Time-Resolved Fluorescence Study of VU-9 Calmodulin, an Engineered Calmodulin Possessing a Single Tryptophan Residue†

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ABSTRACT: An engineered calmodulin (VU-9 calmodulin), which possesses a single tryptophan residue at position 99 in calcium binding domain III, was studied by time-resolved fluorescence. At least two exponential terms are needed to describe the tryptophan fluorescence decays, either in the presence or in the absence of calcium. The characteristics of the fluorescence decays are strongly dependent upon the number of calcium ions bound per molecule of VU-9 calmodulin until half of the calcium sites are occupied, i.e., three in the absence of magnesium and two in the presence of 5 mM magnesium. A clear time-dependent spectral shift is observed in the presence of calcium. The existence of an isosbestic point in the time-resolved spectra is in agreement with a two-state model. The biexponential analysis of the 340-nm fluorescence decay during calcium titration gives parameters consistent with a two-state model in which tryptophan 99 interconverts between two different conformations, characterized by a different lifetime value, with rates altered by calcium binding. This model explains the decrease in the protein quantum yield induced by calcium binding [Kilhoffer, M. C., Roberts, D. M., Adibi, A. O., Watterson, D. M., & Haiech, J. (1989) *Biochemistry* (preceding paper in this issue)].

Calmodulin is a small acidic protein involved in the calcium regulation of numerous metabolic pathways in all eukaryotic cells [for reviews see Van Eldik et al. (1982), Cox et al. (1984), and Stoclet et al. (1987)]. Upon calcium binding, this protein undergoes conformational changes that enable it to interact specifically with target enzymes and to activate them. As native calmodulins do not contain tryptophan residues, the engineering of isofunctional derivatives in which a strategically placed tryptophan can be used as a site-labeling group may be very useful to gain insight into the calcium binding mechanism and the associated conformational changes (Kilhoffer et al., (1989).

The very sensitivity of tryptophan fluorescence to its surroundings, however, often makes interpretation of fluorescence data very difficult. The fluorescence decay of tryptophan is especially complex because of the great number of nonradiative excited-state processes that can compete with fluorescence [for review see Creed (1984) and Beechem and Brand (1985)]. Moreover, the protein structural fluctuations that can occur on the nanosecond to picosecond time scale (Karplus & McCammon, 1981) can affect the decay rates (Alcala et al., 1987a). The availability of structural information on calmodulin in the absence and in the presence of calcium makes engineered calmodulins with a single tryptophan residue very interesting as model systems to study the time-resolved fluorescence of tryptophan in proteins, and its relationship to the conformation and/or the dynamics of its environment.

In this paper, we report a detailed study of the time-resolved fluorescence of an engineered calmodulin (VU-9 calmodulin) upon calcium titration. This protein possesses a single tryptophan residue at position 99 in calcium binding domain III (Kilhoffer et al., 1988, 1989). We discuss the decay data in terms of a two-state model in which tryptophan 99 interconverts between two different conformations with rates altered by calcium binding and show that the tryptophan time-resolved fluorescence can reveal details of the protein dynamics.

Materials and Methods

All chemicals were high grade commercial products. High-purity magnesium acetate and terbium chloride hexahydrate (gold label) were purchased from Aldrich Chemical Co., and ultrapure guanidine hydrochloride (Gdn·HCl) was purchased from Schwarz-Mann. Ultrapure water (Milli Q instrument from Millipore Corp.) was used throughout the experiments. Buffers were stored in acid-washed plasticware to minimize Ca^{2+} contamination (less than 5×10^{-7} M Ca^{2+}).

VU-9 calmodulin was prepared as described elsewhere (Kilhoffer et al., 1988). Protein homogeneity was assessed by SDS[1]–polyacrylamide gel electrophoresis, amino acid composition analysis, amino-terminal amino acid sequence analysis, and UV spectroscopy (Kilhoffer et al., 1989). VU-9 calmodulin was decalcified by using trichloroacetic precipitation as described previously by Haiech et al. (1981). The protein concentration was determined by UV absorption spectroscopy taking a molar extinction coefficient at the maximum (279.5 nm) of 7400 M^{-1} cm^{-1} for the protein in the absence of calcium (Kilhoffer et al., 1989).

All the experiments were performed in 50 mM Hepes, pH 7.5, at 20 °C. Steady-state fluorescence experiments were performed on a MPF 66 spectrofluorometer (Perkin-Elmer). Absorption spectra were obtained with a Cary 219 instrument.

