Tyrosine Fluorescence of Ram Testis and Octopus Calmodulins. Effects of Calcium, Magnesium, and Ionic Strength†

Marie-Claude Kilhoffer, Jacques G. Demaille,* and Dominique Gérard

ABSTRACT: The effects of calcium and magnesium on the conformation of calmodulin have been studied by intrinsic fluorescence. A complete investigation of the tyrosine emission characteristics was carried out on two calmodulins, ram testis calmodulin, which contains two tyrosine residues at positions 99 and 138, and octopus calmodulin, which contains only Tyr-138. Analysis of fluorescence quantum yield and decay time values provided information on the quenching mechanism occurring in Ca^{2+}-free calmodulins. In particular, Tyr-138 is characterized by a very low emission yield ($\Phi = 0.016$) due to a drastic static quenching process. Monovalent cations (150 mM Na^+ or K^+) induce an additional quenching of the fluorescence. By contrast, binding of 2 calcium ions per mol of calmodulin in the high-affinity sites of domains I and II [Kilhoffer, M. C., Demaille, J. G., & Gerard, D. (1980) *FEBS Lett. 116*, 269–272] results in a large "dequenching" of the fluorescence of both Tyr residues. No further increase in the quantum yield occurs upon binding of Ca^{2+} to the lower affinity sites. Similarly, the variation of the molar ellipticity of tyrosines at 280 nm is complete after binding of 2 Ca^{2+} per mol, confirming the existence of two steps in the calcium-induced conformational transition [Seamon, K. B. (1980) *Biochemistry 19*, 207–215; Crouch, T. H., & Klee, C. B. (1980) *Biochemistry 19*, 3692–3698]. In the Ca^{2+}-loaded protein, the rather high emission yield of Tyr-99 ($\Phi = 0.11$) may perhaps be accounted for by the vicinity of α-helical structures. The magnesium-induced fluorescence change is not as large as that observed with calcium. Moreover, 150 mM KCl or NaCl abolishes Mg^{2+} effects. This may suggest that calmodulin sites are not saturated by Mg^{2+} in the cytosol of unstimulated cells. Other indications, in particular on the degree of exposure to solvent of both Tyr residues, were also obtained from experiments carried out in the presence of ionic quenchers (Cs^+) and from fluorescence polarization. Tyrosine-99 was found to be largely exposed, while tyrosine-138 is more exposed to solvent in the absence than in the presence of Ca^{2+}. Finally, the greater stability of Ca^{2+}-loaded vs. Ca^{2+}-free calmodulin was confirmed by the study of both intrinsic fluorescence and circular dichroism.

Calmodulin, the ubiquitous and multifunctional calcium-dependent regulator, plays a pivotal role in all eukaryotic cells [see Vincenzi (1979), Klee et al. (1980) Cheung (1980), and Means & Dedman (1980) for reviews]. It confers Ca^{2+} sensitivity to a number of Ca^{2+}-dependent enzymes including cyclic nucleotide phosphodiesterase (Teo et al., 1973; Lin et al., 1974; Lin & Cheung, 1976; Stevens et al., 1976), brain and pancreatic islet adenylate cyclase (Cheung et al., 1975; Brostrom et al., 1975; Valverde et al., 1979), erythrocyte (Ca^{2+}, Mg^{2+})-ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), myosin light chain kinases (Yagi et al., 1978; Dabrowska et al., 1978; Walsh et al., 1979), and several other protein kinases (Cohen et al., 1978; Le Peuch et al., 1979). Calmodulin is a heat-stable, low molecular weight (M_r 16 700) monomeric protein. Its amino acid composition is characterized by the absence of tryptophyl and cysteinyl residues and its high ratios of Phe/Tyr[1] and of acidic to basic residues. Calmodulin is evolutionarily related to troponin C, the calcium switch of sarcomeric muscles, and contains four calcium binding sites among which sites III and IV contain one tyrosine residue each, at positions 99 and 138, respectively (Watterson et al., 1980).

Calcium binding to calmodulin induces conformational changes which have been observed by circular dichroism (Klee, 1977; Dedman et al., 1977) and ultraviolet differential spectra (Klee, 1977), tyrosine fluorescence (Drabikowski et al., 1977; Dedman et al., 1977; Jarrett & Kyte, 1979) and NMR studies (Seamon, 1980), susceptibility to tryptic cleavage (Ho et al., 1975), and reactivity toward group-specific chemical reagents (Walsh & Stevens, 1977; Richman, 1978; Richman & Klee, 1978, 1979). The results reveal an increase in α-helical content and perturbations in the environment of residues such as tyrosine-138, lysine-77, and methionines-71, -72, and -76. Moreover, calcium binding to calmodulin seems to occur in at least two steps (Seamon, 1980). The first step corresponds to calcium binding to the two high-affinity sites recently identified as domains I and II of calmodulin (Kilhoffer et al., 1980a) while the second one is induced by Ca^{2+} binding to site III and finally to the lower affinity site IV (Kilhoffer et al., 1980b). These results are in agreement with ion binding studies which show that Ca^{2+} binding to calmodulin is sequential and ordered and that Ca^{2+}, Mg^{2+}, and K^+ ions compete for each site (Haiech et al., 1981).

