remains partially exposed, while all other Met residues are fully buried. Only the Met\(^{72}\) methyl group is partially exposed, while all other Met methyl groups are fully buried in the Ca\(^{2+}\)-CaM-cNOS peptide and the Ca\(^{2+}\)-CaM-CaMKI peptide complexes. Upon cNOS peptide binding, Met\(^{109}\) remains partially exposed, while all other Met residues are fully buried. Only the Met\(^{72}\) methyl group is partially exposed, while all other Met methyl groups are fully buried in the Ca\(^{2+}\)-CaM-CaMKI peptide complex. Furthermore, we have found that all Met methyl groups become completely buried in a complex between Ca\(^{2+}\)-CaM and peptides derived from a plant glutamate decarboxylase (22).

**DISCUSSION**

In this work, we have studied the surface exposure of the Met methyl groups of CaM in different physiologically relevant states by using the soluble spin label TEMPOL. TEMPOL has no specific interactions with CaM and thus is suitable to probe the exposed hydrophobic surface in CaM (Fig. 1). In Ca\(^{2+}\)-CaM, we found that all Met methyl groups are fully exposed

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**TABLE III**

Carbon-13 spin-lattice relaxation rate \(R_1\) (s\(^{-1}\)) for the Met methyl groups in apo-CaM, Ca\(^{2+}\)-apo-CaM, and Ca\(^{2+}\)-peptide complexes in the presence of 4, 2, and 2 eq of TEMPOL, respectively.

|               | Met\(^{36}\) | Met\(^{51}\) | Met\(^{71}\) | Met\(^{72}\) | Met\(^{109}\) | Met\(^{124}\) | Met\(^{144}\) | Met\(^{145}\) |
|---------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| **Apo-CaM**   |             |             |             |             |             |             |             |             |
| \(R_1\) (0 eq)\(^a\) | 0.56        | 0.63        | 0.75        | 0.63        | 0.56        | 0.60        | 0.61        | 0.60        |
| \(R_1\) (4 eq) | 0.55        | 0.70        | 0.70        | 0.68        | 0.71        | 1.15        | 1.00        | 0.91        |
| \(R_1\) (4 eq)/\(R_1\) (0 eq) | 0.98        | 1.11        | 0.93        | 1.08        | 1.27        | 1.92        | 1.78        | 1.49        |
| **Ca\(^{2+}\)-apo-CaM** |             |             |             |             |             |             |             |             |
| \(R_1\) (0 eq) | 0.54        | 0.55        | 0.68        | 0.62        | 0.61\(^b\) | 0.47        | 0.46        | 0.60        |
| \(R_1\) (2 eq) | 0.92        | 0.82        | 0.97        | 1.29        | 0.79\(^b\) | No\(^c\)     | No          | No          |
| \(R_1\) (2 eq)/\(R_1\) (0 eq) | 1.70        | 1.49        | 1.43        | 2.08        | 1.30\(^c\) | 1.00        | No          | No          |
| Ca\(^{2+}\)-CaM-TR\(_1\)C/CT\(_2\)C |             |             |             |             |             |             |             |             |
| \(R_1\) (0 eq) | 0.56        | 0.58        | 0.46        | 0.74        | No          | 0.51        | 0.38        | 0.65        |
| \(R_1\) (2 eq) | 0.54        | 0.73        | 0.52        | No          | No          | No          | No          | No          |
| \(R_1\) (2 eq)/\(R_1\) (0 eq) | 0.96        | 1.25        | 1.13        | 0.60        | No          | No          | No          | No          |

\(a\) Assignments taken from Siivari et al. (39).
\(b\) Average values compared in this study are underlined.
\(c\) Only for the Ca\(^{2+}\)-form of Met\(^{36}\) resonance; the apo-form of Met\(^{76}\) resonance is partially overlapped with Met\(^{124}\) resonance.
\(d\) No, Resonance not observable due to line broadening.

