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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 ϵ-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 Gln residue does not alter the ability of the protein to 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 ϵ-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 Means *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 quantitate 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 Gln 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^{ϵ}-Monomethyl- and

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N^{ϵ}-dimethyl-L-lysines were products of Serva. N^{ϵ}-Trimethyl-L-lysine was obtained from Calbiochem. ^{13}C-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 *Escherichia 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 *et al.* (1989), except that the calbindin D_{9K} gene was replaced by the synthetic calmodulin gene. The expressed calmodulin was purified following the methods previously described by Brodin and Grundström (1986) for calbindin D_{9K}.

Assay of Phosphodiesterase Activity—Cyclic nucleotide phosphodiesterase activity was assayed 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 mM Tris, pH 8.0, 20 mM imidazole, 3 mM $CaCl_2$, 3 mM $MgCl_2$, and variable quantities of calmodulin or its mutant. The reaction was initiated by the addition of cAMP at a 1 mM 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 µl 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 µl containing 25 mM Tris-HCl, pH 7.5, 60 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 µg/ml myosin light chain kinase, 0.5 mg/ml myosin, and 1 mM [γ-^{32}P]ATP (≈200 cpm/pmol).

^{13}C-Methylation of Calmodulin Samples—The reductive methylation of calmodulin samples using ^{13}C-enriched (99%) formaldehyde has been described previously (Jentoft and Dearborn, 1983). Briefly, 10 mg of calmodulin was dissolved in 4 ml of 50 mM HEPES, 10 mM $CaCl_2$, pH 7.5. ^{13}C-Labeled formaldehyde and freshly prepared $NaCNBH_3$ (1 M solution) were added to this solution, and the mixture was shaken gently and incubated overnight at 4 °C. Both $NaCNBH_3$ and [^{13}C]formaldehyde were added at a 5–10-fold molar excess over the free amino groups of the protein. The reaction was stopped by extensive dialysis against 10 mM NH_4HCO_3 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% (Huque and Vogel, 1993).

Site-directed Mutagenesis of Lys-115 to Gln in Bacterially Expressed Calmodulin—A 23-mer oligonucleotide with the sequence 5′-CAAACCTTGGTGAG**CAG**CTGACA-3′ was constructed to generate the Lys → Gln mutation at position 115. A *Sty*I 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 ^{14}N NMR spectra were recorded in a 90% H_2O, 10% D_2O solution containing 0.15 M KCl. The ^{14}N spectra were obtained at ambient temperature at 28.9 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 (T_1) was measured by the standard inversion recovery method. The concentration of calmodulin samples was ~1.0 mM, whereas those of L-lysine and N^{ϵ}-methylated L-lysine derivatives were ~20 mM. The pH values of the samples were adjusted by the addition of microliter amounts of diluted KOH or HCl.

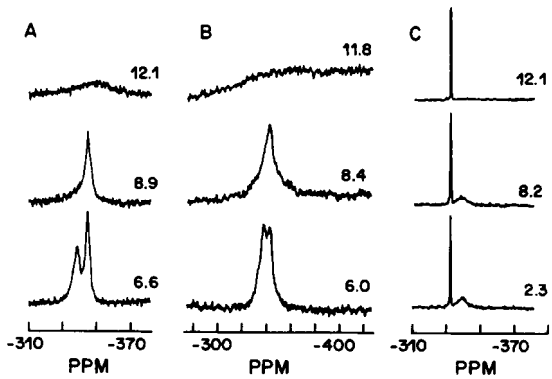

FIG. 1. **^{14}N NMR spectra of L-lysine (*A*), N^{ϵ}-monomethyl-L-lysine (*B*), and N^{ϵ}-trimethyl-L-lysine (*C*) in aqueous solution as function of pH.** The pH values are indicated.

^1H-^{13}C HMQC[1] 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 proportional phase increment technique (Marion and Wüthrich, 1983). Typically, each HMQC spectrum was recorded with 128 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 $^{13}CH_3OH$ using 49.5 and 3.36 ppm as the chemical shifts for its methyl carbon and proton, respectively.

