Characterization of Trimethyllysine 115 in Calmodulin by $^{14}$N and $^{13}$C NMR Spectroscopy*

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In this paper, we describe three approaches to study the single trimethyllysine 115 in calmodulin. First, $^{14}$N NMR spectroscopy has been used as a novel spectroscopic tool. Because of the unique symmetrical tetrahedral substitution of its side chain, the trimethyllysine residue gives rise to a sharp $^{14}$N NMR resonance; hence, this has allowed the detection and quantitation of the level of trimethylation. Trimethyllysine side chains of bovine testis calmodulin and yeast cytochrome c were shown to have a high mobility in aqueous solution as determined by $^{14}$N NMR relaxation measurements. Second, we have purified mammalian calmodulin from an overproducing Escherichia coli strain. By comparison of the $^{1}$H-$^{13}$C heteronuclear multiple quantum coherence spectra of $^{13}$C-dimethylated calmodulin samples from bovine testis and E. coli, the resonance for Lys-115 in bacterially expressed calmodulin could be identified. pH titration experiments showed that the e-NH$_2$ group of Lys-115 has a normal pK$_a$ value both in the apo and Ca$^{2+}$ forms of the protein and in a complex of calmodulin with a 22-residue calmodulin-binding peptide derived from myosin light chain kinase. Third, we have shown that mutation of Lys-115 to the uncharged Gin residue does not alter the ability of the protein to nesitil stimulate the enzymes cyclic nucleotide phosphodiesterase and myosin light chain kinase. These results show that the trimethylation of Lys-115 is not caused by an unusual pK$_a$ and reactivity of its e-NH$_2$ group and that its side chain remains flexible. Moreover, our data suggest that the introduction of a permanent positive charge on Lys-115 by trimethylation is also not the major reason for this specific post-translational modification.

Calmodulin is a ubiquitous, highly conserved Ca$^{2+}$-binding protein. It activates numerous target enzymes via a Ca$^{2+}$-dependent pathway (for recent review, see Meena et al. (1991)). Calmodulin purified from most sources has a single trimethyllysine residue at position 115 of its amino acid sequence (Klee and Vanaman, 1982). The enzyme responsible for this specific post-translational modification is $S$-adenosyl-$L$-methionine: lysine $N$-methyltransferase (Morino et al., 1987; Rowe et al., 1986). However, calmodulins purified from organisms such as Chlamydomonas reinhardtii (Lukas et al., 1985), Dictyostelium discoideum (Marshak et al., 1984), and Trypanosoma Ruben et al., 1984) have a normal lysine instead of trimethyllysine at position 115. In other instances, Lys-115 in the protein is only partially methylated (Oh and Roberts, 1990; Oh et al., 1992; Rowe et al., 1986; Molla et al., 1981, Sauter et al., 1993). The key factors that determine the specific methylation of Lys-115 while the other 7 lysine residues in calmodulin remain unmodified remain unclear.

Several studies concerned with the function of this post-translational modification have been reported. Unmethylated calmodulins are not significantly different from methylated calmodulins in terms of their ability to bind Ca$^{2+}$ and to stimulate most target enzymes in vitro (Putkey et al., 1985, 1986; Roberts et al., 1985; Rowe et al., 1986). However, unmethylated calmodulin activates plant NAD kinase three times better than methylated calmodulin (Roberts et al., 1986), and methylation could function as another level of regulation of NAD kinase activity. By introduction of a mutant calmodulin, which cannot be trimethylated, into tobacco plants, Roberts et al. (1992) showed that trimethylation plays an important role in vivo because these plants had markedly reduced growth rates. Another important function of the methylation is probably the regulation of the level of calmodulin in specific tissues by controlling the protein turnover rate via the ubiquitin-dependent degradation pathway (Hershko, 1988, 1991). It has been observed that unmethylated calmodulin is a good substrate for ubiquitinylation and that the reaction takes place specifically at Lys-115 (Gregori et al., 1985, 1987). This again raises the question, why is only Lys-115 modified? Is it perhaps because it has an unusual reactivity?