Additional information on this problem was obtained in this study which describes, on the one hand, the complete investigation of the fluorescence characteristics of mammalian calmodulin tyrosyl residues and, on the other hand, the comparative fluorescence properties of mammalian and octopus calmodulins. Octopus calmodulin differs from the mammalian

† From the Laboratoire de Biophysique, ERA CNRS 551, UER des Sciences Pharmaceutiques, Université Louis Pasteur, BP 10, 67048 Strasbourg Cedex, France (M.-C.K. and D.G.), and Faculté de Medecine, Unité 249 INSERM and Centre de Recherches de Biochimie Macromoléculaire du CNRS, BP 5051, 34033 Montpellier Cedex, France (J.G.D.). *Received December 4, 1980.* This work was supported in part by grants to D.G. from CNRS, INSERM CRL 80.3.007, and Université Louis Pasteur and to J.G.D. from CNRS (Modulation de l'action des hormones au niveau cellulaire), INSERM (CRL 78-4-086-1, 79-4-151-3, and 80-5-032, ATP 63.78.95 Fibre musculaire cardiaque), DGRST (ACC Biologie et Fonction du Myocarde et Parois artérielles), NATO Grant 1688, Muscular Dystrophy Association, and Fondation pour la Recherche Médicale Francaise. M.-C.K. acknowledges receipt of a DGRST fellowship.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Mops, morpholinopropanesulfonic acid; Cam, calmodulin; Phe, phenylalanine; Tyr, tyrosine; pCa = $-\log [Ca^{2+}]$; pMg = $-\log [Mg^{2+}]$.

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protein by some amino acid substitutions: it lacks stoichiometric amounts of trimethyllysine and contains only a single tyrosyl residue in a position homologous to tyrosine-138 of mammalian calmodulins (Molla et al., 1981). The octopus protein, however, is at least as effective as the mammalian one in activating myosin light chain kinase (Molla et al., 1981). In addition, the presence of a single tyrosyl residue facilitates the interpretation of fluorescence experiments.

In this paper, we studied the effects of Ca^{2+} and Mg^{2+} ions on calmodulin conformation and the influence of ionic strength on these effects. Fluorescence modifications were shown to be complete after binding 2 mol of Ca^{2+} per mol of calmodulin. Ca^{2+} binding affects the microenvironment of both tyrosines-99 and -138. Finally, under physiological conditions (150 mM K^+), Mg^{2+} binding does not seem to exert a major influence on calmodulin conformation, in line with the inability of Mg^{2+} to activate calmodulin-dependent enzymes. This furthermore suggests that, in the unstimulated cell, calmodulin ion binding sites are not occupied by Mg^{2+}, as a result of the competition between K^+ and Mg^{2+} ions (Haiech et al., 1981).

Experimental Procedures

Materials

All chemicals were high grade commercial products. Ultrapure water (Milli Q Instrument from Millipore Corp.) and acid-washed plastic ware were used throughout to minimize calcium contamination. Stock solutions were passed through a Chelex-100 column and stored in plastic ware. Ram testis and octopus calmodulins were prepared as previously described (Autric et al., 1980; Molla et al., 1981). All fluorescence measurements were performed on air-equilibrated solutions contained in quartz vessels placed in a thermostated metallic holder. Temperature within the cell was measured by a thermocouple with a precision of 0.5 °C.

Methods

Ram testis calmodulin concentrations were determined by ultraviolet spectroscopy using $\epsilon_M = 3300\ M^{-1}\ cm^{-1}$ (Klee, 1977; Autric et al., 1980). Correction of scattered light was made as indicated by Shih & Fasman (1972). Octopus calmodulin concentrations were measured by amino acid analysis of aliquots withdrawn from the cuvette after each experiment. Calmodulin was freed of Ca^{2+} by trichloroacetic acid precipitation (Haiech et al., 1981).

Residual calcium was <0.04 mol/mol of protein. Calcium concentration was measured by atomic absorption spectrophotometry by using a Varian Model 1150 apparatus.

Fluorescence Measurements. Fluorescence spectra were obtained with an absolute spectrofluorometer (FICA 55) and quantum yields determined as previously described (Gerard et al., 1975), taking free L-tyrosine as a reference ($\Phi = 0.14$) (Chen, 1967). Quantum yields were corrected to account for the screening effect of scattered light (Helene et al., 1971). Solutions (absorbance <0.12) were excited at 275 ± 2.5 or 270 ± 2.5 nm. Lifetimes (τ) were measured by the single photoelectron technique (Gerard et al., 1972). Excitation was performed at 275 ± 5 nm, and fluorescence was observed at 310 ± 5 nm. The fluorescence decay profiles were analyzed statistically by using the residuals distribution form (Grinwald & Steinberg, 1974; Grinwald, 1976).

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

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are respectively the parallel and perpendicular components of the fluorescence.

Measurements were carried out by using an apparatus built in our laboratory, according to the general principle described by Steinberg & Gafni (1972) for circular fluorescence polarization measurements. For measurement of linear polarization, the instrument was transformed as previously described (Erlich et al., 1980). The accuracy in the determination of p is 10^{-2}.

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

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

where ρ is the relaxation time of the unit carrying the Tyr, τ is the fluorescence lifetime, and p and p_0 are the measured and the fundamental polarization degrees, respectively.

The relaxation time (ρ_0) for a spherical protein can be calculated by using the relation

$$\rho_0 = \frac{4\pi\eta r^3}{kT}$$

where η is the medium viscosity, k is Boltzmann's constant, T is the absolute temperature, and r is the radius of the spherical protein.

Fluorometric Titrations. Titration of metal-free calmodulin by Ca^{2+} or Mg^{2+} was performed by adding aliquots of $CaCl_2$ or $MgCl_2$ stock solutions and correcting for dilution. The stoichiometries of the Cam–Ca^{2+} complex were determined from experiments performed at protein concentrations more than 10-fold higher than the dissociation constant. Under these conditions, added Ca^{2+} was assumed to be entirely protein bound.

Circular Dichroism Spectra. Near-ultraviolet circular dichroism spectra were recorded on a Jobin-Yvon dichrograph III. Samples were prepared at a concentration of 10^{-4} M. The molar ellipticity $[\theta]_M$ values were expressed in deg cm^2 $dmol^{-1}$ and corrected for solvent blank.

