# Tyrosine and Tyrosinate Fluorescence of Bovine Testes Calmodulin: Calcium and pH Dependence†

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ABSTRACT: At physiological pH, bovine testes calmodulin (t-CaM) upon excitation at 278 nm shows typical tyrosine fluorescence at 305 nm and a spectral band characteristic of emission from tyrosinate, at 330–350 nm. In addition, a new band at 312–320 nm appears upon excitation at 288 nm. The pH dependence of the excitation spectra demonstrates that the intense tyrosinate emission at 330–355 nm originates from direct excitation of ground-state tyrosinate. The tyrosinate emission shows complex pH dependence and reaches its highest intensities at pH 7.0 and 8.5, in both apo (Ca free) and holo (Ca saturated) t-CaM. The evidence suggests that the major contribution to the tyrosinate emission at 330–350 nm originates from Tyr-99. In holo t-CaM, the tyrosine emission at 305 nm is quenched at basic pH values and exhibits a sigmoidal pH titration curve with p$K$(app) 7.0. The tyrosine emission in apo t-CaM is weaker and is almost insensitive to changes in pH. The pH dependence of the emission at 316 nm is the same as the pH dependence of the tyrosine emission in both apo and holo t-CaM. The differences between the fluorescence of apo and holo t-CaM are attributed to a $Ca^{2+}$-induced shift in the p$K_a$ of carboxylic side chains located in the immediate vicinity of the tyrosine residues. The enhancement of the fluorescence by $Ca^{2+}$ is pH dependent and is maximal at pH 6.5. Above pH 8.0, there is almost no $Ca^{2+}$ effect on the fluorescence. Conformational changes induced by variations in pH in the physiological range, pH 6.0–8.0, as detected by the fluorescence measurements, suggest that changes in the levels of both $Ca^{2+}$ and the proton regulate the conformation and also the biological function of calmodulin.

Calmodulin is a ubiquitous $Ca^{2+}$-binding protein and plays a central role in cell regulation (Cheung, 1980a). Previous studies of this protein [for reviews see Cheung (1980b), Kakiuchi et al. (1981), and Klee & Vanaman (1982)] have established the following. CaM[1] is a small ($M_r$ 16 700), heat- and acid-stable protein characterized by a high Asp and Glu content, lack of cysteine and tryptophan, one histidine, two tyrosines (Tyr-99 and -138), and an $\epsilon$-trimethyllysine residue at position 115. Tyr-99 is replaced by Phe in CaM from plants and invertebrates. The sequences of CaM from different mammalian sources differ only in the amidation states of some carboxyl side chains (Sasagawa et al., 1982). It is not clear whether these differences are real or result from different purification procedures.

The binding of $Ca^{2+}$ induces large conformational changes in CaM. These changes facilitate the interaction of CaM with its target proteins and have been the subject of many studies (see the reviews cited above). Most previous studies focused on the role of the bound $Ca^{2+}$ in modulating the structure of CaM and neglected the role of the proton in this process. Since CaM is highly acidic (p$I$ = 4.1) and carries a large negative charge above its p$I$, it is reasonable to infer that variations in pH will cause conformational changes in this protein. To investigate such pH-dependent conformational changes, we have examined the intrinsic tyrosine fluorescence of apo and holo t-CaM over the pH range 5.0–10.0. Tyrosine fluorescence is a sensitive probe for conformational changes in class A (tryptophan-free) proteins (Cowgill, 1976). In CaM, the tyrosyl residues, Tyr-99 and Tyr-138, are in the putative $Ca^{2+}$-binding sites III and IV, respectively (Jamieson et al., 1980). Recent NMR studies reveal that these sites are the high-affinity $Ca^{2+}$-binding sites in the case of CaM from bovine brain (Ikura et al., 1983b) and bovine testes (Andersson et al., 1983). Therefore, changes in the tyrosine fluorescence caused by variations in pH are directly related to conformational changes in the high-affinity $Ca^{2+}$-binding sites of CaM.