In this work, we have used various approaches to study trimethyllysine 115. First, we used $^{14}$N NMR as a novel spectroscopic tool to detect and quantify the trimethylation of Lys-115 in calmodulin from bovine testis. The dynamic properties of trimethyllysine were studied by $^{14}$N NMR relaxation measurements. For comparison, the single trimethyllysine 77 of yeast cytochrome c was also studied by $^{14}$N NMR. To the best of our knowledge, this is the first useful application of $^{14}$N NMR for the study of a protein. Second, by using bacterially expressed calmodulin, we have been able to study the pH titration behavior of Lys-115 and to obtain its pK$_a$ values in both the apo and Ca$^{2+}$ forms and in a complex of calmodulin with a calmodulin-binding domain peptide (Ikura et al., 1992). Finally, we have studied the capacity of Lys-115 mutants, in which the positively charged lysine side chain is replaced by the polar uncharged Gin residue, to activate two typical target enzymes. These experiments have allowed us to determine whether Lys-115 in calmodulin is characterized by an unusual side chain mobility or an unusual reactivity or whether its positive charge is an absolute requirement for enzyme activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trifluoperazine dihydrochloride, mellitin, and sodium cyanoborohydride were purchased from Sigma. N$^1$-Monomethyl- and
N°-dimethyl-L-lysines were products of Serva. N°-Trimethyl-L-lysine was obtained from Calbiochem. 13C-Enriched (99%) formaldehyde was purchased from MSD Isotopes (Montreal). Bovine calmodulin was purified from bovine testis (Pel-Freez Biologicals) following standard procedures (Andersson et al., 1983; Vogel et al., 1983). Cyclic nucleotide phosphodiesterase was kindly provided by Dr. J. Wang. Chicken gizzard myosin and smooth muscle myosin light chain kinase were gifts of Dr. M. Walsh. Other proteins were purchased from Sigma and were used without further purification. The peptide from skeletal muscle myosin light chain kinase encompasses residues 577–598 (Zhang et al., 1993). It was synthesized by the Core Facility for Protein/DNA Chemistry at Queen’s University (Kingston, Canada) and was >95% pure as judged by high pressure liquid chromatography.

Bacterial Expression of Bovine Calmodulin—Calmodulin was expressed in Esherichia coli cells harboring a “runaway” plasmid containing a synthetic bovine calmodulin gene with codons optimized for E. coli. The description of the plasmid and the expression of calmodulin were exactly the same as described by Brodin and Grundstrom (1986) for calbindin D9K.

Assay of Phosphodiesterase Activity—Cyclic nucleotide phosphodiesterase activity was measured colorimetrically by measuring the phosphate released from CAMP as described by Sharma and Wang (1979) with minor modifications. Reaction mixtures in volumes of 4 ml each were incubated for 3 min at 30 °C in 20 m Tris, pH 8.0, 20 m imidazole, 3 m CaCl2, 3 m MgCl2, and variable amounts of calmodulin or its mutant. The reaction was initiated by the addition of CAMP to a 1 m final concentration. At 3-min intervals, 0.45 ml of the reaction mixture was withdrawn, and the reaction was stopped by the addition of 50 m of 55% trichloroacetic acid, followed by assay of the amount of free phosphate released. The total reaction time was 21 min for each reaction. The phosphodiesterase activity in each reaction was deduced from the slope of the curve giving the amount of liberated phosphate versus reaction time.

Assay of Myosin Light Chain Kinase Activity—Myosin light chain kinase activity was assayed by the filter paper method (Blumenthal and Stull, 1980) with chicken gizzard myosin as the substrate. The reaction was carried out in a volume of 50 m containing 385 m Tris-HCl, pH 7.5, 50 m CaCl2, 4 m MgCl2, 0.01 m CaCl2, 10 m/g mL myosin light chain kinase, 0.5 mg/mL myosin, and 1 m [γ-32P]ATP (~200 cpm/pmol).