Fluorescence decay experiments were performed with the pulse fluorometry technique. The excitation source was a

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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frequency-doubled rhodamine 6G laser, dumped and synchronously pumped by a mode-locked argon laser (Spectra Physics). The excitation wavelength was set at 297 nm. The emission was detected by using the single photon counting method, with a Phillips XP 2020 photomultiplier at right angle to the excitation beam, through a polarizor set at the magic angle (54.7°) to the direction of the excitation polarization. The emission wavelength was selected with an 8-nm bandpass monochromator (Jobin-Yvon H10). Adding a filter (Schott WG 320) in front of the monochromator to eliminate any residual diffused light did not affect the data. The decay was recorded on a multichannel analyzer (Ortec 7100). The decay data were transmitted to an IBM computer and analyzed as sums of exponentials

$$I(t,\lambda) = \sum_i \alpha_i(\lambda) e^{-t/\tau_i(\lambda)} \quad (1)$$

with

$$\sum_i \alpha_i(\lambda) = 1 \quad (2)$$

by using an iterative, nonlinear, least-squares convolution procedure based on the Marquardt algorithm. To take into account the wavelength dependence of the photomultiplier, the apparatus response function, whose half-width was around 600 ps, was determined for each emission wavelength by using a solution of *p*-terphenyl in a cyclohexane–CCl_4 mixture (4:3). In this mixture, the *p*-terphenyl lifetime was less than 20 ps and gave a negligible error on the real apparatus response function (Kolber & Barkley, 1986).

The time-resolved spectrum, $F(\lambda,t)$, was reconstructed from the decay parameters obtained at a set of wavelengths, regularly spaced, spanning the spectrum (typically 10), by using

$$F(\lambda,t) = F(\lambda)\left(\sum_i \alpha_i(\lambda) e^{-t/\tau_i(\lambda)}\right) / \left(\sum_i \alpha_i(\lambda)\tau_i(\lambda)\right) \quad (3)$$

where $F(\lambda)$ is the steady-state fluorescence intensity at the wavelength λ.

The emission wavenumber barycenter at time t, $\langle\bar{\nu}(t)\rangle$, was determined from the time-resolved spectrum by

$$\langle\bar{\nu}(t)\rangle = \int_0^\infty F(\bar{\nu},t)\bar{\nu}\, d\bar{\nu} \Big/ \int_0^\infty F(\bar{\nu},t)\, d\bar{\nu} \quad (4)$$

where $F(\bar{\nu},t)$ represents the fluorescence intensity at the wavenumber $\bar{\nu}$.

The average number of calcium ions bound per molecule of calmodulin (Ca_b) and the distribution of the VU-9·Ca_n species were determined from the association constants determined elsewhere (Kilhoffer et al., 1988).

Results

Decay Analysis. VU-9 calmodulin contains two fluorescent residues: tryptophan 99 and tyrosine 138. The excitation wavelength was set at 297 nm to excite the tryptophan residue selectively and to avoid further complications due to tyrosine 138 → tryptophan 99 energy transfer when the data are interpreted. The fluorescence decays were recorded at several emission wavelengths spanning the spectrum from 310 to 400 nm. The number of components in the fitting procedure was progressively increased until the fit no longer improved significantly. The adequacy of the curve-fitting to the observed profile was judged from the χ^2 values and the visual inspection of the randomness of the weighted residual and autocorrelation function plots. Figure 1 shows the decay of VU-9 calmodulin in the absence of calcium at 340 nm. The decay was obviously not monoexponential. Biexponential analysis gave a χ^2 of 1.8. The addition of a third component to the fit slightly reduced the χ^2 to 1.6 but did not markedly improve the residual plot