RESULTS

^{14}N NMR Studies of Lysine and Its N^{ϵ}-Mono-, N^{ϵ}-Di-, and N^{ϵ}-Trimethylated Derivatives—For comparison, the free amino acid L-lysine and its N^{ϵ}-mono-, N^{ϵ}-di-, and N^{ϵ}-trimethylated derivatives were included in these studies. In ^{14}N NMR spectra (Fig. 1*A*), 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_a is ≈8.0. The other resonance shifts and broadens at higher pH (Fig. 2), indicating that it has a pK_a of ≈10.5. The pK_a values reported for lysine are 3.2 (for the carboxyl group), 8.0 (for the α-nitrogen), and 10.5 (for the ε-nitrogen) (Keim *et al.*, 1974). Thus, from their pH titration behavior, the ^{14}N NMR resonances at −336 and −343 ppm can be assigned to the α- and ε-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 ^{14}N nucleus; obviously, the protonated nitrogen has higher symmetry ($R\text{-}NH_3^+$ 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 ^{14}N NMR for N^{ϵ}-mono- and N^{ϵ}-dimethyl-L-lysines in aqueous solution. Two overlapping resonances (for the α- and ε-nitrogens) are observed below pH 8 (Fig. 1*B*). In both cases, the peak for the α-nitrogen broadens very much around pH 8, its reported pK_a. 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.

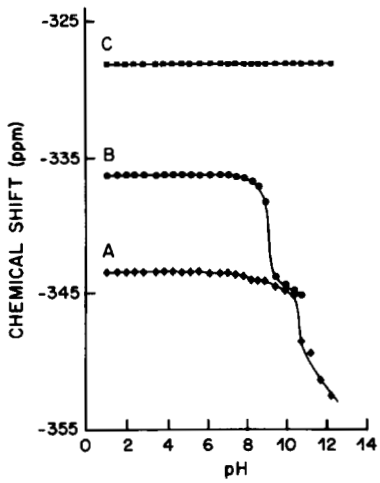

FIG. 2. **pH titration curves of α- and ε-nitrogens in L-lysine and N^{ϵ}-trimethyl-L-lysine.** *Curve A*, ε-nitrogen of L-lysine; *curve B*, α-nitrogen of L-lysine; *curve C*, N^{ϵ}-trimethyl group. Because of the severe peak broadening when the pH of the sample is higher than the pK_a value for a given resonance, the titration curves are not very smooth.

consistent with the expected titration behavior of mono- and dimethyllysines (Huque and Vogel, 1993). Unlike the mono-, dimethyl-, and unsubstituted L-lysines, the N^{ϵ}-trimethyl-L-lysine in aqueous solution shows one narrow resonance (−328 ppm at pH 8.2; see Fig. 1*C*). 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.

^{14}N 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 Ca^{2+} and apo forms of the protein in aqueous solution, four sharp resonances were observed in the ^{14}N NMR spectrum (Fig. 3*A*). The lowest field (0 ppm) resonance is due to KNO_3, 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 (p$K_a \approx 9.3$) 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^{ϵ}-trimethyl-L-lysine. Interestingly, this resonance is absent in ^{14}N 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.*, 1980). The ^{14}N 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 ^{14}N 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 KNO_3 and trimethyllysine resonances in a ^{14}N NMR spectrum of calmodulin acquired under fully relaxed conditions suggests that each calmodulin molecule contains 1 trimethyllysine residue.

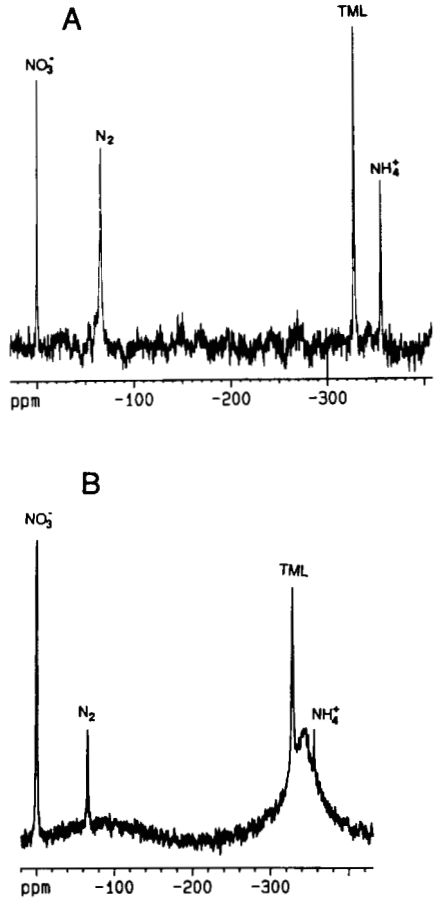

FIG. 3. *A*, ^{14}N NMR spectrum of 1 mM Ca^{2+}-calmodulin in aqueous solution at pH 7.3. Under the same conditions, apocalmodulin gives rise to a spectrum identical to that of Ca^{2+}-calmodulin. *B*, ^{14}N spectrum of 4.2 mM cytochrome *c* in aqueous solution at pH 7.8. *TML*, trimethyllysine.