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\(^2\)There are alternative explanations for the observation of the changes in exposure in the N-terminal lobe, upon binding of calcium to the C-terminal lobe of CaM. For example, the addition of more than 2.0 eq of Ca\(^{2+}\) could lead to partial exposure of the N-terminal Met residues. Care was taken to avoid this in our experiments. In addition, when 1.2 eq of Ca\(^{2+}\) were added to apo-CaM, slight changes in the chemical shifts of some of the N-terminal Met resonances could already be seen in the HMQC spectra (data not shown). These subtle changes were missed in original proton NMR studies concerning Ca\(^{2+}\) binding to CaM (38, 64), since the shift change is primarily in the \(^{13}\)C dimension of the HMQC spectra. Interestingly, these changes were largest for residues Met\(^1\) and Met\(^{71}\), which are part of the central linker of CaM. It should also be realized that with a approximate 10-fold difference in dissociation constants for calcium binding to the two lobes of CaM, transient binding of calcium to the two N-terminal sites can never be completely excluded. Nevertheless, our suggestion that information about Ca\(^{2+}\) binding to the C-lobe may be transmitted to the N-lobe in Ca\(^{2+}\)-apo-CaM is in agreement with a number of recent studies.

**FIG. 5. Two-dimensional \(^1\)H, \(^{13}\)C HMQC NMR spectra of Ca\(^{2+}\)-CaM in the absence and presence of 2 eq of TEMPOL.** The four Met resonances from the C-lobe of CaM have identical chemical shift values compared with Ca\(^{2+}\)-apo-CaM. The four Met resonances from the N-lobe of CaM have similar (but not identical) chemical shift values as in apo-CaM. The broadening effects are obvious for all the Met resonances (except for Met\(^{76}\) in the linker region). Compare with Fig. 4, A and B.
except Met\textsuperscript{76} (Fig. 3, Table I). This observation is consistent with the x-ray structures of Ca\textsuperscript{2+}-CaM, which indicate that two Met-rich hydrophobic patches are exposed in Ca\textsuperscript{2+}-CaM and ready for target protein binding (7, 8). The result obtained for the Met\textsuperscript{76} residue is somewhat surprising, since the x-ray structure shows that the central α-helix region is fully exposed (7, 8). Moreover, NMR structural and dynamic measurements have shown that the region between Lys\textsuperscript{77} and Ser\textsuperscript{81} is flexible (9). The absence of significant broadening of the Met\textsuperscript{76} resonance upon the addition of spin label is probably related to the fact that TEMPOL is more suitable for probing the hydrophobic surfaces rather than the charged surfaces in proteins, as noted by Petros et al. (27). Indeed, there are many charged residues around Met\textsuperscript{76} in the amino acid sequence of CaM (\textsuperscript{74}RKMKDTP\textsuperscript{80}) (7, 8), and these would limit the approach of the polar uncharged TEMPOL molecule.

The Met methyl groups in the isolated lobes of Ca\textsuperscript{2+}-CaM, Ca\textsuperscript{2+}-TR\textsubscript{1}C, and Ca\textsuperscript{2+}-TR\textsubscript{2}C were found to be fully exposed in the presence of Ca\textsuperscript{2+} (Fig. 3, Table I). This observation is consistent with several reports where it was shown that Ca\textsuperscript{2+}-TR\textsubscript{1}C alone or combined with Ca\textsuperscript{2+}-TR\textsubscript{2}C could bind to and activate some CaM target enzymes (43–46). Interestingly, oxidation of the Met residues in Ca\textsuperscript{2+}-TR\textsubscript{2}C abolished its capacity to activate the erythrocyte Ca\textsuperscript{2+}-ATPase, illustrating the importance of exposed hydrophobic Met side chains in the binding and subsequent activation process (44).