Results

Among the experiments presented under Results, those concerning octopus calmodulin were restricted to the determination of the major fluorescence characteristics only, due to the scarcity of available pure protein.

Spectral Characteristics. Absorption spectra of ram testis and octopus calmodulins are shown in Figure 1. The spectrum of both proteins is characteristic of phenylalanine-rich proteins which lack tryptophyl residues. Octopus calmodulin contains only one tyrosyl residue; its spectrum therefore presents a stronger participation of the phenylalanyl residues and a lower extinction coefficient at 277 nm. The fluorescence spectra are typical of class A proteins, i.e., tyrosine-containing proteins which lack tryptophyl residues with maxima at 303 nm (Figure 1). As expected, the fluorescence spectra are not affected upon denaturation by 6 M guanidinium chloride.

Fluorescence Quantum Yield and Decay Times. Values of emission quantum yield and decay times have been determined for both proteins under different experimental conditions: native or denatured protein, absence or presence of divalent cations, and high or low ionic strength. The main results are indicated in Table I and in Figures 2 and 3.

In the absence of divalent cations, the quantum yields (Φ) are rather low ($\Phi \approx 0.03$ for ram testis calmodulin and $\Phi = 0.016$ for octopus calmodulin) but in the same range as most of class A proteins (Longworth, 1971). The ram testis cal-

Table I: Fluorescence Characteristics of Ram Testis and Octopus Calmodulins

conditions	$-Ca^{2+}$ Φ[a]	τ (ns)[a]	$(\Phi/\tau) \times 10^{-7}$ s^{-1}	ω[a]	$+Ca^{2+}$ Φ	τ (ns)	$(\Phi/\tau) \times 10^{-7}$ s^{-1}	ω
Ram Testis Calmodulin[b]								
10 mM Tris	0.030	1.7	1.75	0.56	0.078	2.3	3.4	0.15
100 mM Tris	0.027	1.7	1.60	0.60				
10 mM Tris, 150 mM KCl	0.024	1.7	1.40	0.65	0.083	2.3	3.6	0.10
100 mM Tris, 150 mM KCl	0.025	1.7	1.45	0.63	0.076	2.3	3.3	0.17
100 mM Tris, 5 mM $MgCl_2$	0.046	1.9	2.40	0.40	0.082	2.3	3.6	0.10
100 mM Tris, 5 mM $MgCl_2$, 150 mM KCl	0.030				0.075	2.3	3.2	0.19
denatured (6 M Gdn·HCl)	0.047	1.8	2.60	0.35				
Octopus Calmodulin								
100 mM Tris	0.016	2.5	0.65	0.9	0.048	2.5	1.9	0.5
100 mM Tris, 5 mM $MgCl_2$	0.035							
Free Tyrosine, Water (pH 7)								
	0.14	3.5	4	0				

[a] Φ = fluorescence quantum yield (±0.002), τ = fluorescence lifetime (±0.2 ns), and ω = fraction of static quenching. [b] All protein solutions were at pH 7.5 ± 0.1.

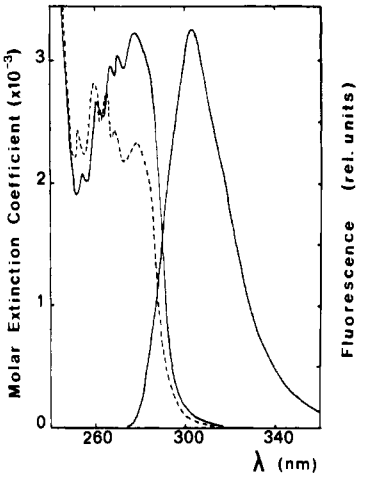

FIGURE 1: Absorption and fluorescence of ram testis calmodulin (—) and of octopus calmodulin (---). The fluorescence spectrum is identical for both proteins, in the native and denatured states.

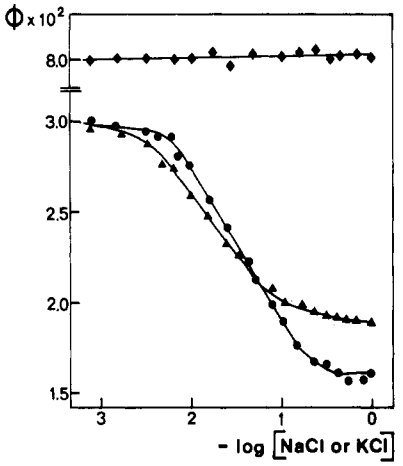

FIGURE 2: Effect of ionic strength on the fluorescence quantum yield of ram testis calmodulin (0.3 mg/mL, 10 mM Tris, pH 7.6). NaCl (●) and KCl (▲) were added in the absence of calcium; similar experiments were performed by addition of KCl or NaCl (♦) in the presence of 1 mM $CaCl_2$.

modulin quantum yield is slightly dependent on the ionic strength in the absence of Ca^{2+}, as shown by the decrease of Φ with increasing amounts of KCl or NaCl (Figure 2) or high Tris concentration (see Table I). Decay times of both proteins are quite different (2.5 ns for octopus Cam and a mean value