We have also recorded a ^{14}N NMR spectrum of cytochrome *c* from *Saccharomyces cerevisiae*, which is known to have a single residue of N^{ϵ}-trimethyllysine at position 77 (Delange *et al.*, 1970; Paik *et al.*, 1989). This protein gives rise to virtually the same spectrum as bovine calmodulin (Fig. 3*B*); 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 -NH_3^+ groups between −328 and −356 ppm.

The longitudinal relaxation times (T_1) measured for the trimethyllysine ε-nitrogen in the Ca^{2+} and apo forms of calmodulin were 0.038 and 0.050 s, respectively. However, to be able to calculate the correlation time (τ_c) from the T_1 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 ($\omega_0\tau_c << 1$), the longitudinal relaxation time for a quadrupolar nucleus is given by the following equation (Harris, 1986): $1/T_1 = 3/10(\pi^2\chi^2\tau_N)((2I + 3)/I^2(2I - 1))$. For ^{14}N, $I = 1$, and after rearrangement, χ can be expressed as follows: $\chi = (14.8044 \times T_1 \times \tau_N)^{-1/2}$. The T_1 values of the ^{14}N nucleus in the free amino acid were measured by the inversion recovery method. The correlation time (τ_N) was considered to be the same as that of the methylene carbon atom

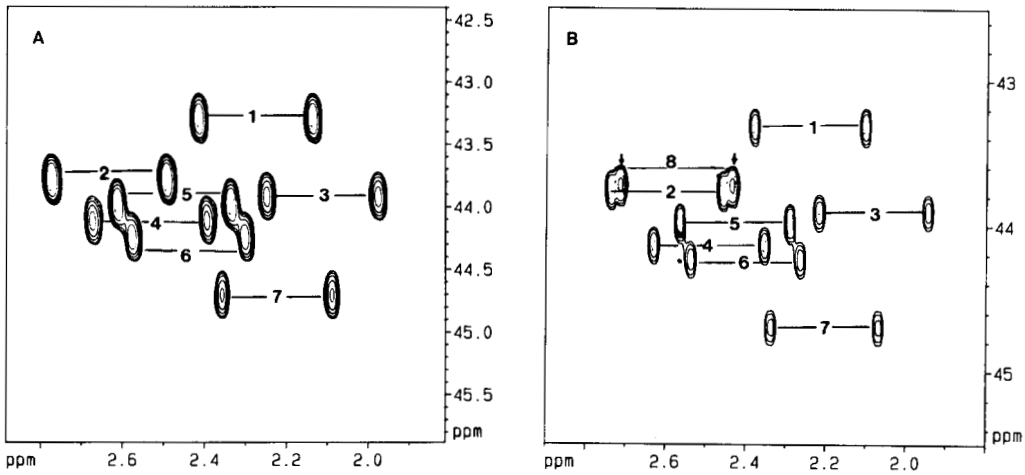

FIG. 4. **^{1}H-^{13}C HMQC spectra of ^{13}C-methylated calmodulin samples.** *A*, calmodulin purified from bovine testis; *B*, calmodulin expressed in *E. coli*. The spectra were recorded for a 1 mM ^{13}C-methylated Ca^{2+}-calmodulin sample at pH 10.0. Since there was no decoupling applied during 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 τ_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 χ 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 χ 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 × 10^{-11} and 11–40 × 10^{-11} s, respectively. Titration 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 convert Lys-115 into trimethyllysine, all 7 lysine residues that are present in bovine calmodulin plus Lys-115 could become labeled. Trimethyllysine 115 in calmodulin purified from bovine testis is no longer reactive and therefore cannot be labeled; thus, it is not detected by this technique. We have shown elsewhere that heteronuclear ^{13}C-^{1}H HMQC spectra provide a sensitive and convenient means to study the [^{13}C]dimethyllysine residues (Zhang and Vogel, 1993). The HMQC spectra shown in Fig. 4 for the two ^{13}C-methylated calcium-saturated calmodulin samples are essentially the same, except that there is one extra resonance in the protein sample from *E. coli* (Fig. 4). This extra resonance, which is labeled as *peak 8* in Fig. 4*B*, is undoubtedly due to Lys-115 in the protein. The resonances for the other 7 lysines are all separated in these two-dimensional spectra, and they appear in identical positions for the two proteins. In the same fashion, we have also been able to observe and assign the Lys-115 resonance in apocalmodulin (spectra not shown). Site-specific mutagenesis of Lys-115 to Gln served to confirm the assignment of this resonance. The HMQC spectrum of the Ca^{2+} form of the methylated K115Q mutant (Fig. 5)