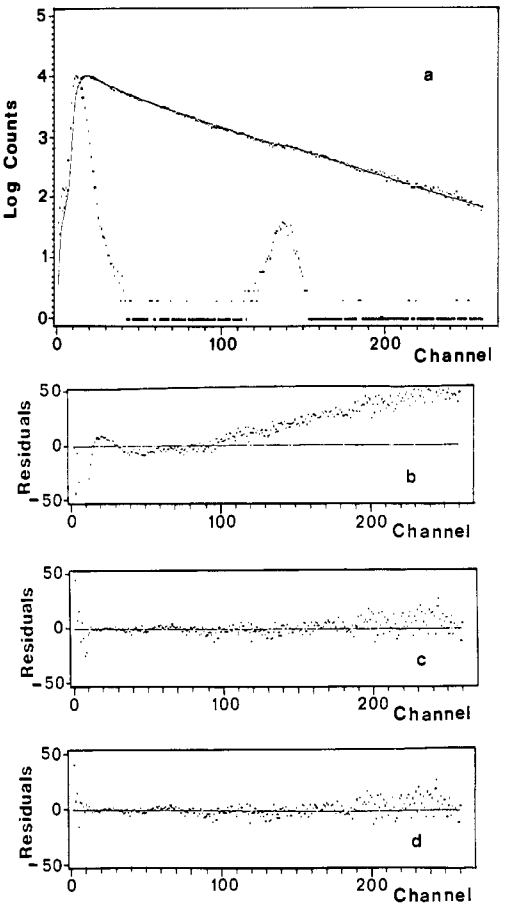


FIGURE 1: Fluorescence decay of VU-9 calmodulin in the absence of calcium at 340 nm. Data were recorded as outlined under Materials and Methods. VU-9 calmodulin concentration was 32 μM in 50 mM Hepes, pH 7.5. The solid line in (a) is the computed curve reconvolved by using the optimal biexponential fit. The weighted residuals are plotted for the optimal monoexponential (b), biexponential (c), and triexponential (d) fits. The linearity was 0.108 ns per channel.

(Figure 1c,d). Depending upon both the experimental conditions and the emission wavelength, two or three components were sufficient to obtain an optimal fit of the decay data. It should be noted that a high-frequency oscillation pattern was observed in the residual plots, the origin of which is discussed by Van Hoek and Visser (1985). Although it did not appear to alter the quality of the fit, it increased the χ^2 value significantly above unity, even when the residuals appeared "random".

VU-9 Calmodulin Decay in the Absence and in the Presence of Calcium. Similar to the reference VU-1 calmodulin, VU-9 calmodulin has four calcium binding sites and two cation sites that do not discriminate between calcium and magnesium (Kilhoffer et al., 1988). In 50 mM Hepes, pH 7.5, VU-9 calmodulin can bind six calcium ions. The fluorescence decay of Trp 99 was strongly modified by the presence of calcium. In the absence of calcium, whatever the emission wavelength, the fluorescence intensity decay could be fitted by a biexponential function. The lifetimes were approximately independent of the wavelength and averaged around 1.3 and 5.3 ns, whereas the amplitude of the longer lifetime increased from 0.45 at 310 nm to 0.63 at 400 nm (Figure 2). In the presence of a 10-fold excess of calcium, two components were sufficient to describe the decay when the emission wavelength was equal to or greater than 340 nm, but an additional subnanosecond component was required at wavelengths below 340 nm. The lifetimes were approximately independent of the wavelength with average values of about 7.0, 1.7, and 0.6 ns (Figure 3a),

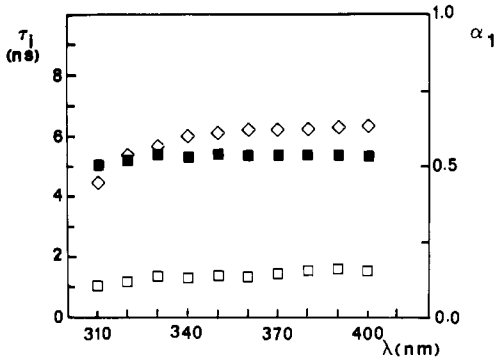


FIGURE 2: Wavelength dependence of the fluorescence decay parameters of VU-9 calmodulin in the absence of calcium. A biexponential analysis was used throughout the spectrum. (■) τ_1; (□) τ_2; (◇) α_1. VU-9 calmodulin concentration was 52 μM in 50 mM Hepes, pH 7.5.

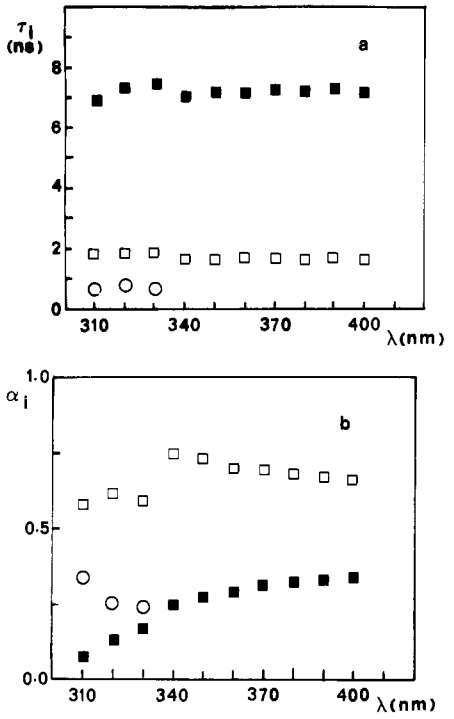


FIGURE 3: Wavelength dependence of the fluorescence decay parameters of VU-9 calmodulin in the presence of a saturating calcium concentration. A triexponential analysis was used up to 330 nm and a biexponential above that. (a) Lifetimes τ_1 (■), τ_2 (□), and τ_3 (○); (b) amplitudes α_1 (■), α_2 (□), and α_3 (○). VU-9 calmodulin and Ca^{2+} concentrations were 43 and 430 μM, respectively, in 50 mM Hepes, pH 7.5.