Unusual tyrosine emissions at wavelengths longer than the normal one near 305 nm have been reported in a number of class A proteins. They include cattle adrenodoxin (Kimura et al., 1971, 1972; Lim & Kimura, 1980), parsley plastocyanin (Graziani et al., 1974), Indian cobra cytotoxins (Szabo et al., 1978, 1979), human serum albumin (Longworth, 1981), and fruit fly histone H1 (Jordano et al., 1983). It has been suggested (Szabo et al., 1978, 1979; Jordano et al., 1983) that the emission in the range 330–350 nm arises from excited-state tyrosinate, produced by excited-state proton transfer to adjacent acceptor groups. The long wavelength emission in all the above cases was weak and appeared as a shoulder on the tyrosine band. In contrast, we have found that t-CaM shows intense tyrosinate emission (Pundak & Roche, 1983). Recently, we have found that variations of pH in the physiological range modulate the $Ca^{2+}$–CaM stimulated activity of brain cyclic nucleotide phosphodiesterase (PDE) (S. Pundak and R. S. Roche, unpublished results). The latter effect could be accounted for by the dissociation of a single proton from the $Ca^{2+}$–CaM–PDE complex in the range pH 5.5–7.0. The mechanism by which the variation in pH causes changes in the activity of the $Ca^{2+}$–CaM–PDE complex is not yet clear. One possibility is that the binding of the proton and $Ca^{2+}$ are coupled. The latter possibility could provide for a pH-dependent fine tuning of $Ca^{2+}$–CaM modulated processes in cell regulation.

In this paper, we present the results of a detailed study of the pH and $Ca^{2+}$ dependence of the tyrosine–tyrosinate

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[1] Abbreviations: CaM, calmodulin; t-CaM, testes CaM; DEAE-cellulose, diethylaminoethylcellulose; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PDE, phosphodiesterase; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography.

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fluorescence of bovine testes CaM. This study provides insights into the origins of tyrosinate emission in class A proteins and the dependence of the fluorescence on the binding of $Ca^{2+}$ and the proton. The interplay of $Ca^{2+}$ and the proton in modulating the structure of calmodulin, as revealed by the fluorescence measurements, suggests that the biological role of calmodulin as the molecular coupler of the two intracellular second messengers cAMP and $Ca^{2+}$ is pH dependent.

## Experimental Procedures

*Materials.* DEAE-cellulose (DE-52) was obtained from Whatmans (England), Sepharose CL-48 from Pharmacia (Sweden), and EGTA from Sigma. Fluphenazine was a gift from Squibb (Canada) Ltd. All other reagents were analytical grade. Fluphenazine–Sepharose was synthesized according to Porath et al. (1974).

*Purification of Bovine Testes CaM.* Fresh bovine testes were obtained from 4–8-month-old calves and immediately frozen. After removal of the scrotum, the testicular tissue was homogenized in 1:2 (w/v) 100 mM Tris–2 mM EGTA–2 mM β-mercaptoethanol, pH 8.0, buffer. The homogenate was centrifuged for 20 min at 10000*g* and the supernatant collected by filtration through cheesecloth. Ethanol was added to the filtrate to a final concentration of 20%, and then the pH was adjusted to an apparent value of 6.0. The 20% alcohol mixture was recentrifuged under the same conditions, and the supernatant was collected. A batch of DEAE-cellulose (200 g/L) was added to the supernatant, and the mixture was stirred for 20 min at room temperature. After removal of the liquid phase by vacuum filtration, the gel was washed with 3 L of 0.1 NaCl–20 mM Tris–2 mM EGTA, pH 6.0. The calmodulin was eluted from the gel by washing with four portions of 400 mL of 0.5 M NaCl–20 mM Tris–5 mM $CaCl_2$, pH 7.0. The eluted calmodulin solution was concentrated to 200 mL by reverse osmosis using an Amicon TCF-10 system. The concentrated calmodulin solution was applied to the fluphenazine–Sepharose column (30 mL bed volume) preequilibrated with the same buffer. The affinity column was washed with 1 L of the same buffer. Then the calmodulin was eluted by a pulse of 10 mM Tris–2 mM EGTA, pH 7.5. The eluted calmodulin fraction was loaded on to a DEAE-cellulose column (20 mL) preequilibrated with the same buffer and, after washing, was eluted by an increasing NaCl gradient. The latter additional DEAE-cellulose column step was introduced to ensure the removal of any trace of tryptophan-containing proteins which would vitiate the fluorescence experiments. The calmodulin sample was dialyzed against distilled water and lyophilized. The calmodulin powder was stored at −20 °C.