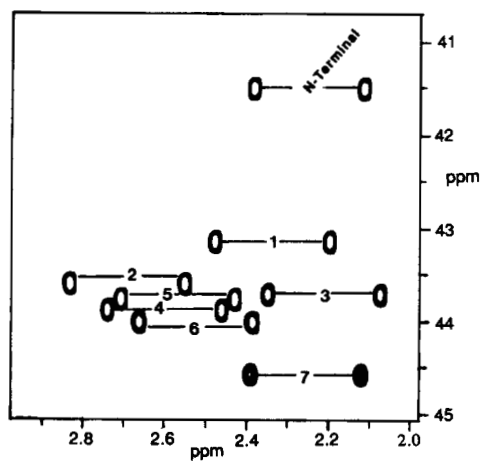

FIG. 5. **^{1}H-^{13}C HMQC spectrum of ^{13}C-methylated K115Q calmodulin mutant at pH 10.0.** The experimental conditions and labeling of resonances are the same as described for Fig. 4.

is exactly the same as that of calmodulin purified from bovine testis. The ^{1}H NMR spectrum of the K115Q mutant is also identical to that of the bacterially expressed protein (data not shown). The above results not only confirm the assignment, but also show that the specific point mutation (K115Q) does not change the structure of calmodulin.

By performing pH titration experiments on ^{13}C-methylated calcium-saturated calmodulin and apocalmodulin samples purified from *E. coli*, we were able to obtain the pK_a values for the ϵ-NH_2 groups of Lys-115 and the other 7 Lys residues. Fig. 6 shows the pH titration curves of Lys-115 and the lysine residue labeled as *peak 2* in Fig. 4 in Ca^{2+}-calmodulin. It is clear that these 2 lysine residues have almost identical pH titration curves and the same pK_a values. In addition, pH titration of a 1:1 complex of calmodulin with a peptide encompassing the calmodulin-binding domain of skeletal muscle myosin light chain kinase revealed that Lys-115 has a normal pH titration curve and a pK_a value similar to those for most of the other lysine residues in the protein. Table I lists the pK_a values for the ϵ-NH_2 groups of lysine 115 in the three different samples[3];

[2] The line width of the trimethyllysine resonance of yeast cytochrome *c* (≈30 Hz) is almost identical to that of calmodulin, indicating that it has similar motional characteristics.

[3] 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-

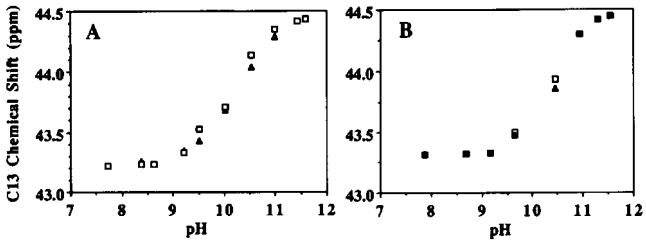

FIG. 6. **pH titration curves of Lys-115 (▲) and peak 2 (□) in Fig. 4.** The titration curves were obtained by monitoring both the ^{13}C and ^{1}H chemical shift changes as a function of pH for ^{13}C-methylated calmodulin samples, but only the ^{13}C shifts are shown here. *A*, pH titration curves of Ca^{2+}-calmodulin; *B*, pH titration curves of apocalmodulin.

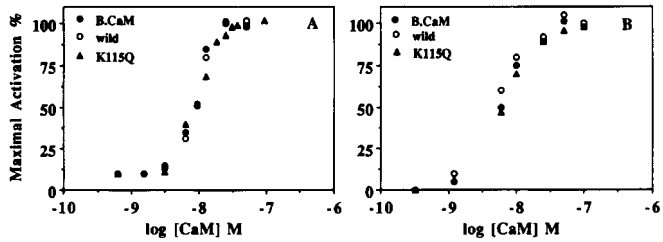

FIG. 7. Shown is the activation of phosphodiesterase (*A*) and smooth muscle myosin light chain kinase (*B*) by bovine testis calmodulin (*B.CaM*; ●), bacterially expressed calmodulin (○), and K115Q calmodulin (▲).