Table I: Analysis of the 340-nm Fluorescence Intensity Decay of VU-9 Calmodulin under Various Experimental Conditions

exptl condition	τ_1 (ns)	α_1	τ_2 (ns)	α_2
50 mM Hepes, pH 7.5	5.3	0.61	1.3	0.39
+1 mM Ca^{2+}	6.9	0.22	1.6	0.78
+5 mM Mg^{2+}	5.2	0.60	1.3	0.40
+1 mM Ca^{2+} + 5 mM Mg^{2+}	7.1	0.22	1.5	0.78
+5.5 M Gdn·HCl	3.0	0.61	1.0	0.39

while the amplitudes were highly dependent on the wavelength (Figure 3b).

When VU-9 calmodulin was denatured in the presence of 5.5 M guanidine hydrochloride, the properties of the fluorescence decay were greatly modified (Table I). Whatever the wavelength, the decay was biexponential with a shorter lifetime of 1.0 ns and a longer one of 3.0 ns.

Time-Resolved Spectra of VU-9 Calmodulin. Calcium titration of VU-9 calmodulin showed that the fluorescence

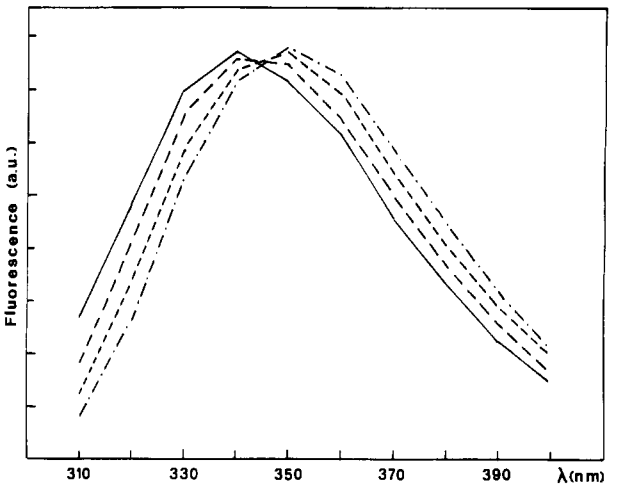


FIGURE 4: Normalized time-resolved emission spectra of VU-9 calmodulin in the presence of calcium at 0.1 (—), 2.2 (– –), 6.5 (- - -), and 16.2 ns (–·–) after the excitation pulse. The spectra were normalized to obtain the same area. VU-9 calmodulin and Ca^{2+} concentrations were 43 and 120 μM, respectively, in 50 mM Hepes, pH 7.5.

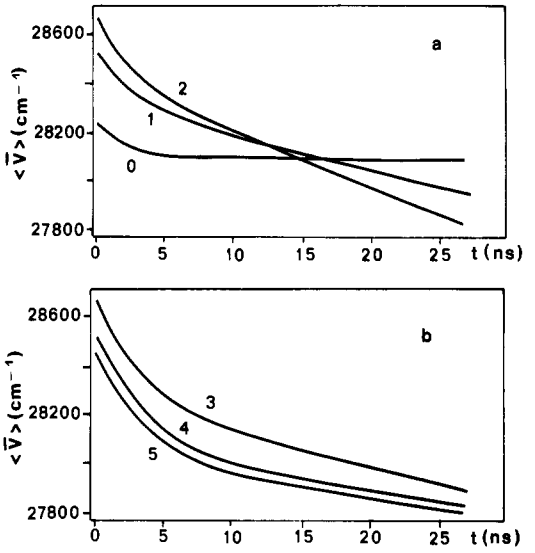


FIGURE 5: Calcium dependence of the emission wavenumber barycenter relaxation of VU-9 calmodulin. The emission wavenumber barycenters were determined as described under Materials and Methods for an average number of calcium ions bound to VU-9 calmodulin equal to (a) 0 (curve 0), 1.1 (curve 1), 1.8 (curve 2), (b) 2.7 (curve 3), 3.9 (curve 4), and 5.9 (curve 5) in 50 mM Hepes, pH 7.5.

