Fluorescence Characterization of VU-9 Calmodulin, an Engineered Calmodulin with One Tryptophan in Calcium Binding Domain III†

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ABSTRACT: Absorption and fluorescence properties of VU-9 calmodulin, an engineered calmodulin in which a tryptophan residue has been introduced in position 99, have been investigated. Tryptophan 99 fluoresces with a maximum around 348 nm and is easily quenched by fluorescence quenchers such as acrylamide, indicating that the chromophore is in a polar environment and well exposed to the solvent, a location which has been reported previously for tyrosine 99 in mammalian calmodulin [Kilhoffer, M. C., Demaille, J. G., & Gérard, D. (1981) *Biochemistry 20*, 4407–4414]. The quantum yields of tryptophan 99 were found to be 0.19 in the absence of calcium and 0.15 in its presence. These values indicate that the chromophore is in a particular microenvironment where it is protected from the quenching mechanisms normally occurring in proteins. Steady-state fluorescence polarization measurements indicate that the protein exhibits segmental mobility both in the absence and in the presence of calcium. Binding of calcium decreases the mobility of the chromophore, a good indication for a rigidification of the protein structure. A quite rigid structure of at least the carboxy-terminal part of VU-9 calmodulin in the presence of Ca^{2+} is also suggested by Förster energy-transfer measurements.

Calmodulin is a ubiquitous multifunctional calcium binding protein present in all eukaryotic cells. In the presence of calcium, calmodulin activates a great variety of intracellular enzymes [for a review see Klee and Vanaman (1982) and Stoclet et al. (1987)] and is therefore involved in many cellular functions such as motility, secretion, glycogen metabolism, and synaptic transmission. In its structure, the protein exhibits four calcium binding domains numbered I–IV starting from the amino-terminal part of the molecule. Binding of calcium to calmodulin induces a conformational change that allows the protein to interact with and then to activate its target proteins.

One approach in the study of calmodulin structure to function relationship is to introduce reporter groups in different areas of the protein to monitor the microenvironment and changes in this microenvironment during calcium binding. This approach was rendered possible by the availability of the calmodulin synthetic gene and the techniques of cassette mutagenesis and protein engineering, which allow the production of high quantities of well-defined calmodulin mutants (Roberts et al., 1985; Craig et al., 1987; Kilhoffer et al., 1988). We choose to introduce tryptophan residues, the fluorescence properties of which have been shown to be very useful in the study of protein structure and dynamics. Indeed, tryptophan is highly fluorescent compared to the other aromatic amino acids, and its fluorescence is very sensitive to a wide variety of environmental conditions (Creed, 1984).

VU-9 calmodulin is a recombinant protein in which a tryptophan residue has been introduced in position 99 in calcium binding domain III. The mutation has been shown to be isofunctional (Kilhoffer et al., 1988), and the study of VU-9 calmodulin has already brought important information concerning ion binding to calmodulin (Kilhoffer et al., 1988).

In the present study, we present a quantitative description of the fluorescence properties of the single tryptophan residue of VU-9 calmodulin. Analysis of these properties in the absence and in the presence of calcium brings information on the protein conformation and on the conformational changes induced by calcium binding.

Materials and Methods

Materials

Trichloroacetic acid (CCl_3COOH) RH Normapur was obtained from Prolabo. Solutions were prepared fresh before use. All other chemicals were high-grade commercial products. Ultrapure water (Milli Q instrument from Millipore Corp.) was used throughout the experiments. Buffers were stored in acid-washed plasticware to minimize calcium contamination (calcium contamination was less than 5×10^{-7} M). Fluorescence experiments were performed on air-equilibrated solutions contained in quartz vessels placed in a thermostated metallic holder. Temperature within the cell was measured by a thermocoupler with a precision of 0.5 °C. Experiments were all performed in 50 mM Hepes,[1] pH 7.5. The refractive index was measured by using an OPL refractometer.

Methods

Production of Calmodulin Mutants. VU-1 calmodulin was obtained as described previously (Roberts et al., 1985). VU-9 calmodulin, which differs from VU-1 calmodulin by the re-

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CaM, calmodulin.

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placement of phenylalanine 99 by tryptophan, was produced as described elsewhere (Kilhoffer et al., 1988).

Fluorescence Measurements. Steady-state fluorescence spectra were obtained with a Perkin-Elmer MPF66 spectrofluorometer interfaced to a Perkin-Elmer 7500 computer. Excitation wavelength was set either at 295 nm for selective excitation of tryptophan or at 280 nm where both tryptophan and tyrosine residue excitation can be achieved. Solution absorbance was always less than 0.11 at the excitation wavelength. Quantum yields (ϕ) of VU-9 calmodulin were determined by taking L-tryptophan in water as a reference (ϕ = 0.14 at 20 °C) and were corrected to account for the screening effect of scattered light (Hêlène et al., 1971). Quantum yields of VU-1 calmodulin were determined similarly by taking L-tyrosine as reference (ϕ = 0.14 at 20 °C).