The purified calmodulin migrated as a single band on SDS gel electrophoresis and exhibited calcium-dependent mobility. On a calibrated HPLC size-exclusion column (TSK 3000 SW, Toyo Soda, Japan) equilibrated with 6.0 M Gdn·HCl–0.01 M Tris–1 mM EDTA, pH 7.0, the calmodulin appeared as a single peak with an apparent molecular weight of 17 000. The activity of calmodulin was tested with the Ca–Mg-ATPase system from human red blood cells, according to the method of Vincenzi et al. (1980).

*Fluorescence Measurements.* Samples of $2.5 \times 10^{-5}$ M (0.417 mg/mL; OD < 0.1) t-CaM–15 mM Tris–1 mM $CaCl_2$ at a variety of pH values were prepared in 1-mL flasks. To chelate the calcium, 40 μL of 0.1 M EGTA–15 mM Tris, at the appropriate pH, was added. Fluorescence measurements were performed at 22 °C on an SPF-500 spectrofluorometer (Aminco Bowman) with an attached X–Y recorder. Corrected emission and excitation spectra were recorded in the ratio mode with 4-nm band-pass on both monochromators; the scan rate was 50 nm/min and time constant, 1 s. The emission spectra were corrected for the buffer base line.

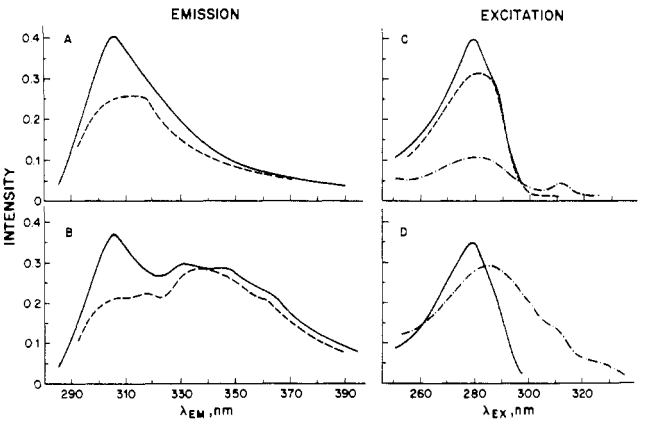


FIGURE 1: Corrected emission and excitation spectra of holo t-CaM (0.4 mg/mL) in 15 mM Tris and 1 mM $CaCl_2$. At pH 6.5, (A) and (C); at pH 7.0, (B) and (D). The emission spectra were obtained by excitation at 278 ± 4 nm (—) and at 288 ± 4 nm (---). The excitation spectra were monitored by emission at 305 (—), 316 (---), and 345 nm (···).