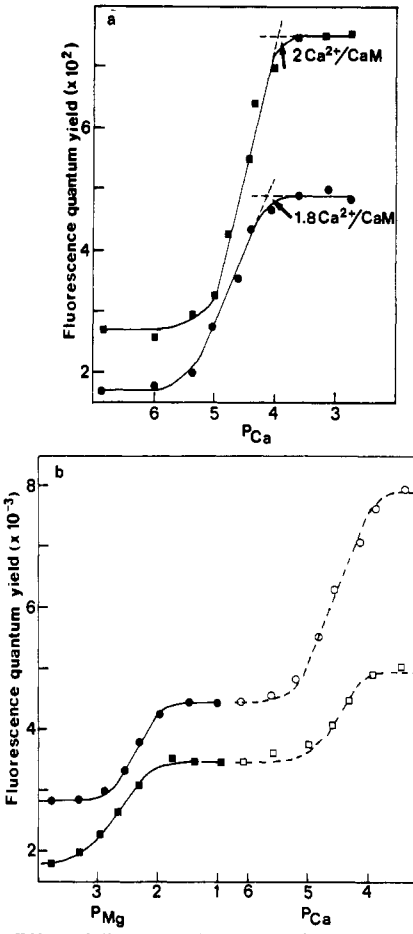

FIGURE 3: Effect of divalent cations on the fluorescence of calmodulins. (a) Effect of calcium on the fluorescence of ram testis calmodulin (45 μM in 100 mM Tris, pH 7.55) (■) and of octopus calmodulin (35 μM in 100 mM Tris, pH 6.9) (●). In both cases the plateau was reached upon binding 1.8–2 mol of Ca^{2+} per mol of calmodulin. (b) The effect of magnesium on the fluorescence of ram testis calmodulin [18 μM in 100 mM Tris, pH 7.6 (●)] and octopus calmodulin [10 μM in 100 mM Tris, pH 7.3 (■)] is represented by the solid line. The dashed line indicates the effect of further addition of Ca^{2+} to the Mg^{2+}-loaded ram testis (○) and octopus calmodulins (□). CaM = Cam = calmodulin.

of 1.7 ns for ram testis Cam). The value obtained with octopus calmodulin is rather high for a class A protein.

Denaturation of ram testis calmodulin wth 6 M guanidinium chloride induces a quantum yield increase without any im-

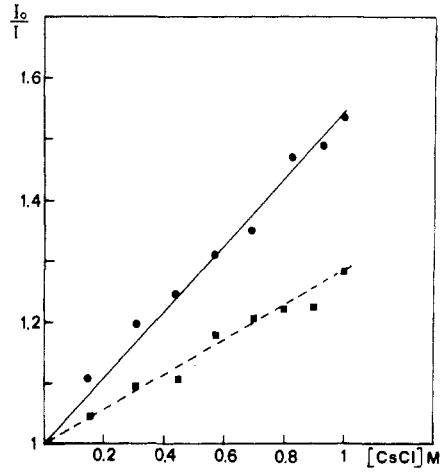

FIGURE 4: Quenching of calmodulin fluorescence by CsCl. Stern–Volmer plots of Ca^{2+}-loaded ram testis (●) and octopus (■) calmodulins. I and I_0 correspond to the fluorescence intensities in the presence and in the absence of CsCl, respectively. Trivial ionic strength effects were corrected for by measuring I_0 in the presence of the same concentrations of monovalent ions (Na^+) which do not exhibit quenching properties toward free tyrosine in water. Ram testis calmodulin was in 10 mM Tris, 1 mM $CaCl_2$, and 0.1 M NaCl, pH 7.5; 20 mM Mops was used instead of Tris for the octopus protein. Experiments were carried out by adding increasing amounts of 3 M stock solutions of CsCl or NaCl in the same buffer.

portant variation of the excited-state lifetime (Table I).

Calcium titrations of octopus and ram testis calmodulins were carried out as illustrated in Figure 3a. Calcium binding induces an increase in tyrosine fluorescence, as already observed by Dedman et al. (1977), Drabikowski et al. (1977), and Jarrett & Kyte (1979). The quantum yield corresponding to the plateau, obtained after binding of 2 mol of Ca^{2+} per mol of calmodulin, is 0.08 for ram testis calmodulin and 0.048 for the octopus protein. When calcium is bound to calmodulin, the fluorescence quantum yield is no longer affected by the ionic strength (Figure 2), in agreement with the observations of Richman & Klee (1979). Emission decay time analyses show an important variaton of τ for the ram testis protein from 1.7 ns in the absence of Ca^{2+} to 2.3 ns in the presence of Ca^{2+}. By contrast, no such modification was observed for octopus calmodulin, with τ remaining equal to 2.5 ns.

Addition of magnesium to Ca^{2+}-free calmodulin also leads to a fluorescence quantum yield increase (Figure 3b). However, the total increase is not as large as that observed with Ca^{2+}. For ram testis calmodulin, the plateau corresponds to a quantum yield of 0.046. The concentration of Mg^{2+} required to reach this plateau depends on the ionic strength of the medium. The higher the ionic strength is, the higher the concentration of Mg^{2+} necessary to reach this plateau. Subsequent addition of Ca^{2+} to the calmodulin–magnesium complex led to an additional increase of the quantum yield up to 0.08 at saturating calcium concentration. Similar observations were made with octopus calmodulin (Figure 3b); saturating amounts of Mg^{2+} increased the quantum yield from 0.016 to 0.035, and subsequent Ca^{2+} addition further increased the quantum yield up to 0.046.

Accessibility to Ionic Quenchers. Some ions (Cs^+, I^-) have been shown to quench the fluorescence of free tyrosine in solution. For evaluation of the exposure of calmodulin-emitting residues to the aqueous medium, the effect of Cs^+ on the protein fluorescence has been investigated. No significant experiments could be carried out with I^- due to the large negative charge of the protein at neutral pH. Experiments were performed in the presence and absence of Ca^{2+} ions. However, the results obtained with the metal-free protein showed an unexplained great dispersion and could not be interpreted. Therfore, only results obtained for Ca^{2+}-loaded calmodulins from ram testis and octopus are presented.