TABLE I

pK_a values for Lys-115 in apo- and Ca^{2+}-calmodulins and in the calmodulin-peptide complex

The pK_a values measured for other resonances are listed under "Range of pK_a values."

Protein	Lys-115 pK_a			Range of pK_a values
	From ^{13}C	From ^{1}H	Mean	
Apo-CaM[a]	10.52	10.48	10.50	9.9–10.5
Ca^{2+}-CaM	10.23	10.17	10.20	9.3–10.2
CaM-peptide	10.14	10.09	10.12	9.6–10.5

[a] Calmodulin.

in addition, for comparison, the range of pK_a values measured for peaks 1–7 are listed as well. The pK_a 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 Gln 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.

DISCUSSION

^{14}N NMR of Trimethyllysine—Nitrogen atoms are found in many biological compounds, and the ^{14}N isotope has a natural abundance of 99.6%. Furthermore, since ^{14}N is a quadrupolar nucleus, it has short relaxation times compared to ^{15}N, thus allowing a faster pulse repetition rate. Therefore, ^{14}N 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 Loewenthal, 1974; Cohen *et al.*, 1975; Richards and Thomas, 1975; Gerothanassis *et al.*, 1987). In this paper, we have used ^{14}N NMR spectroscopy to study the specific N^{ϵ}-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^{ϵ}-mono-, N^{ϵ}-di-, and N^{ϵ}-trimethyllysines. Only the ε-nitrogen of trimethyllysine gives a very sharp ^{14}N resonance; this is because of its very highly symmetrical structure, which is almost perfectly tetrahedral. The trimethylated ε-nitrogen is always positively charged and does not have a titratable proton, so the ^{14}N 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 ^{14}N resonance at −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 ≈−340 ppm for their ε-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 ^{14}N 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 ^{14}N NMR spectroscopy provides a fast, simple, and straightforward alternative. Additional advantages of ^{14}N NMR for this purpose are that it is noninvasive and that further information about the mobility of the side chain can be obtained from ^{14}N relaxation measurements. A correlation time of $9–40 \times 10^{-11}$ s for trimethyllysine 115 in calmodulin indicates the presence of fast motions of the ε-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 ^{14}N 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_a for Lys-115 Determined by ^{13}C 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 unmethylated Lys-115 is also located on the surface of the protein and that it has a high solvent accessibility similar

pared to the peaks from the lysine residues. It has a normal pK_a value of ≈7.5.

[4] 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_a. By comparing the ^1H-^{13}C 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_a compared to the other Lys residues in both the apo and Ca^{2+} forms (Table I). Thus, the specific trimethylation of Lys-115 does not result from an unusual pK_a, which in turn could lead to an unusual reactivity of this residue (Zhang and Vogel, 1993). It has been reported that Ca^{2+} stimulates the methylation of Lys-115 when calmodulin and calmodulin *N*-methyltransferase are incubated together *in vitro* (Oh and Roberts, 1990). In Ca^{2+}-calmodulin, all the Lys residues have lower pK_a values than in the apo form, and the pK_a change of Lys-115 follows virtually the same pattern displayed by the other Lys residues (Table I). Thus, our data also exclude the possibility that the Ca^{2+}-stimulated methylation arises from an unusual reactivity change in the -NH_2 group of Lys-115 upon the removal of Ca^{2+}. Moreover, even in a complex with a peptide derived from myosin light chain kinase, the Lys-115 pK_a 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^{2+} 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 calmodulin can give rise to methylation of Lys-13 as well (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.

Removal of Positive Charge of Lys-115—Bacterially expressed 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-^{13}C 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 Trimethylation and Ubiquitinylation—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^{2+} (Gregori *et al.*, 1985, 1987; Jennissen *et al.*, 1992). From the pK_a results reported herein 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 ubiquitinylation results from an unusual pK_a 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 specificity 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 linkage between the lysine 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 ubiquitinylation 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^{2+}, the activity of the *N*-methyltransferase, and the efficiency of the ubiquitin-dependent machinery.

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