decay parameters of tryptophan 99 are very sensitive to the molar ratio of calcium to VU-9 calmodulin. The mean lifetime, defined as $\langle\tau(\lambda)\rangle = (\sum_i \alpha_i(\lambda)\tau_i(\lambda)^2)/(\sum_i \alpha_i(\lambda)\tau_i(\lambda))$, decreased from 4.3 to 3.1 ns at 310 nm and increased from 4.8 to 5.5 ns at 400 nm when the molar ratio of calcium to VU-9 calmodulin increased from 0 to 10 (data not shown). The mean lifetime at 340 nm remained independent of calcium. From the decay parameters at a set of wavelengths spanning the spectra, time-resolved spectra were reconstructed as described under Materials and Methods. A typical time-resolved spectrum for VU-9 calmodulin in the presence of calcium is shown in Figure 4. The spectra obtained at various times after the excitation pulse were normalized to obtain the same area. An isosbestic point was observed at 345 ± 1 nm. Figure 5 shows the relaxation of the emission wavenumber barycenter as a function of time after the excitation pulse for various average numbers of calcium ions bound per molecule of calmodulin. In the absence of calcium, a 140-cm^{-1} shift

in the emission wavenumber barycenter, which corresponds to a 2-nm shift in wavelength, was completed after 5 ns. Striking changes appeared in the kinetics and the amplitude of the emission barycenter relaxation as soon as one calcium ion was bound to VU-9 calmodulin. The relaxation phenomenon was not completed even 20 ns after the excitation pulse. The low fluorescence intensity at times over 20 ns did not allow further insight into relaxation. The amplitude and the kinetics of the relaxation depended upon the average number of calcium ions bound to the protein, up to three. In this case, a 600-cm^{-1} (9-nm) red shift was observed between 0.1 and 20 ns. All the changes in the barycenter relaxation were completed after the binding of the first three calcium ions. Further binding of calcium did not affect the relaxation phenomenon, but induced a small red shift in the whole spectrum. Although changes in the steady-state fluorescence spectra were observed during calcium titration, spectra in the absence of calcium and in the presence of 1 mM calcium, when all the calcium binding sites were saturated, were very similar with the emission maxima around 348 nm (Kilhoffer et al., 1989). Time-resolved fluorescence clearly shows that the similarity in the emission maxima does not mean identity in the polarity of the tryptophan environment in the calcium-free and calcium-saturated proteins but results from the conjugated effects of (at least) two phenomena with opposite effects on the spectrum position.

Calcium Titration in the Presence and in the Absence of Magnesium. In the presence of 5 mM magnesium, the number of calcium binding sites in VU-9 calmodulin decreased to four, with a 10 times lower affinity (Kilhoffer et al., 1988). The steady-state spectrum of VU-9 calmodulin in the presence of 5 mM magnesium was 1 nm blue shifted, compared to that of the protein in the absence of magnesium. Adding calcium induced less than 5% change in the steady-state fluorescence intensity (data not shown). Either in the absence of calcium or in the presence of a saturating calcium concentration, the addition of 5 mM magnesium did not markedly change the fluorescence decay (Table I).

Calcium titration was monitored from changes in the 340-nm fluorescence decay parameters of VU-9 calmodulin, either in the absence or in the presence of 5 mM magnesium (Figure 6). In every case, the 340-nm decay could be described by a biexponential function. In the absence of magnesium, the longer lifetime showed a sigmoidal increase when the number of calcium ions bound increased from 0 to 4, with a midpoint for an average of calcium ion bound equal to 2.5. In the presence of magnesium, the curve shifted to lower mean saturation and the midpoint was around 1.5 calcium ions bound to the protein. Changes in τ_1 appeared to follow the formation of the species containing at least 2 calcium ions in the presence of magnesium and 3 in its absence. Changes in the amplitude α_1 of τ_1 could not be directly related to the appearance of a precise species. However, it should be noted that they occurred at average saturations lower than those for the lifetime τ_1. In every case, changes in the decay parameters are shifted, as compared to the binding isotherms, and completed when half of the calcium sites are occupied, i.e., three in the absence of magnesium and two in the presence of 5 mM magnesium. These results are consistent with previous studies showing that the calcium binding sites in calmodulin are not independent and do not have equal affinities for calcium (Kilhoffer et al., 1988).