Table II: Fluorescence Polarization Data of Ram Testis Calmodulin[a]

conditions	$-Ca^{2+}$ p	$-Ca^{2+}$ τ (ns)	$-Ca^{2+}$ ρ (ns)	$+Ca^{2+}$ p	$+Ca^{2+}$ τ (ns)	$+Ca^{2+}$ ρ (ns)
100 mM Tris, 150 mM KCl (pH 7.6)	0.20	1.7	7.2	0.21	2.3	11.5
10 mM Tris, 5 mM $MgCl_2$ (pH 7.6)	0.21	1.9	10	0.21	2.3	11.5

[a] Excitation and observation were carried out at 270 and 320 nm, respectively. p = degree of polarization, τ = fluorescence lifetime, and ρ = fluorophore relaxation time. For calculation of ρ (see Experimental Procedures), the fundamental polarization value of tyrosine residues was $p_{0(270nm)} = 0.32$.

Figure 4 shows that, in both cases, calmodulin quenching is diffusion controlled and obeys the Stern–Volmer law:

$$I_0/I = I + K[Q] = I + k_q\tau_0[Q]$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher at concentration [Q] and K is the Stern–Volmer constant which is the product of the kinetic constant of the quenching process (k_q) by the mean lifetime of the protein in the absence of quencher (τ_0).

For ram testis calmodulin (τ_0 = 2.3 ns), $K = 0.54\ M^{-1}$ and $k_q = 2.35 \times 10^8\ M^{-1}\ s^{-1}$ (see Table I). The quenching is less efficient for octopus calmodulin (τ_0 = 2.5 ns): $K = 0.32\ M^{-1}$ and $k_q = 1.4 \times 10^8\ M^{-1}\ s^{-1}$.

More quantitative information on the exposure of the residue can be obtained by calculation of the solid angle A under which a quencher can freely approach an excited residue. Values of A can be deduced from the expression of k_q

$$k_q = A(N/1000)(D_F + D_Q)R[1 + R[(D_F + D_Q)\tau_0]^{-1/2}]_p$$

where N is the Avogadro number, R is the minimal interaction distance between the fluorophore and the quencher (~5 Å), D_F is the diffusion constant of the fluorophore (i.e., 0.7×10^{-5} $cm^2\ s^{-1}$ for free tyrosine and ≃0 for the residue), D_Q is the diffusion constant of the quencher ($0.9 \times 10^{-5}\ cm^2\ s^{-1}$ for Cs^+), τ_0 is the lifetime of the fluorophore in the absence of quencher, and p is the probability of deactivation during an encounter.

For tyrosine in solution where $A = 4\pi$, the probability of deactivation p can be calculated from the experimental value of k_q by using the above relation. For the couple tyrosine–Cs^+, p has been shown to be equal to 0.12 (Lux et al., 1977).

With the assumption that this value of p remains constant for the tyrosyl residue, the solid angles calculated for octopus and ram testis Ca^{2+}-loaded calmodulins, respectively, were $A = \pi$ and $A = 1.8\pi$, the latter being characteristic of an exposed residue (Lux et al., 1977; Gérard et al., 1972).

Fluorescence Polarization Data. To obtain information on the mobility of the emitting residues, the degree of fluorescence polarization (p) was measured for ram testis calmodulin. Values of p and of the relaxation times (ρ) which can be deduced from p (see Methods) are indicated in Table II. Relaxation times deduced from fluorescence polarization measurements (7.2 and 11.5 ns) are significantly lower than those calculated for a fluorophore rigidly attached to the protein; i.e., ρ_0 = 29 ns for a globular protein of 21-Å radius (Klee et al., 1980). The tyrosyl residues therefore appear to exhibit their own rotational mobility with respect to the protein motion. In addition, saturating concentrations of Ca^{2+} or Mg^{2+}

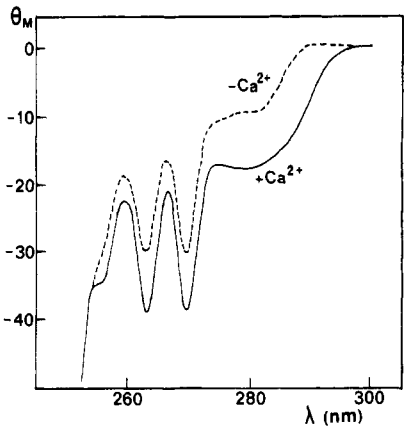

FIGURE 5: Near-ultraviolet circular dichroic spectra of ram testis calmodulin (0.1 mM in 100 mM Tris, 150 mM KCl, and 5 mM $MgCl_2$, pH 7.6) in the absence of calcium (- - -) and in the presence of 1 mM $CaCl_2$ (—). The mean residue ellipticity $[\theta]_M$ is expressed in deg cm^2 $dmol^{-1}$.

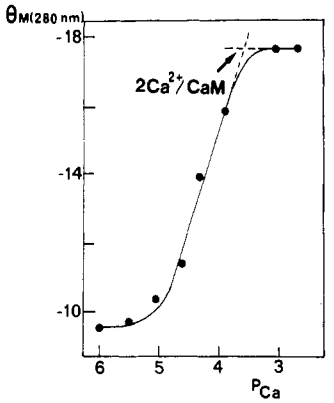

FIGURE 6: Variation of the molar ellipticity at 280 nm ($[\theta]_{M(280)}$) as a function of Ca^{2+} concentration. Ram testis calmodulin (0.1 mM) was dissolved in 100 mM Tris, 150 mM KCl, and 5 mM $MgCl_2$, pH 7.6. The plateau is reached for two Ca^{2+} per calmodulin.

induce a small decrease of the emitting residues mobility, which could be interpreted as a partial burial of one or two chromophores after divalent ion binding.

Near-Ultraviolet Circular Dichroism. The near-ultraviolet circular dichroism spectra of Ca^{2+}-free and Ca^{2+}-loaded ram testis calmodulin are represented in Figure 5.