Fluorescence lifetime measurements were obtained by time-correlated single photon counting, using an argon ion synchronously pumped, cavity-dumped dye laser, frequency doubled to give <20-ps pulses at 297 nm, the excitation wavelength used in the experiment. Details of the device are given elsewhere (Chabbert et al., 1989). Emission was monitored at 348 nm, at a right angle to the excitation beam. Temperature was maintained at 20 °C. The experimental decay data were stored in a multichannel analyzer. The kinetic traces were fitted to a mono- or a biexponential decay function that was convoluted with the instrument response function by an iterative, nonlinear least-squares procedure based on the Marquardt algorithm. Acceptability of the fit was judged from the reduced χ^2 value, the randomness of the weighted residuals, and the autocorrelation function. In all cases where multiexponential fits were reported, they were necessary to lower the χ^2 value and to randomize the residuals.

Linear Fluorescence Polarization Measurements. Fluorescence polarization is characterized by the degree of polarization

$$p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are, respectively, the parallel and perpendicular components of the fluorescence. Measurements were carried out at 20 °C under continuous illumination by using a T-format device (SLM 8000SC spectrofluoropolarimeter). With this apparatus, $I_{\parallel}$ and $I_{\perp}$ are detected simultaneously on two separate channels with vertically polarized excitation. The two channels are balanced by using horizontally polarized excitation. Excitation was set at 295 nm (monochromator) and the emission at 342 nm (interference filter with a band pass of 8 ± 0.5 nm).

Correlation times (ρ) were deduced from the fluorescence polarization degree values by using Perrin's relation

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{\tau}{\rho}\right)$$

where ρ is the correlation time of the unit carrying the chromophore, τ is the fluorescence lifetime, and p and p_0 are the measured and the fundamental polarization degrees, respectively. The fundamental polarization degree of tryptophan for excitation at 295 nm is 0.36 (Gĕrard, 1975).

Tryptophan fluorescence quenching experiments were carried out by adding aliquots of an acrylamide stock solution to VU-9 calmodulin in the absence and in the presence of saturating calcium concentrations. As acrylamide did not alter the overall shape of the fluorescence spectrum (no shift in the maximum, no spectrum enlargement), changes in the fluorescence intensity at 348 nm were recorded. Data were corrected for the dilution and for the screening effect due to the absorption of acrylamide at 295 nm, according to (Hêlène et al., 1971)

$$I_p = I_m[(d_p + d_s)(1 - 10^{-d_p})]/[d_p(1 - 10^{-(d_p+d_s)})]$$

where I_m represents the fluorescence intensity measured at 348 nm, I_p the fluorescence intensity corrected for light scattering and for the screening effect of acrylamide, d_p the protein absorbance at the excitation wavelength, and d_s the absorbance due to the light scattering of the protein molecules and to acrylamide absorption.

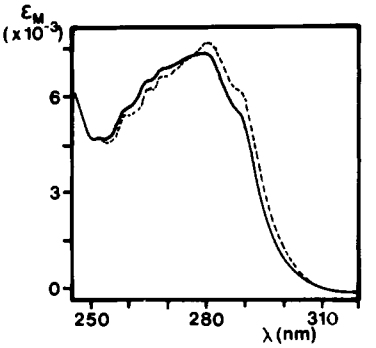

FIGURE 1: Absorption spectra of VU-9 calmodulin in the absence (—) and the presence (- - -) of 1 mM calcium. After decalcification, the protein was dissolved in 50 mM Hepes buffer, pH 7.5. Calcium was added subsequently to a final concentration of 1 mM.

Ultraviolet absorption spectra were recorded on a Cary 219 spectrophotometer in quartz cuvettes with a light path of 1 cm and a speed of 0.2 nm/s. Correction for scattered light was made according to the method of Gĕrard et al. (1975).

Protein Concentration. The extinction coefficients of VU-1 and VU-9 calmodulins were determined by taking the absorption spectrum of a given protein solution and measuring the protein concentration of the same solution by amino acid composition analysis. Subsequently, protein concentrations were measured by UV absorption spectroscopy taking a molar extinction coefficient at 279.5 nm of 7400 M^{-1} cm^{-1} for VU-9 calmodulin in the absence of calcium and of 1560 M^{-1} cm^{-1} at 276.5 nm for VU-1 calmodulin in the absence of Ca^{2+}.

Results

VU-9 calmodulin exhibits one tryptophan residue (Trp 99 in calcium binding domain III), one tyrosine residue (Tyr 138 in calcium binding domain IV), and eight phenylalanine residues. Absorption and fluorescence of VU-9 calmodulin are mainly due to tryptophan 99, which covers the absorption and emission of tyrosine 138. Information concerning this chromophore can be obtained by the study of VU-1 calmodulin, which has only tyrosine 138, tryptophan 99 being replaced by a phenylalanine (Roberts et al., 1985). As certain absorption and fluorescence parameters of tyrosine 138 are required in the study of VU-9 calmodulin, some spectroscopic properties of VU-1 calmodulin will be presented in this section.