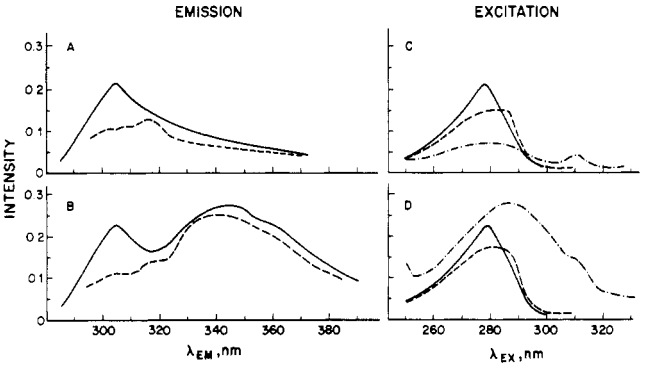


FIGURE 2: Corrected emission and excitation spectra of apo t-CaM. The same conditions and symbols as in Figure 1, except that the buffer contains 2 mM EGTA.

## Results

The excitation and emission spectra of holo and apo bovine testes calmodulin at pH 6.5 and 7.0 are shown in Figures 1 and 2, respectively. Excitation of calmodulin at pH 6.5 results in typical tyrosine emission spectra (Figures 1A and 2A, solid line), both of which exhibit a maximum at 305 nm. A comparison of these two spectra reveals a calcium-dependent enhancement of the tyrosine fluorescence. In contrast, excitation of calmodulin at pH 7.0 produces double-peaked emission spectra (Figures 1B and 2B, solid line). The first peak (305 nm) corresponds to tyrosine emission while the second one is due to tyrosinate emission. Comparison of the spectra for holo and apo t-CaM reveals that the intensity of the tyrosine emission is preferentially enhanced by the binding of $Ca^{2+}$; the tyrosinate emission remains virtually unchanged. The only significant effect of calcium removal on the tyrosinate fluorescence is to red shift the peak. By measuring the excitation spectra as a function of pH, we can determine whether the tyrosinate emitter arises from direct excitation of ground-state ionized tyrosine or by excited-state proton transfer from singlet excited tyrosine. If the latter mechanism dominates, we would expect to find the excitation spectrum for the tyrosinate emission to be independent of pH below the $pK_a$ of ground-state tyrosine. In both the holoprotein and apoprotein, the excitation spectra at pH 6.5 (Figures 1C and 2C) and 7.0 (Figures 1D and 2D) show big increases in the intensity of the excitation for the emission at 345 nm (tyrosinate)