13C-Methylation of Calmodulin Samples—The reductive methylation of calmodulin samples using 13C-enriched (99%) formaldehyde has been described previously (Jentoft and Dearborn, 1983). Briefly, 10 mg of calmodulin was dissolved in 4 ml of 50 m HEPES, 10 m Tris-HCl, pH 7.5, 50 m CaCl2, pH 7.5. 13C-Labeled formaldehyde and freshly prepared NaCNBH3 (1 m solution) were added to this solution, and the mixture was shaken gently and incubated overnight at 4 °C. Both NaCNBH3 and [13C]-formaldehyde were added at a 5–10-fold molar excess over the free amino group of the protein. The reaction was stopped by extensive dialysis against 10 m NH4HCO3 buffer, and the solution was subsequently freeze-dried and stored at −20 °C prior to NMR studies. This procedure results in the dimethylation of all lysine residues in calmodulin to >95%.

Site-directed Mutagenesis of Lys115 to Gln in Bacterially Expressed Calmodulin—A 23-mer oligonucleotide with the sequence 5'-CAAACCTTGTGAGCAGCTGACA-3' was constructed to generate the Lys115 to Gln mutation at position 115. A Sty1 restriction enzyme site (underlined) is included in the oligonucleotide, and the Gln codon is highlighted by boldface letters. The calmodulin gene was first subcloned into the pBS plasmid vector (Stratagene). The mutation was generated by the polymerase chain reaction following published procedures (Kadowaki et al., 1989). The other primer used to generate the mutation was the universal primer for the pBS plasmid vector. The mutation was identified by sequencing the individual clone that carried the polymerase chain reaction-generated DNA fragment. DNA sequencing was also employed to rule out potential misincorporations in the polymerase chain reaction.

NMR Spectroscopy—All 14N NMR spectra were recorded in a 90% H2O, 10% D2O solution containing 0.15 m KCl. The 14N spectra were obtained at ambient temperature at 28.2 MHz on a Bruker AM400 wide-bore spectrometer equipped with a 10-mm broad-band probe. The spectra were recorded with 8K of data points covering a 20,000-Hz sweep width, a 0.2-s acquisition time, a 0.4-s pulse repetition rate, and a 25-μs pulse width corresponding to a 90° pulse angle. The longitudinal relaxation time (T1) was measured by the standard inversion-recovery method. The concentration of calmodulin samples was ~1.0 m, whereas those of L-lysine and N°-methylated L-lysine derivatives were ~20 m. The pH values of the samples were adjusted by the addition of microliter amounts of diluted KOH or HCl.

13C NMR Spectra—13C-1H HMQC NMR spectra were recorded on a Bruker AMX-500 spectrometer equipped with digital phase shifters, an inverse-detected 5-mm broad-band probe, and an X32 computer using the pulse sequence described by Bax et al. (1983). The phase-sensitive mode of the spectra was obtained by using the time fractional phase increment technique (Marion and Wuthrich, 1986). Typically, each HMQC spectrum was recorded with 200 experiments, with eight scans/experiment. All spectra were processed using the Bruker UXNMR software package. The data were zero-filled once in the F2 dimension and twice in the F1 dimension before Fourier transformation. The chemical shifts of the resonances were referenced to internal 13CH3OH using 49.5 and 3.36 ppm as the chemical shifts for its methyl carbon and proton, respectively.

RESULTS

14N NMR Studies of Lysine and Its N°-Mono-, N°-Di-, and N°-Trimethylated Derivatives—For comparison, the free amino acid L-lysine and its N°-mono-, N°-di-, and N°-trimethylated derivatives were included in these studies. In 14N NMR spectra (Fig. 1A), L-lysine shows two broad partially overlapping resonances at low pH (at ~336 and ~343 ppm, line widths of 150 and 84 Hz, respectively). Upon raising the pH, the downfield resonance broadens and overlaps further with the ~343 ppm peak; its pK° is ~8.0. The other resonance shifts and broadens at higher pH (Fig. 2), indicating that it has a pK° of ~10.5. The pK° values reported for lysine are 3.2 (for the carboxyl group), 8.0 (for the ε-nitrogen), and 10.5 (for the ε-nitrogen) (Kent et al., 1974). Thus, the N°-trimethylated derivative of L-lysine was studied at pH 8, where the ε-nitrogen proton at pH 8 is desprotonated, thus allowing for the determination of the pK° values of the ε-nitrogen. The 14N NMR resonances at ~336 and ~343 ppm can be assigned to the ε- and γ-nitrogens of the free lysine, respectively. Line broadening as a result of deprotonation has been reported previously (Tzalmona and Loewenthal, 1974; Cohen et al., 1975). It probably results from a decrease in the symmetry of the surrounding of the quadrupolar 14N nucleus; obviously, the protonated nitrogen has higher symmetry (R-NH3+ is nearly tetrahedral). The broadening at high pH may also arise in part from the high exchange rate of protons by base catalysis (Blomberg et al., 1976).