Absorption Characteristics of VU-9 and VU-1 Calmodulins. Absorption spectra of VU-9 calmodulin are shown in Figure 1. In the absence of calcium, the spectrum exhibits a maximum at 279.5 nm, a shoulder at 287 nm attributable to the single tryptophan residue, and secondary peaks at 252, 259, 265, and 268.5 nm due to the vibronic absorption bands of phenylalanine residues. Addition of 1 mM calcium induces a 1-nm red shift of the maximum and of the shoulder at 287 nm. However, the positions of the secondary peaks are essentially unchanged. The molar extinction coefficient at the maximum was found to be 7400 ± 200 M^{-1} cm^{-1} in the absence and in the presence of calcium. This value is close to

Table I: Spectroscopic Characteristics of Calmodulin Mutants

	$-Ca^{2+}$						$+Ca^{2+}$					
	ϵ_M (M^{-1} cm^{-1})	Tyr or Trp absorption max (nm)	λ_{max} emission (nm)	$\Delta\lambda_{1/2}$ (nm)	ϕ	τ	ϵ_M (M^{-1} cm^{-1})	Tyr or Trp absorption max (nm)	λ_{max} emission (nm)	$\Delta\lambda_{1/2}$ (nm)	ϕ	τ
VU-1, 50 mM Hepes, pH 7.5	1560 (276)	276.5	303	32	0.031	-	1500 (280)	280	305.5	32	0.061	-
VU-9, 50 mM Hepes, pH 7.5	7400 (280)	280	348	56	0.19	τ_1 = 1.35 ns, 14% τ_2 = 5.30 ns, 86%	7400 (281)	281	348	57	0.15	τ_1 = 1.55 ns, 38% τ_2 = 6.84 ns, 62%
6 M guanidine hydrochloride		281	352	61	0.11 (exc 295)	-						
L-Trp/H_2O	5800 (280)	279	352	61	0.14	τ_1 = 0.47 ns, 5.5% τ_2 = 2.7 ns, 94.5%						

[a] Experiments were performed in 50 mM Hepes without Ca^{2+} ($-Ca^{2+}$) or in the presence of 1 mM Ca^{2+} ($+Ca^{2+}$). ϵ_M corresponds to the molar extinction coefficient at the wavelength indicated in parentheses, λ_{max} to the wavelength of maximum fluorescence, $\Delta\lambda_{1/2}$ to the fluorescence spectrum width at half maximum, ϕ to the fluorescence quantum yield, and τ to the lifetime. Excitation wavelength was 297 nm unless indicated otherwise.

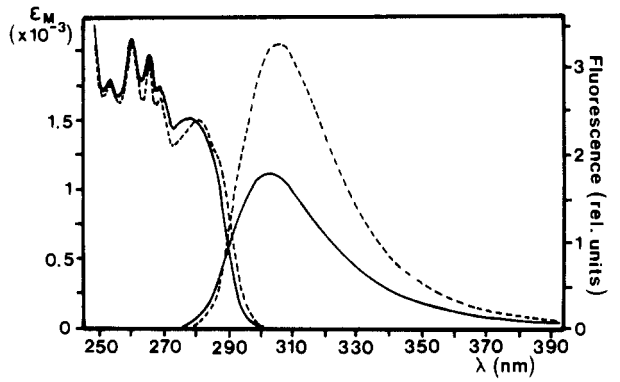

FIGURE 2: Absorption and fluorescence spectra of VU-1 calmodulin in the absence (—) and the presence (- - -) of 1 mM calcium. The protein was dissolved in 50 mM Hepes buffer, pH 7.5. ϵ_M corresponds to the molar extinction coefficient. For the fluorescence spectra, the excitation wavelength was set at 280 nm.

the one expected for a protein containing one tryptophan and one tyrosine.

Figure 2 shows the absorption spectra of VU-1 calmodulin. In the absence of calcium, VU-1 calmodulin exhibits maxima at 276.5, 268.5, 265, 259, and 252 nm. The maximum at 276.5 nm is characteristic of the absorption of tyrosine residues, although it is flattened. The other maxima correspond to the vibronic absorption of phenylalanines. The molar extinction coefficient at 276.5 nm is 1560 M^{-1} cm^{-1}. In the presence of calcium, the tyrosine maximum shifts toward 280 nm and a shoulder appears at 286 nm. Such spectroscopic characteristics are quite unusual for tyrosine and to our knowledge have not been reported in the literature. The molar extinction coefficient at the tyrosine maximum is 1500 M^{-1} cm^{-1}.