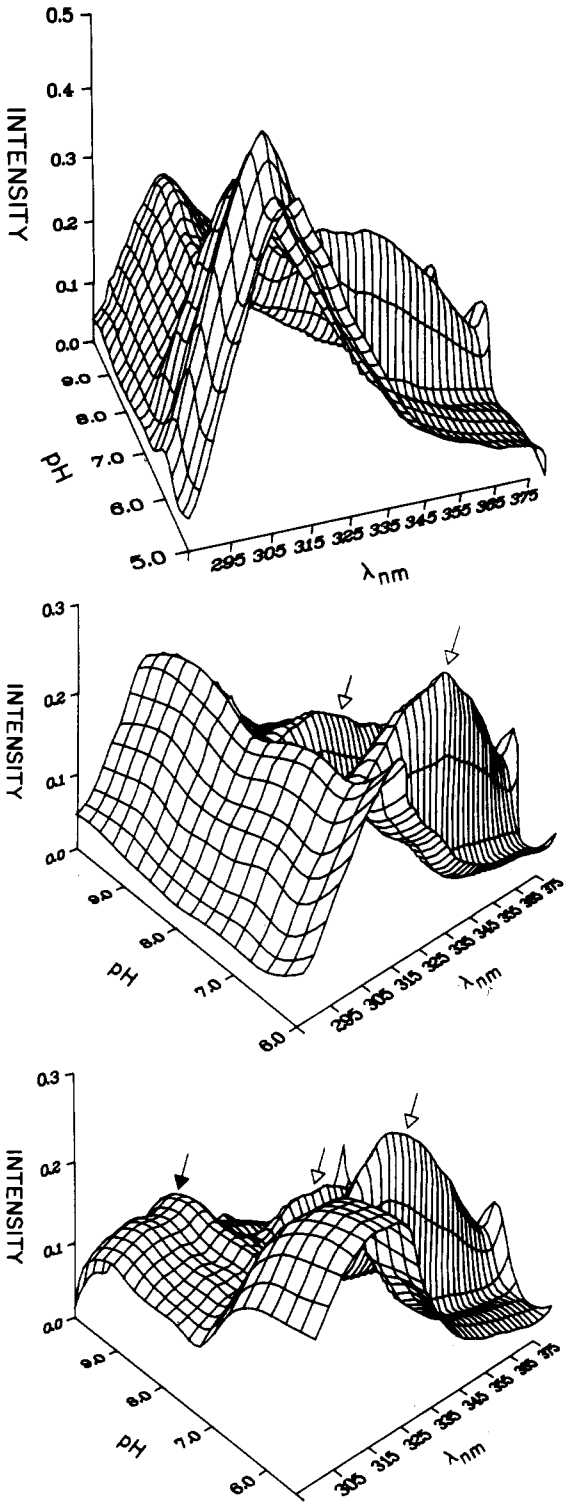


FIGURE 3: Emission surface, intensity vs. pH vs. wavelength, of CaM (0.4 mg/mL) in 15 mM Tris. (Top) Excitation of holo CaM at 278 ± 4 nm. (Middle) Excitation of apo CaM at 278 ± 4 nm. (Bottom) Excitation of holo CaM at 288 ± 4 nm.

and a decrease in the intensity of the excitation for the emission at 305 nm (tyrosine) when the pH is raised from pH 6.5 to 7.0. This indicates clearly that the emission band at 330–350 nm originates from excitation of ground-state tyrosinate. The possible mechanism for this phenomenon will be discussed later.

Excitation of apo t-CaM at 288 nm results in a new band with a maximum near 316 nm (Figure 2A,B, broken line). In holo t-CaM, the 316-nm band overlaps the 305-nm peak (Figure 1A,B, broken line) whereas in apo t-CaM it is more

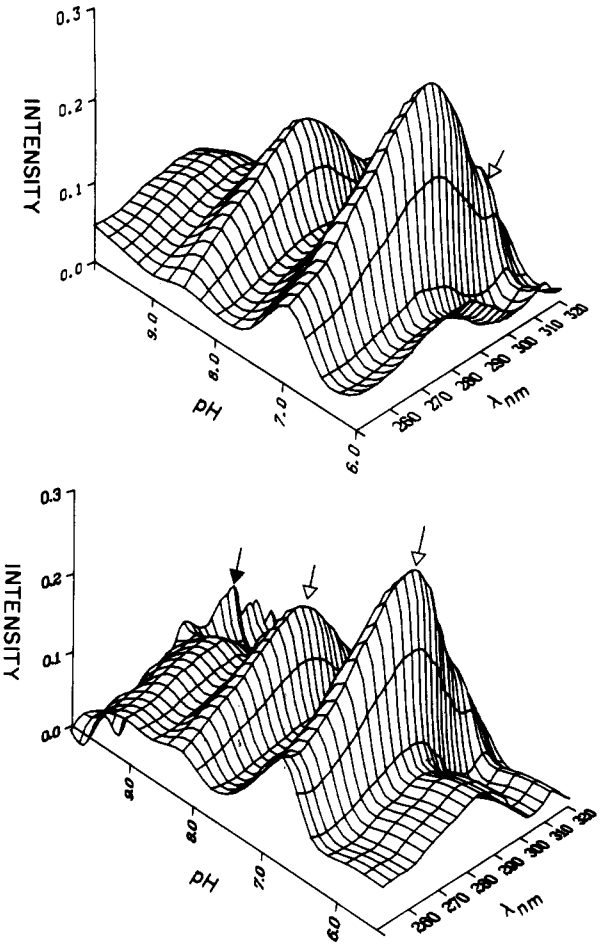


FIGURE 4: Excitation surface of CaM (0.4 mg/mL) in 15 mM Tris. (Top) Emission of holo CaM at 305 ± 4 nm. (Bottom) Emission of holo CaM at 345 ± 4 nm.

resolved (Figure 2A,B, broken line).