Protonation and deprotonation behavior similar to that for L-lysine has been observed by 14N NMR for N°-mono- and N°-dimethyl-lysines in aqueous solution. Two overlapping resonances (for the ε- and γ-nitrogens) are observed below pH 8 (Fig. 1B). In both cases, the peak for the ε-nitrogen broadens very much around pH 8, its reported pK°, Hence, only one broad resonance is observed around pH 9, which is assigned to the protonated ε-nitrogen. Again, the ε-nitrogen peaks broaden into the base line when the pH is raised above 10, which is 1

The abbreviation used is: HMQC, heteronuclear multiple quantum coherence.
consistent with the expected titration behavior of mono- and dimethyllysines (Huque and Vogel, 1993). Unlike the mono-, di-, and unsubstituted L-lysines, the N'-trimethyl-L-lysine in aqueous solution shows one narrow resonance (−328 ppm at pH 8.2; see Fig. 1C). The latter absorption has a line width of only 3 Hz and is assigned to the quaternary nitrogen having tetrahedral electronic distribution. As expected, the chemical shift for the quaternary trimethylated nitrogen does not change in the course of the pH titration (between 1.0 and 12.2) (Fig. 2), thus indicating that there is no titratable proton in the quaternary substituted nitrogen and that it remains positively charged at all pH values.

14N NMR of Calmodulin and Cytochrome c—Calmodulin purified from bovine testis is reported to contain 1 trimethyllysine residue at position 115 in its amino acid sequence. For both the Ca2+ and apo forms of the protein in aqueous solution, four sharp resonances were observed in the 14N NMR spectrum (Fig. 3A). The lowest field (0 ppm) resonance is due to KNO2, which was added as a chemical shift and concentration reference compound. The resonance at −66 ppm is assigned to dissolved atmospheric nitrogen since bubbling of the solution with argon results in its disappearance (McIntyre et al., 1989). The resonance at −356 ppm can be assigned from its chemical shift and pH titration behavior (pKa ~ 9.3) to contaminating ammonium ions. The protein in the final stage of the purification is dialyzed extensively against an ammonium bicarbonate buffer, and clearly, some ammonium is left after lyophilization. The remaining signal at −328 ppm (line width of 23 Hz) can be attributed to the quaternary nitrogen of the trimethyllysine residue. The chemical shift of this resonance is identical to that observed for the corresponding free amino acid N'-trimethyl-L-lysine. Interestingly, this resonance is absent in 14N NMR spectra of bacterially expressed protein. Obviously, E. coli lacks the enzymes required for trimethylation of calmodulin, although methylation of elongation factor Tu occurs in this organism (Arai et al., 1989). The 14N resonance also does not move or broaden as a function of pH. Neither the other nitrogen atoms of calmodulin that are part of the protein’s backbone or side chains nor the acetylated terminal nitrogen is observed in the 14N NMR spectrum. The absence of a sharp resonance at −328 ppm was also noted for a number of other small proteins (data not shown), which do not contain trimethyllysine residues. Integration of the KNO3 and trimethyllysine resonances in a 14N NMR spectrum of calmodulin acquired under fully relaxed conditions suggests that each calmodulin molecule contains 1 trimethyllysine residue.

We have also recorded a 14N NMR spectrum of cytochrome c from Saccharomyces cerevisiae, which is known to have a single residue of N'-trimethyllysine at position 77 (Delange et al., 1970; Paik et al., 1989). This protein gives rise to the same spectrum as bovine calmodulin (Fig. 3B); again, the sharp resonance for trimethyllysine was observed at −328 ppm, and it was notably absent in spectra taken from equine cytochrome c, which is known to be devoid of this modified lysine. Because of the large number of lysine residues in this protein and the higher concentration of the protein used in the experiment, a broad resonance was observed for their side chain -NH3+ groups between −328 and −356 ppm.