Discussion

The fluorescence intensity decay of most proteins containing a single tryptophan residue can be described adequately by two or three exponential terms [for a review see Beechem and Brand (1985)]. However, the number of exponential components required to generate an analytical function that describes the decay measured is not necessarily equivalent to the number of physical components undergoing decay. A limited number of exponentials can conceal complex distributions of decay times (James & Ware, 1985, 1986; Alcala et al., 1987b). When fluorescence stems from different molecular or conformational species, the decay times observed depend not only upon the individual intrinsic lifetimes but also upon their rates of interconversion (Lakowicz, 1983; Alcala et al., 1987a). Tryptophan fluorescence is very sensitive to its environment (e.g., distance from quenching groups such as carbonyls in peptide bonds). Because of the complexity of this environment in a protein, slight differences in the conformation of the tryptophan or in its surroundings may induce large changes in the nonradiative deexcitation rates. Internal motions in proteins occur on time scales ranging from the pico- to the microsecond and more (Karplus & McCammon, 1981). Different microenvironments around a residue can thus interconvert during the excited-state lifetime, and distributions of decay times, depending upon both protein conformation and dynamics, may be expected. However, although the interpretation of the fluorescence decay in conformational terms is by no means straightforward, comparative studies can give important qualitative information on the tryptophan environment, as summarized below.

The amplitudes of the two lifetime components of calcium-depleted VU-9 calmodulin were similar, whether the protein was native or denatured. However, the lifetimes markedly decreased, from 5.3 to 3.0 ns for the longer component and from 1.3 to 1.0 ns for the shorter one, when the secondary structure of the protein was disrupted by guanidine hydrochloride (Table I). Clearly, the environment of tryptophan 99 in the native protein protects it from nonradiative deex-

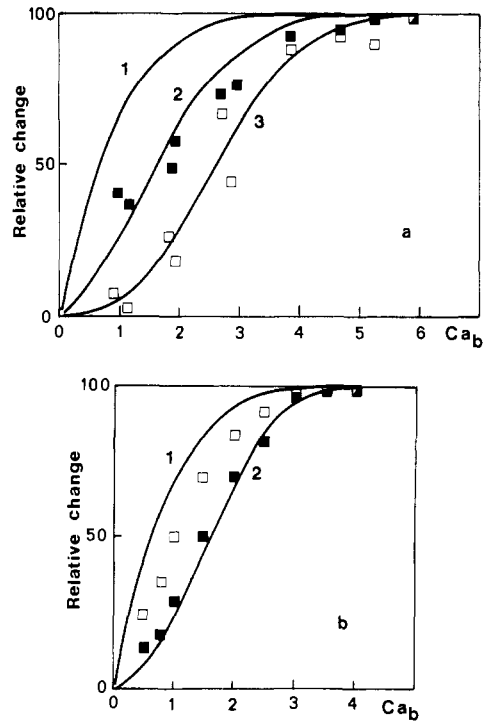
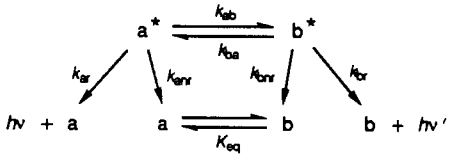


FIGURE 6: Changes in the 340-nm fluorescence decay parameters of VU-9 calmodulin as a function of the average number of calcium ions bound to VU-9 calmodulin. Changes are expressed as percent of maximum changes. (a) In the absence of magnesium, changes in α_1 (■) and in τ_1 (□); (b) in the presence of 5 mM magnesium, changes in α_1 (□) and in τ_1 (■). The solid lines represent the appearance of the VU-9·Ca_n species. The experiments were performed in 50 mM Hepes, pH 7.5.

Scheme I



citation pathway that occur when the structure of the protein is disrupted and when the tryptophan residue can freely sample its local environment. This result agrees with the high quantum yield (ϕ = 0.19) of VU-9 calmodulin in the absence of calcium (Kilhoffer et al., 1989). As discussed previously (Kilhoffer et al., 1989), calmodulin structure is stabilized by numerous H-bonds involving among other residues Gly 98 and Ile 100. Such hydrogen bonds could affect the electrophilicity or the mobility of quenching groups and thus reduce the nonradiative deexcitation rates. The mean rotational correlation time of tryptophan 99, i.e., 3.0 ns in the absence of calcium (Kilhoffer et al., 1989), is also consistent with a hindrance of fast, large-amplitude motions of Trp 99.

Calcium binding to VU-9 calmodulin induced a further increase in the lifetime values and a striking change in their relative weight (Table I), which might be related to an increase in the rigidity of calmodulin structure in the presence of calcium. NMR data are consistent with the hydrogen bonds of Ile 100 and Gly 98 being tightened by calcium binding (Ikura et al., 1985, 1987). The substantial increase in the mean rotational correlation time of Trp 99 from 3.0 to 4.7 ns (Kilhoffer et al., 1989) and the appearance of a clear relaxation phenomenon in the time-resolved emission spectrum (see below) upon calcium binding to VU-9 calmodulin are also in agreement with a slowing down of the internal mobility of the protein. Because atoms from the α-carbon backbone and amino acid side chains are involved in the coordination of the calcium ions, it is not surprising that calcium binding stabilizes the structure of the protein.