Fluorescence Properties of VU-1 Calmodulin. VU-1 calmodulin emission spectra in the absence and in the presence of calcium are shown in Figure 2. In the absence of calcium, the emission spectrum of VU-1 calmodulin exhibits a maximum at 303 nm and a width at half-maximum ($\Delta\lambda_{1/2}$) of 32 nm, characteristic of tyrosine. Under the same conditions, the quantum yield of VU-1 calmodulin is 0.031. Such a value is common for tyrosine residues in proteins where the chromophore is generally quenched by quenching groups. Addition of calcium to VU-1 calmodulin induces a 2-nm red shift of the whole spectrum with a maximum located at 305 nm, the $\Delta\lambda_{1/2}$ remaining unchanged. Although unusual, such spectral characteristics have been reported in the literature for histones (Khrapunov et al., 1984) and would correspond to tyrosine residues located in apolar environments. Calcium binding to

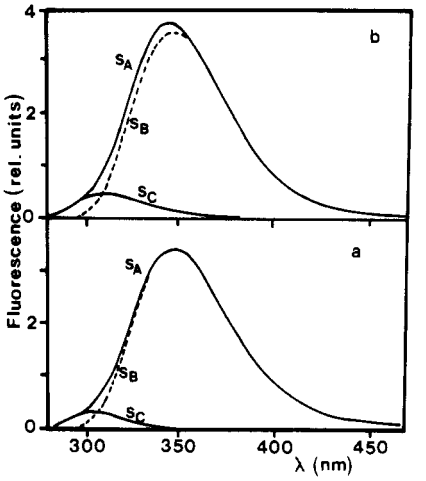

FIGURE 3: Fluorescence spectra of VU-9 calmodulin in the absence (a) and the presence (b) of 1 mM calcium. The spectra obtained for excitation at 280 and 297 nm were normalized at 380 nm, where fluorescence only originates from tryptophan. S_A corresponds to 280 nm excited normalized fluorescence spectrum and S_B to the 297 nm excited normalized fluorescence spectrum. S_C is the differential spectrum between S_A and S_B and corresponds to the emission spectrum of tyrosine 138. Buffers conditions: 50 mM Hepes, pH 7.5.

VU-1 calmodulin also enhances the quantum yield to 0.061. This study shows that calcium binding to VU-1 calmodulin affects the properties of tyrosine 138. The exact nature of the environment cannot be assessed from this study. However, the change in the absorption and fluorescence spectra induced by calcium would go along with the buried nature of tyrosine 138 observed for mammalian calmodulin (Richman, 1978; Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1985) in the presence of calcium. Since VU-1 calmodulin stands for the reference mutant, knowledge of certain values such as the quantum yield and the molar extinction coefficient of tyrosine 138 was important for further studies on mutants.

Fluorescence Spectra of VU-9 Calmodulin. Fluorescence spectra of VU-9 calmodulin were obtained for excitation at 280 nm, where both tyrosine and tryptophan absorb, and at 297 nm, where tryptophan is selectively excited. The fluorescence spectrum of VU-9 calmodulin excited at 297 nm in the absence of calcium shows a maximum at 348 nm with a half-maximum width ($\Delta\lambda_{1/2}$) of 56 ± 0.5 nm (Figure 3a). These values are close to those of tryptophan in water (Table I), indicating that tryptophan 99 in VU-9 calmodulin is largely exposed to the hydrophilic medium. The small blue shift (4 nm) as compared to tryptophan in water could be explained,

Table II: Tryptophan 99 Emission Maximum (I_{max}) and Relative Increase of Intensity at This Maximum (ΔI_{max}) at Various Molar Ratios of Calcium to VU-9 Calmodulin

Ca/VU-9 CaM	I_{max} (nm)	ΔI_{max}[a] (%)
0	348	0
1	346	0
2	344	5
3	345	11
4	346	10
5 and 6	347	10

[a] ΔI_{max} was calculated relative to the intensity at the maximum in the calcium-free form.

as proposed by Cockle et al. (1978), by the presence of apolar residues near the tryptophan in the amino acid sequence, which increases the hydrophobicity of the tryptophan microenvironment.

Excitation of VU-9 calmodulin at 280 nm keeps the emission maximum and the spectrum width at half-maximum unchanged (Figure 3a); only a small shoulder on the blue edge of the spectrum corresponding to the emission of tyrosine 138 can be seen. Thus, fluorescence of VU-9 calmodulin mostly arises from tryptophan emission, the contribution of tyrosine being very low. Emission spectra obtained for excitation at 280 and 297 nm were normalized at 380 nm, where fluorescence originates only from the tryptophanyl residue (Figure 3a). The difference spectrum with a maximum at 303 nm corresponds to the emission of tyrosine 138. Addition of calcium induces only slight modifications in the steady-state fluorescence spectrum of VU-9 calmodulin. At 1 mM calcium, only a 7% increase in the fluorescence intensity at the maximum was recorded, with no shift of the maximum located at 348 nm. When excitation was set at 280 nm, the emission spectrum was 1 nm blue shifted with a maximum located at 347 nm; $\Delta\lambda_{1/2}$ remains unchanged. Figure 3b shows the 380 nm normalized emission spectra of VU-9 calmodulin excited at 280 and 297 nm. In the presence of calcium, the emission of tyrosine 138 appears to be more important. Also, the difference spectrum shows a maximum that is about 3 nm red shifted, in good agreement with the shift observed for tyrosine 138 in VU-1 calmodulin.