Between 295 and 275 nm, the signal corresponds to the circular dichroism of the tyrosyl residues which are the only absorbing residues in this wavelength range. Then, between 273 and 255 nm, the more important signal should essentially be attributed to phenylalanyl residues. Finally, below 255 nm, the circular dichroism is due to the peptide bonds.

Ca^{2+} binding to calmodulin is accompanied by an increase of the circular dichroism of the aromatic amino acids, indicating that the asymmetric environment of the chromophores is affected by the Ca^{2+}-induced conformational change.

Moreover, by monitoring the variation of the molar ellipticity of tyrosines at 280 nm (Figure 6), the Ca^{2+} effect appears to be complete for 2 mol of Ca^{2+} bound per mol of protein, in agreement with recent studies of Crouch & Klee (1980).

Temperature Effect on the Fluorescence and Circular Dichroic Characteristics of Calmodulin. Figure 7a represents the temperature dependence of ram testis calmodulin fluorescence quantum yield. In the absence of Ca^{2+}, the fusion curve is characterized by a plateau between 30 and 53 °C. On each side of the plateau, the quantum yield decreases linearly with increasing temperature. Upon cooling of the previously heated solution, a linear increase of the quantum yield occurs,

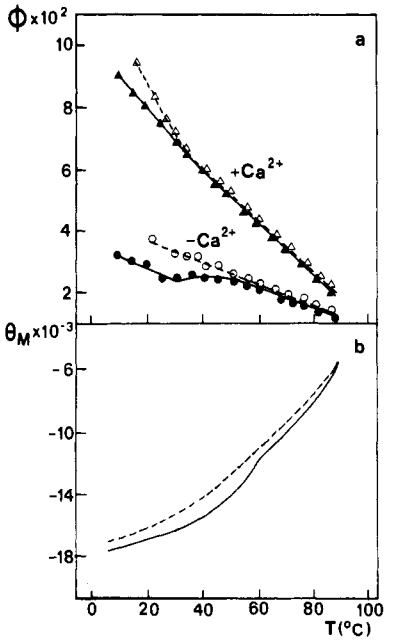

FIGURE 7: Effect of temperature on the conformation of calmodulin followed by intrinsic fluorescence and circular dichroism. (a) Variation of the emission quantum yield upon heating (solid symbols) or cooling (open symbols) of a 10 μM calmodulin solution in 0.1 M Tris, pH 7.3, in the absence (circles) or presence (triangles) of 0.1 mM Ca^{2+}. (b) Variation of molar ellipicity at 222 nm of 10 μM calmodulin in 0.1 mM $CaCl_2$, 0.15 M KCl, and 0.1 M Tris, pH 7.5, upon heating (—) or cooling (- - -).

and the initial quantum yield value is not recovered, indicating that the protein underwent an irreversible thermal denaturation.

Upon heating the Ca^{2+}-loaded protein, a linear quantum yield decrease occurs, the phenomenon being almost entirely reversible upon cooling of the solution. Similar observations were made by monitoring circular dichroism at 222 nm (Figure 7b), in agreement with all previous reports concerning the heat stability of calmodulin in the presence of Ca^{2+}. Ca^{2+} binding is also known to confer to calmodulin a great stability to denaturation (6 M urea; Brzeska et al., 1980) and tryptic digestion (Ho et al., 1975).

Discussion

Some discussion has been presented under Results, allowing the Discussion to be focused on the properties of Tyr-99 and Tyr-138.

Quenching Mechanism Involved in Calmodulin. Comparative analysis of calmodulin quantum yield (Φ) and decay times (τ) with those of free tyrosine (see Table I) indicates that both dynamic and static quenching processes occur in calmodulins.

Dynamic quenching due to a diffusion-controlled encounter between the excited fluorophore and quenching group of the protein is evidenced by lifetime values lower than those of free tyrosine.

Static quenching, related to a permanent contact between the chromophore and the quenching group, can be deduced from the Φ/τ ratios since it induces a quantum yield decrease without modification of decay time values. The efficiency (ω) of this process can be estimated from the Φ/τ ratios of the protein and the monomer model system since the fraction ω of fluorescent residues submitted to this process is given by

$$\omega = 1 - \frac{(\Phi/\tau)_p}{(\Phi/\tau)_m}$$

where subscripts p and m, respectively, refer to the residues and to the corresponding model system, free tyrosine in water (pH 7) in this case (Gérard et al., 1972). Values of ω are given in Table I.

These data show that in Ca^{2+}-depleted ram testis calmodulin, a mean of one (out of two) tyrosine emits ($\omega = 0.5$) whereas in octopus calmodulin, only 0.1 residue participates in the fluorescence ($\omega = 0.9$). This last result indicates that, in the absence of Ca^{2+}, tyrosine-138 is almost totally quenched.

Calcium binding to both octopus and mammalian calmodulins induces a 3-fold enhancement of tyrosine fluorescence quantum yield. In the case of ram testis calmodulin, the enhancement corresponds to a decrease in the dynamic quenching (since τ varies from 1.7 to 2.3 ns) and to the abolition of most of the static quenching process ($\omega \simeq 0.1$). In octopus calmodulin, however, the fluorescence enhancement is only related to a partial cancellation of the static quenching process ($\omega \simeq 0.5$).

In both cases, the fluorescence enhancement must be related to changes in the environment of the tyrosines involving the removal of quenching groups (such as COO^- or CONH). These quenching groups may also be involved in structures where they lose their quenching properties, e.g., involvement of CONH or COO^- in an α helix (Cowgill, 1976). In addition, it is worth noting that calmodulin fluorescence changes occur after binding of 2 mol of Ca^{2+} to domains I and II, which precisely lack tyrosine residues (Kilhoffer et al., 1980a) and must therefore be related to an overall change of the protein conformation induced by Ca^{2+} ions. This is at variance with troponin C, the calcium-binding subunit of the troponin complex, in which tyrosine fluorescence enhancement is due to a local effect following Ca^{2+} binding to the site which contains the tyrosyl residue, namely, site III containing tyrosine-109 (Leavis & Lehrer, 1978).