Figure 3 shows the emission surfaces of holo and apo bovine testes calmodulin, obtained by excitation at 278 and 288 nm, over the pH range 5.0–10.0. The emission surface of holo t-CaM, excited at 278 nm, is shown in Figure 3 (top). Three ridges are easily identified. In the foreground, the pH dependence of the tyrosine fluorescence is clearly seen as a ridge at 305 nm running parallel to the pH axis. In the background, partly obscured by the tyrosine ridge, are two ridges lying parallel to the wavelength axis which correspond to the two pH extrema in the tyrosinate emission at pH 7.0 and 8.5, respectively. Figure 3 (middle) shows the emission surface of apo t-CaM under the same conditions as in Figure 3 (top). Due to the considerable decrease in the intensity of the tyrosine fluorescence at 305 nm, the two ridges in the tyrosinate emission at pH 7.0 and 8.5 (indicated by arrows) are more clearly seen. At pH 7.0, the emission intensity of the tyrosinate is greater than that of the tyrosine. Upon excitation at 288 nm, the tyrosine emission (305 nm) of holo t-CaM is substantially decreased, while the tyrosinate emission is slightly enhanced (Figure 3, bottom). As a result, the two tyrosinate emission peaks at pH 7.0 and 8.5 (indicated by open arrows) are revealed. Above pH 7.0, the 316-nm band (indicated by black arrow) is clearly distinguished from the tyrosine emission band and has higher intensity. The emission surface of apo t-CaM obtained by excitation under the same conditions is similar (not shown). The only differences are that the emission intensities at 305 and 316 nm are almost pH independent and that the emission at 316 nm is higher and separated from the tyrosine emission even in the acidic pH range.

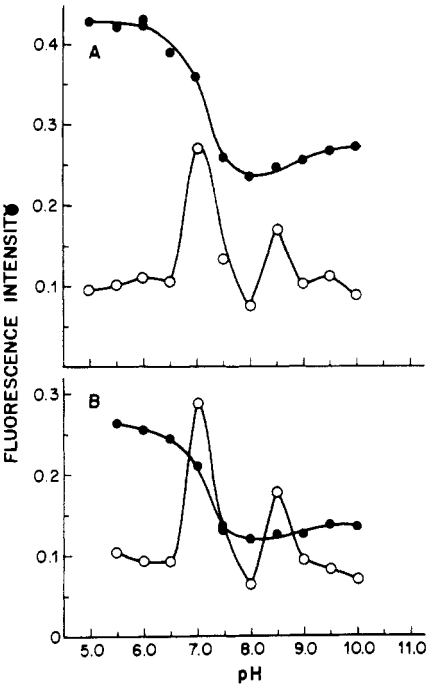


FIGURE 5: Fluorescence intensities of holo CaM as function of pH. (A) Excitation at 278 ± 4 nm. (B) Excitation at 288 ± 4 nm. Emission at 305 nm (●) and at 345 nm (○).