Although the locations of the emission maxima in the absence and in the presence of calcium are similar, changes in the fluorescence maximum and in the intensity at this maximum can be observed when fluorescence spectra were recorded at various molar ratios of calcium to VU-9 calmodulin (Table II), indicating that calcium binding to VU-9 calmodulin alters the microenvironment of tryptophan 99.

When VU-9 calmodulin is denatured with 6 M guanidine hydrochloride, its emission spectrum superimposes with that of free tryptophan in water (λ_{max} = 352 nm; $\Delta\lambda_{1/2}$ = 61 nm).

Fluorescence Quantum Yields. Emission quantum yields of VU-9 calmodulin in the absence and in the presence of molar excess of calcium (1 mM Ca^{2+} final) were determined for excitation at 280 nm (ϕ_p^{280}), where both tyrosyl and tryptophanyl residues absorb, and at 297 nm (ϕ_p^{297}), which corresponds to a selective excitation of the tryptophan residue.

In the absence as well as in the presence of Ca^{2+}, tryptophan quantum yields are high (0.19 and 0.15, respectively, Table I), as compared to the quantum yield of tryptophan in water (ϕ = 0.14). Denaturation with 6 M guanidine hydrochloride lowers the quantum yield to a value commonly observed in denatured proteins (ϕ = 0.11).

For a better insight into the fluorescence properties, it is interesting to determine the fractional quantum yield of the tryptophan residue in the protein excited at 280 nm [ϕ_p^{280}(Trp)]. Since the emission profile of the tryptophan in the protein after excitation at 280 nm could be obtained by normalizing at 380 nm (where only tryptophan fluoresces) the emission spectra corresponding to excitation at 280 and 297 nm, respectively (curves S_A and S_B in Figure 3), ϕ_p^{280}(Trp) can be expressed as

$$\phi_p^{280}(\mathrm{Trp}) = \phi_p^{280}(S_B/S_A)$$

where S denotes the area under the curve indicated by the subscript and ϕ_p^{280} the quantum yield of VU-9 calmodulin upon excitation at 280 nm.

Taking into account the fractional absorption of tryptophan at 280 nm, f_{Trp}^{280}, the yield of tryptophan was given by

$$\phi_{Trp}^{280} = \phi_p^{280}(\mathrm{Trp})/f_{Trp}^{280}$$

f_{Trp}^{280} was calculated to be 0.797, taking the extinction coefficient at 280 nm of VU-9 calmodulin ($\epsilon_{M,280}$ = 7400 M^{-1} cm^{-1}) and of VU-1 calmodulin ($\epsilon_{M,280}$ = 1500 M^{-1} cm^{-1}).

Values of ϕ_p^{280} and ϕ_{Trp}^{280} were found to be 0.163 and 0.204 in the absence of calcium and 0.12 and 0.15 in the presence of calcium, respectively.

Tyrosine → Tryptophan Energy Transfer. Calculation of the energy transfer between the two chromophores is interesting as it may afford information on the distance between these chromophores. Indeed, the energy-transfer efficiency (η) is related to the distance (R) between the chromophores according to

$$R = [(1/\eta) - 1]^{1/6}R_0$$

where R_0 is the Förster critical distance for a given donor and acceptor pair. In our case, R_0 was calculated according to the method of Eisinger et al. (1969)

$$R_0^6 = (8.79 \times 10^{-25})\kappa^2 n^{-4}\phi_D J_{AD} \quad (\mathrm{cm}^6)$$

where $J_{AD} = 4.8 \times 10^{-16}$ M^{-1} cm^6 and n, the refractive index, equals 1.335. The value of $^2/_3$ was taken for κ^2, the orientational factor, as is usual when the donor and acceptor undergo complete dynamic isotropic orientational averaging (Dale et al., 1979). ϕ_D, the quantum yield, is, in the case of VU-9 calmodulin, the quantum yield of tyrosine 138. The value of this quantum yield was obtained from the study of VU-1 calmodulin and was found to be equal to 0.031 and 0.061 in the absence and in the presence of calcium, respectively (Table I). Under these conditions, the calculated distance R_0 was 11.8 Å for VU-9 calmodulin in the absence of calcium and 13.2 Å for the protein in the presence of a saturating calcium concentration (1 mM Ca^{2+} final). The efficiency of the energy-transfer process (η) is given by

$$\phi_p^{280}(\mathrm{Trp}) = \phi_p^{297}(f_{Trp}^{280} + \eta f_{Tyr}^{280}) \quad (1)$$

f_{Tyr}^{280}, the fractional absorption of tyrosine 138 at 280 nm, is equal to $1 - f_{Trp}^{280}$. The values of f_{Trp}^{280} and of ϕ_p^{280}(Trp) have been calculated previously, and that of ϕ_p^{297} is given in Table I. According to eq 1, resonance energy transfer occurs with an efficiency of 0.3 in the absence of calcium and 0.024 in the presence of calcium. The lack of efficient energy transfer from tyrosine to tryptophan was already suggested by the presence of tyrosine fluorescence in the fluorescence spectrum excited at 280 nm. These values lead to calculated distances between tyrosine 138 in domain IV and tryptophan 99 in domain III of 13.5 ± 1.3 and 24.5 ± 2.2 Å in the absence and in the presence of calcium, respectively.