Magnesium ions only partially mimic the effect of calcium by inducing a slight increase of tyrosine quantum yield and ram testis calmodulin decay time. This partial effect of Mg^{2+} ions, with respect to Ca^{2+}, has already been observed (Wolff et al., 1977; Richman & Klee, 1979) and should be related to the fact that Mg^{2+} ions cannot replace Ca^{2+} ions in the activation of calmodulin-dependent enzymes (Wolff et al., 1977). However, the Mg^{2+} effect is specific of divalent cations and different from that induced by K^+ or Na^+. Indeed, monovalent ions caused a decrease of fluorescence yield and led to a more static quenched state of the tyrosyl residues.

Analysis of the Distinct Ca^{2+}-Induced Behaviors of Tyrosine-99 and Tyrosine-138 in Ram Testis Calmodulin. From the comparative analysis of the spectroscopic characteristics of ram testis and octopus calmodulins, the contributions of Tyr-99 and -138 to the fluorescence could be separated, and more quantitative information on the emission properties of both chromophores was obtained.

With the assumption that tyrosine-138 fluorescence characteristics are identical in ram testis and octopus calmodulins, the following conclusions can be drawn: (i) The quantum yield of tyrosine-99 ($\Phi_{(99)}$) is equal to 0.044 in the metal-free calmodulin and 0.11 in the calcium-loaded protein. These results could be determined by

$$\Phi_{(99)} = 2\Phi_p - \Phi_{(138)}$$

where Φ_p and $\Phi_{(138)}$ are respectively the quantum yield of the whole protein and that of tyrosine-138 deduced from the study of octopus calmodulin (see Table I). It is worth noting that $\Phi = 0.11$ is an anomalously high quantum yield when compared to those generally found in native protein and tyrosine peptides (Longworth, 1971).

This indicates that the emitting residue is preserved from the normally occurring quenching mechanism. Similar features have been found for tyrosyl residues in muscle proteins tropomyosin and paramyosin containing 90% of the α helix, in which the tyrosyl residues are favorably situated in an α-helical structure where the quenching by peptide carbonyl groups cannot occur. This may argue for a possible localization of tyrosine-99 of the calcium-loaded calmodulin in close vicinity to α-helical structures. This conclusion is in line with the sensitivity of Tyr-99 resonance to calcium addition (Seamon, 1980) and with the increase in α-helix content upon Ca^{2+} binding to calmodulin (Klee, 1977; Dedman et al., 1977).

(ii) The lifetime of tyrosine-99 excited state is lower than 1.7 ns in the calcium-free protein and higher than 2 ns in the Ca^{2+}-loaded protein. This can be deduced from the analysis of the mean lifetime value of ram testis calmodulin (see Table I), taking into account the tyrosine-138 decay time value (2.5 ns for octopus Cam) and the fact that the contribution of tyrosine-99 to the overall fluorescence emission amounts to about 70%. Hence, analysis of Φ and τ values indicates that Ca^{2+} binding to calmodulin induces very effective static and dynamic "dequenching" of tyrosine-99, indicative of a large change in the microenvironment of this residue.

This is at variance with the results of Richman & Klee (1979) who found that Ca^{2+} binding only affects the microenvironment of tyrosine-138 and with the recent paper of Anderson et al. (1980) in which the Ca^{2+}-induced fluorescence change of pig brain and plant calmodulin, the latter containing a single tyrosyl residue, was related to the modification of the emission properties of a single tyrosyl residue, namely, tyrosine-138. The latter conclusion is only based on the fact that the fluorescence intensity is enhanced 2.5-fold in both proteins. In the present paper, we showed that determination of absolute quantum yield values leads to quite different conclusions.

(iii) In addition, some indications on the accessibility of tyrosine-99 in the Ca^{2+}–calmodulin complex were inferred from the results obtained in the presence of ionic quenchers. In Ca^{2+}-loaded octopus calmodulin, tyrosine-138 is found as a partially buried residue ($A \simeq \pi$). Therefore, to account for the mean accessibility of both tyrosyl residues in ram testis calmodulin ($A \simeq 1.8\pi$), the major emitting residue (tyrosine-99) must be quite largely exposed.

Furthermore, the fluorescence polarization data led us to speculate about the accessibility of tyrosine-138 in Ca^{2+}-free calmodulin. Indeed, the fact that the chromophore mobility increases after removal of Ca^{2+} indicates that chromophores are more exposed to solvent. Since tyrosine-99 cannot become more exposed than it already is, the data indicate that tyrosine-138 is more exposed to solvent in the absence than in the presence of calcium.

Concluding Remarks. The activation of target enzymes by calmodulin includes the binding of calmodulin to the enzyme and the activation per se. The two processes may involve different conformations of calmodulin since Ca^{2+} ions are not required for the δ subunit (endogenous calmodulin) to bind to the other subunits of glycogen phosphorylase *b* kinase (Cohen et al., 1978). In contrast, the other enzymes, such as myosin light chain kinase or cyclic nucleotide phosphodiesterase, bind calmodulin only in the presence of Ca^{2+}. This suggests that the activation of Ca^{2+}-dependent enzymes upon a Ca^{2+} wave may follow different kinetic pathways (Haiech & Demaille, 1981). A first step toward the understanding of the mechanism of activation of calmodulin-dependent enzymes was the demonstration that Ca^{2+} binding is a sequential and ordered process (Haiech et al., 1981). The present study is

focused on the conformational changes induced by the sequential ion binding. The major following conclusions can be drawn.