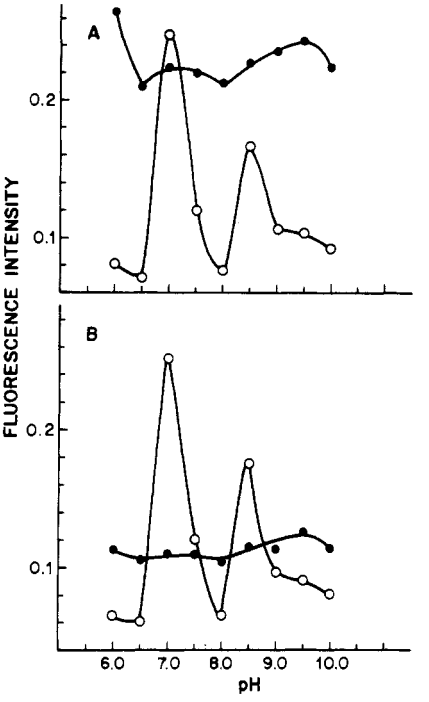


FIGURE 6: Fluorescence intensities of apo CaM as a function of pH. (A) Excitation at 278 ± 4 nm. (B) Excitation at 288 ± 4 nm. Emission at 305 nm (●) and at 345 nm (○).

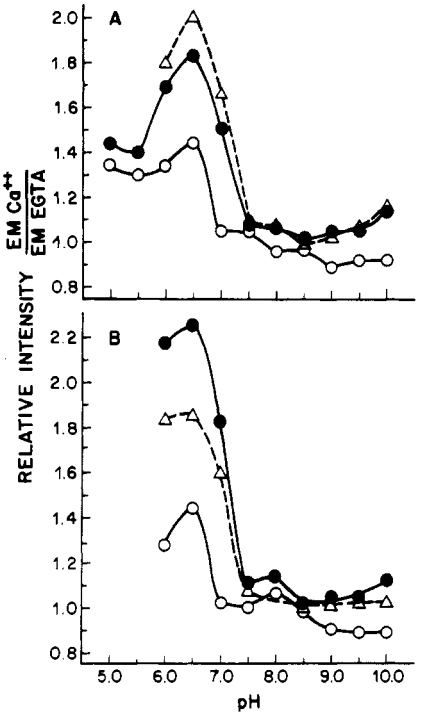


FIGURE 7: Effects of calcium binding on the fluorescence intensities of bovine testes calmodulin. The ratio of the holo CaM emission (Em Ca) to the apo CaM emission (Em EGTA) is plotted as a function of pH. (A) Excitation at 278 ± 4 nm. (B) Excitation at 288 ± 4 nm. The emissions are at 305 (△), 316 (●), and 345 nm (○).

The excitation surfaces of holo t-CaM as a function of pH and wavelength are shown in Figure 4. The tyrosine excitation intensity in the case of apo t-CaM, monitored by the emission at 305 nm (not shown), is insensitive to variation of the pH and has a maximum at 278 nm. On the other hand, in holo t-CaM (Figure 4, top) the intensities of the tyrosine excitation are pH dependent with p$K$(app) around 7.0. The maximum is also at 278 nm. The excitation surface, monitored at 316 nm (not shown), has a pH dependence similar to that of tyrosine. But the maximum is broader, ranging from 278 to 282 nm, and varies inside this range with pH. The tyrosinate excitation surfaces, monitored by the emission at 345 nm, of apo t-CaM (not shown) and holo t-CaM (Figure 4, bottom) both show biphasic pH titrations with maxima at pH 7.0 and 8.5 (marked by open arrows in Figure 4, bottom).

The pH titrations of the tyrosine and tyrosinate fluorescence intensities of holo and apo t-CaM are cross sections at the appropriate wavelength of the surfaces shown in Figure 3. These are shown in Figures 5 and 6. In holo t-CaM, the tyrosine fluorescence titration curves (Figure 5) are sigmoidal with an apparent p$K$ of 7.0 ± 0.1. The first peak of the tyrosinate fluorescence appears at the same pH, while the second one is at pH 8.5. Upon excitation at 278 nm (Figure 5A) the intensities of tyrosine emission are always greater than those of tyrosinate emission. In contrast, upon excitation at 288 nm (Figure 5B) the tyrosine fluorescence intensity is reduced and at pH values greater than 7 is comparable in intensity to the tyrosinate fluorescence. In apo t-CaM (Figure 6), the tyrosine fluorescence intensity is almost unaffected by changes in pH, whereas the tyrosinate fluorescence intensity shows the same biphasic pH titration as in the holoprotein. These results suggest that there is no direct correlation between the quenching of tyrosine fluorescence and the appearance of tyrosinate fluorescence. This may reflect the different contributions of Tyr-99 and -138 to the total emission intensity and also the differences in the pH dependence of their microenvironments. In both holo and apo CaM, the emission at 316 nm (not shown) has the same pH dependence as that of the corresponding tyrosine emission. In addition, the excitation intensities of these three components have identical pH dependencies to the corresponding emission intensities.