The longitudinal relaxation times (T1) measured for the trimethyllysine e-nitrogen in the Ca2+ and apo forms of calmodulin were 0.058 and 0.050 s, respectively. However, to be able to calculate the correlation time (τc) from the T1 values, we need to obtain a value for the quadrupole coupling constant (χ). We have used the free amino acid trimethyllysine as a model compound to estimate χ. For small molecules such as trimethyllysine in the limit of fast motion (ωpτc < < 1), the longitudinal relaxation time for a quadrupolar nucleus is given by the following equation (Harris, 1986):

\[ T1 = \frac{3}{10}k^2\chi^2\tau_N x(2I + 3)y F(2I - 1) \]

For 14N, I = 1, and after rearrangement, χ can be expressed as follows: χ = (14.8044 x T1 x T2)1/2. The T1 values of the 14N nucleus in the free amino acid were measured by the inversion recovery method. The correlation time (τc) was considered to be the same as that of the methylene carbon atom.
Trimethyllysine 115 in Calmodulin

![Diagram](image)

**Fig. 4.** $^1$H-$^1$H HMQC spectra of $^{13}$C-methylated calmodulin samples. **A,** calmodulin purified from bovine testis; **B,** calmodulin expressed in *E. coli.* The spectra were recorded for a 1 nM $^{13}$C-methylated Ca$^{2+}$-calmodulin sample at pH 10.0. Since there was no decoupling applied during the acquisition, each peak appears as a doublet. The resonances resulting from Lys-115 in **B** are labeled as peak 8 and indicated by the arrows.

directly bonded to the quaternary nitrogen in the molecule. Its $\tau_c$ was determined by measuring its $T_1$ value by $^{13}$C NMR and assuming dipole-dipole relaxation with its attached protons as its dominant relaxation mechanism (Harris, 1986). In the pH range of 0.8–7.5, with salt concentrations between 0 and 2 M NaCl, we found that the $\chi$ value for free trimethyllysine lies in the range of 67–125 kHz. These values are consistent with those determined and reported for various other quaternary nitrogen atoms (Huque, 1989). Thus, using this range of $\chi$ values, the correlation times could be calculated for apo- and calcium-calmodulins from their respective $^{14}$N $T_1$ data. The values obtained were $9-30 \times 10^{-11}$ and $11-40 \times 10^{-11}$ s, respectively. Titrination of calmodulin with 0–3 eq of the calmodulin antagonist trifluoperazine dihydrochloride (Vogel, 1987) or 0–2 eq of the model calmodulin-binding peptide mellitin (Vogel, 1987) did not show any change in the $^{14}$N NMR chemical shift or the $T_1$ value of trimethyllysine of the protein (data not shown).²

$^{13}$C NMR Studies of Calmodulin Samples Purified from Bovine Testis and Bacterial Expression System—Calmodulin samples purified from bovine testis and from *E. coli* were labeled with $^{13}$C using a dimethylation reaction that is specific for amino groups. Since *E. coli* cells do not have the ability to be labeled with $^{13}$C using a dimethylation reaction that is specific for amino groups, we were able to obtain the $^{13}$C-$^1$H HMQC spectra of the K115Q mutant at pH 10.0. Since there was no decoupling applied during the acquisition, each peak appears as a doublet. The resonances resulting from Lys-115 in **B** are labeled as peak 8 and indicated by the arrows.