(i) The calcium-induced conformational change of calmodulin when monitored by fluorescence, tyrosine circular dichroism (Crouch & Klee, 1980; this study), and circular dichroism at 222 nm (Klee, 1977) is almost complete after binding of 2 mol of Ca^{2+} per mol of calmodulin at the two high-affinity sites, recently assigned to domains I and II (Kilhoffer et al., 1980a). This shows that the major structural rearrangement of the protein, including domains III and IV which contain the tyrosyl residues, is due to calcium binding to sites I and II. Furthermore, this indicates that calcium binding to sites III and IV no longer affects the emission parameters of both fluorophores, in agreement with the results of Seamon (1980), who found very tenuous changes in the proton resonance of tyrosine residues upon calcium binding to the two lower affinity sites.

(ii) Calcium binding affects the emission characteristics of both tyrosine residues in mammalian calmodulin. The fluorescence study showed that in the absence of calcium the tyrosine residues are located in different microenvironments. It also clearly demonstrated that the environment of both residues, and in particular that of tyrosine-99, is affected by calcium binding, which implies a large conformational change of the protein.

(iii) Mg^{2+} ions only partially mimic the Ca^{2+} effect. In addition, the Mg^{2+} effect is strongly dependent on the ionic strength of the medium and on the competition by K^+ ions (Haiech et al., 1981).

In a Ca^{2+}-free medium containing 150 mM KCl, 5 mM Mg^{2+} has very little effect on the fluorescence (see Table I), probably because of this competition between K^+ and Mg^{2+} ions. The cytosol of unstimulated cells contains 150 mM K^+ and ~0.6 mM free Mg^{2+} (Gupta & Moore, 1980). Under these conditions, calmodulin sites are probably not saturated by magnesium, and the protein conformation is close to, if not identical with, the one observed in the absence of divalent metal. The calcium binding sites of calmodulin appear therefore different from the Ca^{2+}/Mg^{2+} sites of parvalbumins and of troponin C domains III and IV which in the intracellular medium are probably loaded by Mg^{2+} in the absence of Ca^{2+} (Haiech et al., 1979; Potter & Gergely, 1975; Leavis et al., 1978).

Whereas the Ca^{2+}/Mg^{2+} sites will only bind Ca^{2+} after the delay introduced by Mg^{2+} dissociation and therefore be involved in the suppression of the calcium signal, calmodulin sites will bind Ca^{2+} with diffusion-limited kinetics and behave as typical "sensor" sites involved in the triggering of Ca^{2+} effects within the cell (Goodman et al., 1979; Haiech & Demaille, 1981; Demaille et al., 1980).

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A Mouse Temperature-Sensitive Mutant Defective in H1 Histone Phosphorylation Is Defective in Deoxyribonucleic Acid Synthesis and Chromosome Condensation†

Hideyo Yasuda,* Yoh-ichi Matsumoto, Shiro Mita,‡ Tohru Marunouchi, and Masa-atsu Yamada

ABSTRACT: By means of a temperature-sensitive mutant (ts85 strain), we have studied the effect of the decrease in H1 histone phosphorylation on DNA synthesis and chromosome condensation. When ts85 cells were incubated at 39 °C (nonpermissive temperature), the rate of H1 histone phosphorylation was decreased gradually and reached half that at 33 °C (permissive temperature) by 6-h incubation. Wild-type cells, growth-revertant ts85 cells (ts85R-MN3), and other ts mutants which were arrested mainly at the G2 phase at 39 °C had no defects in H1 histone phosphorylation. When ts85 cells were synchronized at the G1/S boundary at 33 °C and released from the blockade at 39 °C, ~70% of cells passed through the S phase and stopped at the G2 phase. The rest were distributed in G1/S to the S phase and mitotic cells were not detected at all. When ts85 cells, synchronized at the G1/S boundary, were further incubated for 8 h at 39 °C with a synchronizing agent, the rate of phosphorylation of H1 histone was decreased and the cells were not able to complete DNA synthesis after release from the blockade. Cytofluorometric analysis revealed that the cells had DNA contents of the S phase. Taken together with our earlier data [Matsumoto, Y., Yasuda, H., Mita, S., Marunouchi, T., & Yamada, M. (1980) *Nature (London) 184*, 181–183], both events, incomplete DNA replication and a defect in chromosome condensation, were thought to be ascribed to the decrease in H1 histone phosphorylation.

When mammalian cells grow, the structural changes of chromatin necessarily take place. The phosphorylation of histone is thought to be important for the changes in chromatin structure. In proliferating cells, the phosphorylation occurs significantly in H1, H2A, and H3 histones (Marks et al., 1973; Gurley et al., 1974a). A lot of studies with cultured cells revealed that the phosphorylation of H1 histone changes most dramatically throughout a cell cycle of that of other histones (Balhorn et al., 1972; Lake & Salzman, 1972; Lake et al., 1973; Gurley et al., 1974b). Since H1 histone phosphorylation begins at the late G1 phase and continues from the S to M phase, this phosphorylation is thought to be related to DNA synthesis and/or chromosome condensation at the M phase. To date, much evidence supporting the relationship between H1 histone phosphorylation and chromosome condensation has been reported (Bradbury et al., 1974a,b; Gurley et al., 1978). On the contrary, only a few data which suggested the role of H1 histone phosphorylation in structural changes of chromatin during the interphase have been reported. H1 histone phos-

† From the Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo 113, Japan (H.Y., Y.M., S.M., and M.Y.), and the Laboratory of Cell Biology, Mitsubishi-Kasei Institute of Life Sciences, Minamiooya, Machida-shi, Tokyo 194, Japan (T.M.). *Received September 2, 1980; revised March 13, 1981.* This work was supported in part by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture, Japan.
‡ Present address: Department of Pharmacology, Keio University School of Medicine, Shinanomachi, Tokyo, Japan.

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