In the absence of Ca\textsuperscript{2+}, we found that the Met methyl groups in apo-CaM are fully buried in the N-lobe and partially exposed in the C-lobe (Fig. 4, Table II). There are no significant differences in the surface exposure properties for the four Met residues in the C-lobe of CaM (Met\textsuperscript{109}, Met\textsuperscript{124}, Met\textsuperscript{144}, and Met\textsuperscript{145}; Table II). For the apo-CaM structure determined by NMR, it was reported that Met\textsuperscript{124} and Met\textsuperscript{144} are partially exposed, while Met\textsuperscript{109} and Met\textsuperscript{145} are fully buried (15). This result seems to contradict our findings. However, it is usually difficult to accurately define the side chains of amino acid residues on the surface of a protein in an NMR structure calculation (47). The flexibility of many amino acid side chains on a protein surface leads to averaging of the nuclear Overhauser effects, which can lead to errors in the structure calculation for such surface residues (48). In contrast, residues in the core of a protein often have a unique conformation, and hence they are much better defined in an NMR structure calculation (47). The difference in the results could also be due to the existence of a semi-open conformation (42). It is also possible that the conformational averaging processes that may exist in the C-terminal lobe of apo-CaM contributes to these measurements by allowing temporary access to the TEMPOL moieties (14, 49). We note, how-
ever, that there are almost no changes in the line width of the methyl protons of Val191, Val105, and Val108 of apo-CaM in the presence of 6 eq of TEMPOL in one-dimensional $^1$H NMR spectra (Fig. 2B); this suggests that several buried side chains in the C-lobe of apo-CaM are not accessible to TEMPOL. Therefore, we feel that the most likely explanation from TEMPOL broadening data is that the C-lobe of apo-CaM adopts a semi-open conformation with all of the Met side chains somewhat exposed. The partially exposed Met methyl groups in the C-terminal lobe of apo-CaM may provide a hydrophobic surface for the binding of some target proteins in the absence of calcium (42, 50).

When CaM is half-saturated with 2 Ca$^{2+}$ ions bound to the C-lobe of CaM, we observed an increased exposure of the Met methyl groups from the N-lobe of CaM (Fig. 5, Table III). These data suggest that information about Ca$^{2+}$-binding to the C-lobe of CaM may be transmitted to the N-lobe. Many studies have illustrated the independent folding of each lobe of calcium-saturated CaM and apo-CaM. Nevertheless, our results reveal a different conformational transition involving both lobes of CaM. This phenomenon was also observed by a fluorescence study using a fluorescent probe attached to Cys26 in spinach CaM (51). Similarly, Mackall and Klee reported that, upon the binding of two Ca$^{2+}$ ions to apo-CaM, the susceptibility of the central linker region to trypsin proteolysis increases 10-fold, compared with that in either apo-CaM or Ca$^{2+}$-CaM. The recent work of Shea’s group as well as Bayley’s group has provided further evidence for the existence of an intermediate state in Ca$^{2+}$-CaM (54–56). Although there is no high resolution x-ray or NMR structure available for Ca$^{2+}$-CaM, some differences have been reported for the N-lobe of skeletal muscle troponin C, a homologous Ca$^{2+}$-binding protein (57). Taken together, the data obtained for Ca$^{2+}$-CaM suggest a structural or dynamic difference between the N-lobe of apo-CaM and the N-lobe of Ca$^{2+}$-CaM. Our data on the exposure of the Met residues in the N-lobe of Ca$^{2+}$-CaM indicate the presence of a partially exposed form, which is intermediate between the apo- and calcium-saturated forms. Interestingly, this effect is only observed for Ca$^{2+}$-CaM and not for an equi-molar mixture of two lobes, TrpC and TrpC, in the presence of 2 eq of Ca$^{2+}$. This suggests that the central linker region of CaM may play a role in transmitting information about structural changes between the two domains of CaM.