Fluorescence Lifetimes of VU-9 Calmodulin. Tryptophan fluorescence in VU-9 calmodulin does not decay monoexpo-

Table III: Fluorescence Polarization and Acrylamide Quenching Parameters of VU-9 CaM[a]

	K_{SV} (M^{-1})	V	k_q (M^{-1} s^{-1})	p	r	$\langle\tau\rangle$ (ns)	ρ (ns)
50 mM Hepes, pH 7.5, without Ca^{2+}	9.8	1.13	2.06×10^9	0.152	0.105	4.7	3
50 mM Hepes, pH 7.5, +1 mM Ca^{2+}	10.7	0.51	2.2×10^9	0.192	0.136	4.8	4.7

[a] Experiments were performed in 50 mM Hepes, pH 7.5, in the absence or presence of 1 mM Ca^{2+}. K_{SV}, V, and k_q correspond to the quenching parameters defined in eq 2. p and r stand for the fluorescence polarization degree and the anisotropy, respectively, $\langle\tau\rangle$ represents the average lifetime calculated as $\langle\tau\rangle = a_1\tau_1 + a_2\tau_2$, and ρ is the rotational correlation time calculated from Perrin's equation. Excitation wavelength was set at 295 nm.

nentially (Table I). Analysis of this problem will be given in the following paper in this issue (Chabbert et al., 1989). Since some parameters we shall discuss later depend on the lifetime, we calculated a mean value of the lifetime $\langle\tau\rangle = a_1\tau_1 + a_2\tau_2$ where a_1 and a_2 are the percent weights associated with τ_1 and τ_2, respectively (Table I).

The mean lifetime of tryptophan 99 in VU-9 calmodulin (4.7 and 4.8 ns in the absence and in the presence of calcium, respectively) is high compared to that of tryptophan in water, indicating that the chromophore is well protected from dynamic quenching due to the diffusion-controlled encounter between the excited fluorophore and some quenching groups of the protein. Whereas calcium binding affected the individual components of the lifetime, the mean lifetime $\langle\tau_0\rangle$ remained unchanged.

Fluorescence Quenching by Acrylamide. Fluorescence quenching, which involves physical contact between the quencher and the excited fluorophore, is described by the modified Stern–Volmer relationship (Birks, 1970; Eftink & Ghiron, 1976)

$$I_0/Ie^{V[Q]} = 1 + K_{SV}[Q] = \langle\tau\rangle/\langle\tau_q\rangle \qquad (2)$$

I_0 and I are the fluorescence intensities at an appropriate emission wavelength in the absence (I_0) and presence (I) of a given quencher concentration [Q], $\langle\tau\rangle$ and $\langle\tau_q\rangle$ are the mean fluorescence lifetimes in the absence and presence of quencher, K_{SV} is the collisional quenching constant, which is the product of the bimolecular quenching rate constant (k_q) and the mean lifetime of the protein in the absence of quencher ($\langle\tau\rangle$), and V is the static quenching parameter.

Quenching of VU-9 calmodulin fluorescence by acrylamide is shown in Figure 4. In the absence as well as in the presence of calcium, the quenching curve shows an upward curvature, pointing to a static quenching contribution. For data obtained in the absence of calcium, the best fit to eq 2 was obtained for $K_{SV} = 9.8$ M^{-1}, with $k_q = 2.6 \times 10^9$ M^{-1} s^{-1} and $V = 1.13$ M^{-1}. In the presence of calcium $K_{SV} = 10.7$ M^{-1}, with $k_q = 2.2 \times 10^9$ M^{-1} s^{-1} and $V = 0.5$ M^{-1}. The dynamic component of the quenching has also been evaluated by plotting $\langle\tau\rangle/\langle\tau_q\rangle$ as a function of [Q]. The plots obtained were linear, and K_{SV}, the Stern–Volmer constants, were similar to those given above (data not shown). Thus, diffusion-controlled encounter between acrylamide and tryptophan is not changed by calcium binding to VU-9 calmodulin, whereas the static quenching process is reduced.

Fluorescence Polarization. Steady-state fluorescence polarization measurements were performed to obtain information on the degree of mobility of tryptophan 99 in VU-9 calmodulin and consequently on the protein dynamics.