² Unlike the bovine protein, bacterially expressed calmodulin also does not become N-acetylated at its NH$_2$-terminal amino group. This free amino group also becomes dimethylated, and this gives rise to resonances at 41.5 ppm ($^{13}$C) and 2.28 ppm ($^1$H) in the HMQC spectrum (see Fig. 5). As expected (Jentoft and Dearborn, 1983; Huque and Vogel, 1993), it shifts in the opposite direction during the pH titration com-
Din samples, but only the 13C chemical

Ranne of curves of Ca2+-calmodulin; the dimethyllysine of Lys-115 are all normal in relation to those wenthal, 1974; Cohen et al., 1975; Richards and Thomas, 1975; the amino acid L-lysine and its derivatives, Ne-mono-, W-di-, and W-trimethyllysines. Only the $\varepsilon$-nitrogen of trimethyllysine Lys-115 in mammalian calmodulin. To obtain a complete characterization of trimethyllysine in calmodulin, we first studied the point mutation of Lys-115 to Gln also has no effect on the ability to activate phosphodiesterase and myosin light chain enzymes.

 DISCUSSION

14N NMR of Trimethyllysine—Nitrogen atoms are found in many biological compounds, and the 14N isotope has a natural abundance of 99.6%. Furthermore, since 14N is a quadrupolar nucleus, it has short relaxation times compared to 15N, thus allowing a faster pulse repetition rate. Therefore, 14N NMR spectroscopy could have some advantages for the study of peptides, proteins, and nucleic acids. However, broad lines, low receptivity, and low resonance frequency, which are problems common to many quadrupolar nuclei, have prevented this spectroscopic tool from being widely applied to biological compounds. Only a few papers dealing with amino acids and their interactions have been published to date (Tzalmona and Lee-wenthal, 1974; Cohen et al., 1975; Richards and Thomas, 1975; Gerothanassis et al., 1987). In this paper, we have used 14N NMR spectroscopy to study the specific N$^\varepsilon$-trimethylation of Lys-115 in mammalian calmodulin. To obtain a complete characterization of trimethyllysine in calmodulin, we first studied the amino acid L-lysine and its derivatives, N$^\alpha$-mono-, N$^\alpha$-di-, and N$^\alpha$-trimethyllysines. Only the $\varepsilon$-nitrogen of trimethyllysinepared to the peaks from the lysine residues. It has a normal pK\textsubscript{w} value of $\sim$7.5.

TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lys-115 pK\textsubscript{w}</th>
<th>Range of pK\textsubscript{w} values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-CaM</td>
<td>10.52</td>
<td>10.50-10.5</td>
</tr>
<tr>
<td>Ca$^{2+}$-CaM</td>
<td>10.23</td>
<td>10.20-10.2</td>
</tr>
<tr>
<td>CaM-peptide</td>
<td>10.14</td>
<td>10.12-10.12</td>
</tr>
</tbody>
</table>

* Calmodulin.

in addition, for comparison, the range of pK\textsubscript{w} values measured for peaks 1–7 are listed as well. The pK\textsubscript{w} values determined for the dimethyllysine of Lys-115 are all normal in relation to those measured for other proteins or model compounds (Jentoft and Dearborn, 1983).

Enzymatic Assays of Bacterially Expressed Calmodulin and Its K115Q Mutant—In agreement with previous observations (Putkey et al., 1986, 1988), calmodulin purified from bovine tissue and bacterially expressed calmodulin show the same ability to activate phosphodiesterase and myosin light chain kinase. This confirms that the trimethylation of Lys-115 does not play a role in the activation of these enzymes (Fig. 7). The point mutation of Lys-115 to Gin also has no effect on the ability of the protein to stimulate these two enzymes (Fig. 7). The latter result indicates that it is not necessary to maintain a positive charge on this residue when calmodulin binds to these enzymes.

FIG. 7. Shown is the activation of phosphodiesterase (A) and smooth muscle myosin light chain kinase (B) by bovine testis calmodulin (B.CaM, ○), bacterially expressed calmodulin (C), and K115Q calmodulin (A).