A time-dependent spectral shift may be due to dipolar relaxation (Bakhshiev et al., 1966; Bagchi et al., 1984) or to the existence of conformers with different spectra that convert into each other with rates slower than or on the same time scale as fluorescence emission. The occurrence of the former phenomenon on the nanosecond time scale has not yet been demonstrated for tryptophan residues in proteins at room temperature. Its observation in the case of model systems (indoles, NATA) requires low temperatures (Gonzalo & Montoro, 1985; Montoro et al., 1988). Because of the resolution of our time-resolved fluoresence apparatus (0.1 ns), the fluorescence emission observed at room temperature probably stems from dipolarly relaxed states. Thus, as the tryptophan emission spectrum is very sensitive to its surrounding (Longworth, 1971), the latter explanation is more likely for the observations described here.

The existence of an isosbestic point in the time-resolved spectra of VU-9 calmodulin (Figure 4) is consistent with the existence of a two-state system. Therefore, we analyzed our results according to a model of interconversion between states a (blue shifted) and b (red shifted) (Scheme I) in which the interconversion rates between the excited states a* and b* (k_{ab}, k_{ba}) and the equilibrium constant between the ground states a and b (K_{eq}) are dependent upon the average number of calcium ions bound (Ca_b), while the radiative (k_{ar}, k_{br}) and nonradiative (k_{anr}, k_{bnr}) decay rates of a and b, respectively, are calcium independent. Because of the red spectral shift observed, the lifetime of species a must be smaller than that of species b. The nature of species a and b cannot be further

Table II: Rate Constants Calculated from the 340-nm Fluorescence Intensity Decay Parameters of VU-9 Calmodulin as a Function of the Average Number of Calcium Ions Bound[a]

Ca_b	τ_1 (ns)	α_1	τ_2 (ns)	α_2	a_0	b_0	k_{ab} ($\times 10^{-9}$ s)	k_{ba} ($\times 10^{-9}$ s)
0	5.26	0.60	1.25	0.40	0.83	0.17	0.24	0.13
1.1	5.28	0.47	1.44	0.53	0.88	0.12	0.16	0.10
1.8	5.64	0.43	1.53	0.57	0.86	0.14	0.13	0.08
2.7	6.30	0.34	1.56	0.66	0.94	0.06	0.13	0.05
3.9	6.62	0.28	1.55	0.72	1.0	0.0	0.13	0.04
4.7	6.68	0.27	1.55	0.73	1.0	0.0	0.13	0.04
5.2	6.63	0.26	1.58	0.74	1.0	0.0	0.12	0.04
5.9	6.82	0.25	1.59	0.75	1.0	0.0	0.12	0.04

[a] The analysis was performed according to Scheme I with $K_a = 0.5 \times 10^9$ s^{-1} and $K_b = 0.12 \times 10^9$ s^{-1}.

specified presently. The radiative rate constant of a fluorophore is a function of both the absorption and emission spectra (Strickler & Berg, 1962). As the emission spectrum shift of VU-9 calmodulin was moderate, which means a large overlapping of the spectra for species a and b, we might assume that $k_{ar} = k_{br}$. In this case, after a δ-pulse excitation, the normalized fluorescence intensity decay at the wavelength of the isosbestic point, which is equal to that of the whole spectrum, was given by

$$I_f(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} \quad (5)$$

with

$$1/\tau_{1,2} = \tfrac{1}{2}(K_a + K_b + k_{ab} + k_{ba} \pm ((K_a + k_{ab} - K_b - k_{ba})^2 + 4k_{ab}k_{ba})^{1/2}) \quad (6)$$

$$\alpha_1 = (a_0(K_a - 1/\tau_2) + b_0(K_b - 1/\tau_2))/(1/\tau_1 - 1/\tau_2) \quad (7)$$

$$\alpha_2 = 1 - \alpha_1 \quad (8)$$

$$a_0 + b_0 = 1 \quad (9)$$

where $K_a = k_{ar} + k_{anr}$, $K_b = k_{br} + k_{bnr}$, and a_0 and b_0 are the fractions of the species a and b at time 0, respectively. The former equations led to

$$a_0 = (\alpha_1/\tau_1 + \alpha_2/\tau_2 - K_b)/(K_a - K_b) \quad (10)$$

$$1/\tau_1 + 1/\tau_2 = K_a + K_b + k_{ab} + k_{ba} \quad (11)$$

$$\tau_1\tau_2 = ((K_a + k_{ab})(K_b + k_{ba}) - k_{ab}k_{ba})^{-1} \quad (12)$$

The isosbestic point was found to be situated at around 345 ± 1 nm. To determine the rate constants, we used the parameters from the 340-nm fluorescence decay analysis. We verified that the biexponential analysis of the fluorescence intensity decay integrated over the whole spectrum gave decay parameters similar to those obtained at 340 nm within 5%.