Values of p, calculated as indicated under Materials and Methods, are shown in Table III. In the absence of calcium, p was equal to 0.152. Addition of molar excess of calcium (1 mM) induces a 21% increase in p, leading to a value of 0.192. In addition, fluorescence polarization excitation spectra

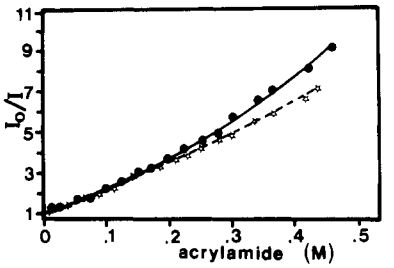

FIGURE 4: Stern–Volmer plot of VU-9 calmodulin quenching by acrylamide. The quenching experiments were performed in 50 mM Hepes, pH 7.5, buffer in the absence (●) and the presence (☆) of 1 mM calcium. Aliquots of a stock solution of acrylamide were added to the protein solution. Excitation wavelength was set at 295 nm and emission recorded at 348 nm. I_0 and I correspond to the fluorescence intensity at 348 nm in the presence of a concentration [Q] of quencher. In the presence of acrylamide, intensities were corrected for the screening effect of acrylamide as indicated under Materials and Methods.

of VU-9 calmodulin in the absence and in the presence of calcium showed a uniform change of p in the 290–300 nm wavelength range (data not shown). Taking into account the fact that the mean fluorescence lifetime, $\langle\tau\rangle$, remains unchanged, the results suggest that the mobility of tryptophan 99 is reduced in the calcium-loaded form of the protein. Rotational correlation times were calculated according to Perrin's equation (see Materials and Methods) by using the mean value of lifetime, $\langle\tau\rangle$. In this calculation, we used for the fundamental polarization degree of tryptophan (p_0) a value of 0.36. This value corresponds to the polarization degree of tryptophan in a rigid medium (glycerol at −70 °C) at an excitation wavelength of 295 nm (which is the excitation wavelength used in our study). By doing so, we assume that the fundamental polarization degree of tryptophan 99 in VU-9 calmodulin is the same as that of isolated tryptophan. Under these conditions, values of 3 and 4.7 ns were obtained for the protein in the absence and in the presence of calcium, respectively. Using fluorescence anisotropy decay of a calmodulin dityrosine derivative to monitor rotational motions of the protein, Small and Anderson (1988) found the rotational correlational times of the whole protein to be 6.48 and 9.9 ns in the absence and in the presence of calcium, respectively. Correlation times found in our study thus indicate that tryptophan 99 has its own segmental motion, independent of that of the whole protein. The higher tryptophan mobility of the calcium-free protein is in agreement with the results of Small and Anderson (1988), which suggested that in the absence of calcium the protein exhibited significantly more segmental mobility than in its presence. Similar conclusions were reached for tyrosine in calmodulin at pH 5 and pH 6.5 (Gryczynski et al., 1988).

Discussion

VU-9 calmodulin is a mutant obtained by site-directed mutagenesis and protein engineering of a synthetic gene coding

for calmodulin. The mutated protein exhibits a tyrosine in position 138 (domain IV) and a tryptophan in position 99 (domain III). This latter residue serves as an intrinsic reporter group that allows specific monitoring of the domain III microenvironment and of the changes in this environment induced either by ion binding to this domain or by a global conformational change taking place when the ions bind to a remote calcium binding domain.

Intrinsic fluorescence studies of VU-9 calmodulin brought information concerning the local microenviroment of the chromophore, its motional properties and solvent accessibility. In addition, its aromatic amino acid composition, one tyrosine and one tryptophan, allowed energy-transfer measurements that convey structural information on the protein. VU-9 calmodulin in the absence or presence of calcium fluoresced with a maximum around 347–348 nm. Although one cannot deduce the exposure of a tryptophan residue from its emission maximum only, these values strongly suggested that the chromophore was in a polar environment. However, tryptophan 99 quantum yields were high compared to those of tryptophan in water or to the values usually encountered in proteins having similar fluorescence maxima and accessibilities to acrylamide. In 1973, Burstein et al. proposed a classification of tryptophan residues in proteins according to their quantum yield and to their exposure to the solvent. These classes include class II, which corresponded to tryptophans immobilized at the protein surface and in limited contact with water with λ_{max} = 342 nm and ϕ = 0.21; and class III, which corresponded to fully exposed tryptophan with λ_{max} = 355 nm and ϕ = 0.14.

Fluorescence properties of tryptophan 99 lie between those of class II and those of class III, suggesting that the chromophore is near the protein surface, well protected from the quenching mechanisms normally occurring in proteins and in a particular solvent microenvironment (i.e., not fully exposed to the solvent). High quantum yields with long-wavelength emission maxima have been reported for tryptophans located in helical structures (Cowgill, 1968). Calmodulin has a high α-helix content [35–50% in the absence of calcium and 43–63% in its presence; for a review see Stoclet et al. (1987)], and the structure of the protein is stabilized by numerous noncovalent forces. Residue 99 is located in the calcium binding loop of domain III, which is surrounded by α-helical structures. In addition to the structure-stabilizing interactions existing between the helices, adjacent calcium binding loops in each half of the protein have been reported to undergo hydrogen bondings. NMR (Ikura et al., 1983a, 1985, 1987) and X-ray crystallographic studies (Babu et al., 1985) showed the residue in position 99 to be close to a β-sheet structure that runs antiparallel to a β-sheet structure formed by residues of the calcium binding loop in domain IV. This structure was reported to be stabilized by hydrogen bonds established between residues in calcium binding loops III and IV, namely, amide and carbonyl groups of the polypeptide bonds (e.g., Ile 100 and Val 136). Other hydrogen bonds involve lateral side chains and peptide bonds of residues from the same calcium binding loop (e.g., Gly 98 and Asp 93). This particular structure of the calcium binding domains and especially of calcium binding domain III with many residues around tryptophan 99 involved in hydrogen bonds may create an environment where the chromophore is shielded from the quenching processes normally occurring in proteins and thus may explain the high quantum yield and high lifetime values of the chromophore.