gives a very sharp 14N resonance; this is because of its very highly symmetrical structure, which is almost perfectly tetrahedral. The trimethylated $\varepsilon$-nitrogen is always positively charged and does not have a titratable proton, so the 14N resonance retains the same line width and chemical shift over a wide pH range (Fig. 2). Our subsequent studies with calmodulin and other proteins showed that a sharp 14N resonance at $\sim$328 ppm can be observed for trimethyllysine in both the apo and Ca$^{2+}$ forms of bovine testis calmodulin and for yeast cytochrome c (Fig. 3). The other lysine residue side chains only give rise to a broad peak at $\sim$340 ppm for their $\varepsilon$-nitrogen atoms. All amide backbone and side chain nitrogen atoms of the protein as well as those in Arg, Trp, and His side chains do not give rise to any detectable signals. Obviously, their substitution pattern is very unsymmetrical, and their motions are slower, leading to extensive broadening of their quadrupolar 14N NMR signals. By comparison to an internal reference compound, we were able to quantitate the amount of trimethyllysine in calmodulin. As expected (Klee and Vanaman, 1982), we obtained a value of 1 trimethyllysine for calmodulin from bovine testis.4 Although it is possible to monitor the levels of trimethylation by amino acid analysis or by using a specific calmodulin N-methyltransferase (Oh and Roberts, 1990), our results show that 14N NMR spectroscopy provides a fast, simple, and straightforward alternative. Additional advantages of 14N NMR for this purpose are that it is noninvasive and that further information about the mobility of the side chain can be obtained from 14N relaxation measurements. A correlation time of 9–40 x 10$^{-11}$ s for trimethyllysine 115 in calmodulin indicates the presence of fast motions of the $\varepsilon$-nitrogen atom of the trimethyllysine residue with respect to the surface of the protein. Similar flexibility of lysine side chains on the surface of a protein is very common. The binding of trifluoperazine dihydrochloride and mellitin does not change the 14N T$_1$ value of trimethyllysine, and since the binding of these two calmodulin antagonists competes with the binding of target proteins (Vogel, 1987), it seems unlikely that the trimethyllysine residue of calmodulin is directly involved in target protein binding.

pK\textsubscript{w} for Lys-115 Determined by 13C NMR—To date, the reason why only Lys-115 becomes trimethylated in calmodulin remains unclear. In the x-ray structure (Babu et al., 1988; Rao et al., 1993), all the lysine residues in Ca$^{2+}$-calmodulin are located on the surface of the protein. Although trimethylated Lys-115 has the highest solvent accessibility, it is unlikely that solvent accessibility is the only reason for the site-specific methylation since Lys-94 and Lys-77 are quite similar in this respect. Recently, the crystal structure of bacterially expressed Ca$^{2+}$-calmodulin has been refined to 1.7 Å, and it shows that the side chain of unmodified Lys-115 is also located on the surface of the protein and that it has a high solvent accessibility similar