Upon binding to its target peptides, almost all of the Met methyl groups become fully buried in Ca$^{2+}$-CaM (Fig. 6, Table IV). Our results are consistent with the high resolution structures of Ca$^{2+}$-CaM-peptide complexes (11–13) as well as site-directed mutagenesis studies (24, 25, 58). The results obtained with the MLCK peptide are also in accordance with enzyme assays of the M124L CaM mutant, which is impaired in the activation of the MLCK enzyme, while mutation of Met72 or Met145 to a Leu residue had little effect (25, 58). It was reported that in a series of Met to Gln mutants, only the M124Q mutant of CaM had a seriously reduced activation potential for CaMKI (24). Our data for the different Ca$^{2+}$-CaM-peptide complexes illustrate the importance of the Met side chains in the binding interface of CaM with its target proteins. The differences reported here for three different Ca$^{2+}$-CaM-peptide complexes strongly suggest that the two Met-rich hydrophobic surface in Ca$^{2+}$-CaM can adjust themselves to a range of target proteins with CaM-binding domains of distinct amino acid sequences. Thus, the unique properties of Met side chains are crucial to CaM’s versatility in binding and activating so many different target proteins.

Recently, Howarth et al. (32) conducted a similar study of the exposure of the Met methyl groups in cTnC, a homologous calcium-binding regulatory protein. In contrast to CaM, troponin C binds only one target protein, troponin I. Troponin C is very similar to CaM both in terms of its amino acid sequence (51% sequence identity and 70% homology (59)) and three-dimensional structure. In addition, it is a Met-rich protein similar to CaM. However, the results obtained by Howarth et al. (32) are quite different from our results with CaM. Although these authors did not acquire data for apo-cTnC, the data for calcium-saturated cTnC clearly showed that the C-lobe Met methyl groups of cTnC are quite exposed, while in the N-lobe only two Met residues are somewhat exposed. These results imply that the Met side chains in cTnC do not become fully exposed upon binding Ca$^{2+}$, and hence they are less of a participant in the exposed hydrophobic binding surface than in Ca$^{2+}$-CaM. The rationale for these differences was recently revealed by the NMR solution structure of calcium-saturated cTnC, in which the N-lobe of cTnC is in a closed conformation compared with the open conformation observed for the C-lobe of cTnC (60, 61). In addition, in the studies by Howarth et al. (32), binding of the target cardiac muscle troponin I peptide to cTnC only protected two Met residues in the C-lobe of cTnC and had no effect on the Met residues in its N-lobe. This result is in

TABLE IV

| Peptide Complex | $R_1$ (0 eq)$^a$ | $R_1$ (4 eq) | $R_1$ (2 eq) | $R_1$ (0 eq)/$R_1$ (4 eq) | $R_1$ (0 eq)/$R_1$ (2 eq) |
|-----------------|-----------------|--------------|--------------|---------------------------|---------------------------|
| 4-CaM-CaMKI     | 0.53            | 0.54         | 0.67         | 0.49                      | 0.52                      |
| 4-CaM-cNOS      | 0.59            | 0.54         | 0.51         | 1.04                      | 0.64                      |
| 4-CaM-MLCK      | 1.11            | 1.00         | 0.76         | 2.12                      | 1.23                      |

$^a$ Assignments taken from Siivari et al. (39).

$^b$ No resonance not observable due to line broadening. $R_1$ (2 eq) is 0.76 and $R_1$ (2 eq)/$R_1$ (0 eq) is 2.24.

$^c$ Ratios with large differences are underlined.

$^d$ Assignments taken from Zhang et al. (19).

$^e$ Assignments taken from Yuan and Vogel (unpublished results).

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stark contrast to the results reported here for CaM, where nearly all Met side chains participate in the binding of the MLCK, cNOS, and CaM Ki peptides (Fig. 6 and Table IV). Clearly, the role of the Met residues is more important in CaM than in cTnC; it remains to be determined whether the Met residues in skeletal muscle troponin C more closely resemble those obtained for cTnC than for CaM.

Overall, our study demonstrates that the calcium-dependent control of the exposure of the Met methyl groups in CaM is a major factor in this protein's versatility. Recently, Beatty et al. (62) showed that very good resolution and sensitivity could be obtained in HMBC spectra of a protein with a molecular mass of 80 kDa that was labeled with [methyl-13C]Met. Thus, the TEMPO broadening approach can probably be extended to higher molecular weight complexes of CaM bound to an intact target protein. Moreover, since Met residues can play an important role in the protein-protein binding interface of other proteins (63), this approach can be applied to other protein-peptide or protein-protein systems as well.

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