The determination of all the constants (K_a, K_b, k_{ab}, k_{ba}, and a_0) with knowledge only of τ_1, τ_2, and α_1 was of course not possible. But, because of the relative importance of the time-dependent emission barycenter shift when the protein was calcium saturated, we first hypothesized that, in this case, the ground-state equilibrium was completely displaced to the a state, i.e., a_0 (Ca_b = 6) = 1. From eq 10, this assumption gave $K_a = 0.5 \times 10^9$ s^{-1}.

Physical solutions of eq 11 and 12 ($k_{ab} \geq 0$, $k_{ba} \geq 0$) with the further assumption $k_{ab} > k_{ba}$ required

$$0.08 \times 10^9 \text{ s}^{-1} \leq K_b \leq 0.16 \times 10^9 \text{ s}^{-1} \quad (13)$$

Values of k_{ab}, k_{ba}, and a_0 obtained with $K_b = 0.12 \times 10^9$ s^{-1} are reported in Table II. Any value of K_b included between the limits defined by eq 13 gave qualitatively similar results. Thus, on the basis of these assumptions, the equilibrium be-

tween ground states a and b is shifted to state a in the presence of calcium, and a marked decrease in the interconversion rates between excited states a* and b* is induced by calcium binding, mainly up to three calcium ions bound per molecule of VU-9 calmodulin. In the presence of magnesium, the analysis of the 340-nm fluorescence decay parameters gave similar results except that changes occurred mainly up to two calcium ions bound.

The quantum yield of the system was given by

$$\phi = k_f(K_b a_0 + k_{ab} + k_{ba} + b_0 K_a)/(K_a(K_b + k_{ba}) + k_{ab}K_b) \quad (14)$$

The radiative rate constant, k_f, was assumed to be equal to that of *N*-acetyl-L-tryptophanamide in aqueous medium, i.e., 0.05×10^{-9} s^{-1} (Werner & Forster, 1979). From the data reported in Table II, we calculated $\phi = 0.18$ in the absence of calcium and $\phi = 0.15$ in the presence of a saturating calcium concentration. The agreement with the quantum yields measured in the absence and in the presence of calcium, 0.19 and 0.15, respectively (Kilhoffer et al., 1989), is quite satisfying.

The lower limit of a_0 ($Ca_b = 6$) was the value obtained in the case of a noninterconverting system [see eq 10 and Alcala et al. (1987a)]. In this latter case, from eq 6 and 7, $a_0(Ca_b = 6) = \alpha_2 = 0.75$, $K_a = 1/\tau_2 = 0.63 \times 10^9$ s^{-1}, and $K_b = 1/\tau_1 = 0.15 \times 10^9$ s^{-1}. The analysis of the decay parameters reported in Table II according to Scheme I with these assumptions led to a decrease of k_{ab} from 0.16×10^9 to 0 s^{-1} and of k_{ba} from 0.06×10^9 to 0 s^{-1} and to an increase of a_0 from 0.60 to 0.75 during calcium titration. The quantum yields calculated from these values were similar to the previous ones.

The decay data thus can be described qualitatively in terms of a two-state model in which calcium binding alters the equilibrium and the interconversion rates between two conformations of the tryptophan residue (or of its environment) characterized by a different lifetime value. Several lines of evidence suggest, however, that Scheme I is oversimplified and cannot account quantitatively for all the results. First, a third subnanosecond component was required to fit the fluorescence decay at the blue edge of the spectrum in the presence of calcium. Because of the slowing down in the kinetics induced by calcium binding, some conformations averaged in the calcium-depleted form of the protein might be resolved in the presence of calcium. The system could require distributions of states or a multistate model to be described quantitatively. Second, the time-resolved spectra of VU-9 calmodulin in the presence of calcium could be decomposed into the sum of the spectra of two species a and b as previously described (Montoro et al., 1988). However, further analysis of the species a and b decays did not give agreement in the lifetimes of the two species (data not shown). Similar behavior has been observed in the case of *p*-(dimethylamino)benzonitrile in mixed solvents and has been interpreted as being due to distributions of reaction rates (Meech & Philipps, 1987). Such a situation might prevail in VU-9 calmodulin.

In conclusion, the analysis presented in this paper represents an initial attempt to interpret the time-resolved fluoresence of VU-9 calmodulin. It shows that tryptophan fluorescence decay can bring information on the protein dynamics. Future studies should disclose in more detail the complexity of the protein structure and dynamics.

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