Using the same strategy, we have also determined that the stoichiometry of trimethylation of yeast cytochrome c is 0.9, in agreement with earlier studies (Delange et al., 1970; Paik et al., 1989).
to that of Lys-94 (Chattopadhyaya et al., 1992). Here, we have studied whether Lys-115 is such a good substrate because it has perhaps an unusual pK<sub>a</sub>. By comparing the 1H-13C HMQC spectra of calmodulin samples purified from bovine testis and E. coli (Fig. 4), we have been able to identify the Lys-115 resonance directly, and subsequent pH titration studies showed that it has a normal pK<sub>a</sub> compared to the other Lys residues in both the apo and Ca<sup>2+</sup> forms (Table I). Thus, the specific tri-methylation of Lys-115 does not result from an unusual pK<sub>a</sub>, which in turn could lead to an unusual reactivity of this residue (Zhang and Vogel, 1993). It has been reported that Ca<sup>2+</sup> stimulates the methylation of Lys-115 when calmodulin and calmodulin N-methyltransferase are incubated together in vitro (Oh and Roberts, 1990). In Ca<sup>2+</sup>-calmodulin, all the Lys residues have lower pK<sub>a</sub> values than in the apo form, and the pK<sub>a</sub> change of Lys-115 follows virtually the same pattern displayed by the other Lys residues (Table I). Thus, our data also allow the possibility that the Ca<sup>2+</sup>-stimulated methylation arises from an unusual reactivity change in the -NH<sub>2</sub> group of Lys-115 when calmodulin and calmodulin to activate phosphodiesterase and myosin light chain kinase, the Lys-115 pK<sub>a</sub> is normal, thus indicating that trimethylation does not serve to protect a residue that is unusually reactive in complexes of calmodulin with target proteins. Single point mutants of calmodulin such as K115R competitively inhibit the calmodulin-N-methyl transferase (Oh and Roberts, 1990); hence, the methyltransferase recognizes features of calmodulin that are distinct from the methylated residue. These results combined suggest that Ca<sup>2+</sup> binding to calmodulin induces a conformational change in the protein, which exposes a surface feature that is recognized by the methyltransferase. However, calmodulin has high internal homology; hence, it could be possible that the methyltransferase can also bind to other site(s) in calmodulin. Indeed, it has been shown that several point mutations in the protein, which in turn could lead to an unusual reactivity of this residue (Schaefer et al., 1987, Ling et al., 1992). Thus, it would be interesting to know which specific residues determine the specificity of post-translational modification of Lys-115 by the methyltransferase. In this respect, mutagenesis studies such as those reported by Lukas et al. (1989) could prove insightful. Examination of Positive Charge of Lys-115—Bacteria normally do not possess calmodulin, which possesses a Lys residue instead of a trimethylated Lys residue at position 115, is known to be indistinguishable from calmodulin purified from bovine tissue in terms of its ability to activate phosphodiesterase and myosin light chain kinase (Fig. 7) (Putkey et al., 1986, 1988). It has also been reported that a K115R mutant activates these enzymes in a normal fashion (Gregori et al., 1987). However, the positive charge is maintained on the side chain of the K115R mutant; hence, this result does not indicate whether maintaining a positive charge in this position is required. For this reason, we replaced Lys-115 with the polar uncharged Gln residue. This mutation does not change the structure of the protein as judged by 1H NMR and 1H-13C HMQC of the mutated protein, which is perhaps not surprising since Lys-115 is located on the surface of the protein. Here, we have shown that mutation of Lys-115 to Gln also does not have any effect on the ability of the protein to stimulate these two enzymes (Fig. 7). The above data suggest that the introduction of a permanent positive charge through trimethylation of Lys-115 does not play a role in the ability of calmodulin to regulate these two enzymes. This is in keeping with the observation that the myosin light chain kinase-binding site and the Lys-115 side chain are on opposite sides of calmodulin (Ikura et al., 1992). However, trimethylation does attenuate the ability of calmodulin to stimulate plant NAD kinase in vitro (Roberts et al., 1986). Therefore, it seems that NAD kinase represents a novel calmodulin-regulated enzyme and that it probably has a calmodulin-binding region that interacts with trimethyllysine 115.

Comparison of Tri-methylation and Ubiquitylation—Gregori et al. (1985, 1987) showed that the trimethylation of Lys-115 serves to protect this important regulatory protein from proteolytic degradation via the ubiquitin-dependent pathway. Ubiquitin can conjugate to unmethylated calmodulin specifically at Lys-115, and this conjugation is also stimulated by Ca<sup>2+</sup> (Gregori et al., 1985, 1987; Jennissen et al., 1992). From the pK<sub>a</sub> results reported here in and from the solvent accessibility data of the side chains of the Lys residues as determined from the x-ray structure of calmodulin, it seems again unlikely that this highly specific ubiquitylation results from an unusual pK<sub>a</sub> or from an abnormal exposure of Lys-115 on the protein surface. Again, some feature of the local structure around Lys-115 is recognized by the enzymes catalyzing the covalent attachment of ubiquitin, and this in turn determines the high specificivity of the reaction. In this respect, it is noteworthy that through the use of deletion mutants, specific regions in the amino acid sequence of the protein cyclin could be identified that seemed to be involved in the binding of the enzymes that form the isopeptide bond between the ubiquitin side chain and ubiquitin (Glotzer et al., 1991). It would be interesting to know whether the features recognized by the enzymes involved in the trimethylation and ubiquitylation are identical since this could provide an elegant way to control intracellular proteolysis. Be that as it may, the proteolytic degradation rate of calmodulin in a specific tissue in vivo depends on a critical balance of multiple factors such as the concentration of Ca<sup>2+</sup>, the activity of the N-methyltransferase, and the efficiency of the ubiquitin-dependent machinery.

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