Further information on the location and exposure of tryptophan 99 was obtained from acrylamide fluorescence quenching studies. Acrylamide has been reported as an uncharged quenching probe that is very sensitive to the solvent exposure of tryptophan (Eftink & Ghiron, 1976a). In the case of calmodulin, which is a highly charged polypeptide at the pH used in the study, acrylamide is preferred to ionic quenchers which may give under- or overestimation of the exposure of the fluorophore. Stern–Volmer plots of VU-9 calmodulin were curved upward, indicating both dynamic and static quenching of the chromophore. Comparison of the parameters that quantitate the two processes (k_q and V) with those reported by Eftink and Ghiron (1976b) for different single-tryptophan-containing proteins suggested that tryptophan 99 was easily accessible to solvent molecules and that there was a good chance that an acrylamide molecule existed within the active volume of the fluorophore to quench it statically. This goes along with a well-exposed tryptophan located near the protein surface, in agreement with the wavelength of fluorescence maximum and the fluorescence spectrum half-maximum width. However, the fact that the quantum yield and lifetime of tryptophan 99 were higher than those of tryptophan in water indicated that the microenvironment of tryptophan 99 was different. The difference might be related to the presence of the protein matrix that creates a particular environment with a specific solvent orientation and polarizability. Our results concerning tryptophan location could be compared to those of tyrosine 99 of mammalian calmodulin. Indeed, in the presence and absence of calcium, this residue has been reported to be easily accessible to chemical reagents (Richman & Klee, 1978; Richman, 1978), and NMR studies indicated that the chromophore is rather well exposed to the solvent (Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1983a,b, 1987). In the presence of calcium, the exposure of tryptophan 99 was not dramatically changed (no significant change in k_q). However, the decrease in the static quenching constant suggested that there is some more steric shielding, which diminished the probability of an acrylamide molecule existing within an active volume element where it can quench the tryptophan instantaneously. This observation fits also with a decrease in the mobility of the chromophore reflected by the increase in the polarization degree p.

Using Förster energy transfer and assuming a value of two-thirds for the orientational factor, we found distances between tyrosine 138 and tryptophan 99 in the absence and in the presence of calcium of 13.5 and 24 Å, respectively. Distance measurements between tyrosine and nitrotyrosine in positions 99 and 138 led to values of 16.7 Å in the absence and 15.5 Å in the presence of calcium (Steiner & Montevalli-Alibadi, 1984), and a value of 13–15 Å can be obtained from the crystal structure of calmodulin in the presence of calcium (Babu et al., 1985). Whereas the calculated distance of 13.5 Å obtained in our study in the absence of calcium appears to be in a range compatible with previous results, the value of 24 Å calculated for the protein in the presence of calcium is completely out of range. One of the major uncertainties in the determination of distances using Förster energy-transfer measurements comes from the estimation of the orientational factor κ^2 (which can take values between 0 and 4). Indeed, relative spatial orientations of tyrosine and tryptophan also contributed to the efficiency of energy transfer. Assuming a distance of 14 Å between the chromophores in positions 99 and 138, we recalculated κ^2. A value of 0.023 was found, suggesting that tyrosine 138 and tryptophan 99 transition moments in the Ca^{2+}-loaded form of VU-9 calmodulin do not sample all orientations with respect to the sub-

strate in a time short compared with the transfer time [isotropic and dynamic averaging conditions of Dale et al. (1979)]. This would go along with a rigidification of the tryptophan microenvironment induced by calcium binding and already suggested by the polarization measurements. From a biological point of view, one could speculate that freezing of some local conformations in the calmodulin structure induced by calcium binding may allow the protein to interact with its target enzymes.

In conclusion, tryptophan 99 in domain III appeared to be in an environment similar to that of tyrosine 99 in mammalian calmodulin, showing that the replacement of a tyrosine by a tryptophan in this position does not alter significantly even the local structure of the protein. Moreover, as combined use of site-direct mutagenesis and protein engineering allows the introduction of tryptophanyl residues at any place in the molecule and the obtainment of high quantities of well-defined modified protein, this system appears to be a model system for the analysis of relationships between the spectroscopic properties of tryptophanyl residues and the location of the chromophore in a specific structural environment.

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