The effect of calcium on the intensities of the tyrosine and tyrosinate emissions is shown in Figure 7, as the ratio of the emission in holo CaM (Em Ca) to that of the apo CaM (Em EGTA). The enhancement of the fluorescence by calcium reaches a maximum at pH 6.5, regardless of whether the fluorescence is excited at 278 nm (Figure 7A) or at 288 nm (Figure 7B). The maximal enhancement of the 305-nm

(tyrosine) and the 316-nm emission bands is 2-fold, whereas that of the tyrosinate 345-nm emission band is only 40%. Above pH 8.0 the binding of calcium causes a quenching of the 345-nm tyrosinate fluorescence and an enhancement of only 10% of the intensity of the other two components.

## Discussion

*Tyrosine–Tyrosinate Fluorescence.* The calmodulins are class A proteins. The protein from vertebrate species contains eight phenylalanines and two tyrosines. Because of the structural relationship of the two tyrosines to the postulated calcium-binding sites III (Tyr-99) and IV (Tyr-138), the fluorescence properties provide useful insights into the microenvironments of these sites and how they may be affected by changes in pH and pCa. The results of the present study clearly demonstrate that bovine testes CaM is an atypical class A protein. The excitation and emission spectra at pH 7.0 (Figures 1 and 2) show that the tyrosinate fluorescence, centered at 330–350 nm, is dominant at this pH. Emission in this wavelength range has been observed in other class A proteins (see the introduction) but usually as a minor component detectable only as a shoulder on the main tyrosine emission band centered at 305 nm. The tyrosinate fluorophore can be generated either by direct excitation of ground-state tyrosinate or by excited-state proton transfer from singlet-excited tyrosine. If the latter mechanism is operative, the excitation spectrum for both the tyrosine and tyrosinate emissions should be identical and show the same dependence on pH. Clearly, this is not the case in both holo and apo t-CaM (Figures 1 and 2). The excitation spectra of the 305- and 345-nm emissions differ significantly, and in the region of pH 7, the excitation spectrum of the 345-nm emission undergoes a large increase in intensity. The maximum of the tyrosinate excitation spectrum lies in the range 287–289 nm at pH 7.0 and above. The absorption maximum of tyrosinate is at 288 nm. These observations indicate that the singlet-excited tyrosinate arises from direct excitation of ground-state tyrosinate. This is the first time that ground-state tyrosinate has been recognized in any protein at pH 7 and suggests that the environment of Tyr-99 or Tyr-138 is sufficiently basic to reduce the p$K$ of tyrosine by at least three units from the value for "normal" tyrosines. In addition to the two major bands at 305 and 345 nm, a band at 312–320 nm is clearly discernible in the emission spectra excited at 288 nm (Figures 1 and 2). In a study of the fluorescence of poly(L-tyrosine) as a function of pH, Longworth & Rahn (1967) assigned a band observed at 312 nm in the highly ionized polymer to Raman scattering by the solvent. All of our spectra have been corrected for solvent background, and we conclude, therefore, that the 316-nm band is a property of the protein rather than a property of the solvent. It may be possible to account for the 316-nm band in terms of a combination of contributions from the tyrosine (305 nm) and tyrosinate (345 nm) emissions.

*pH Dependence of the Tyrosine–Tyrosinate Fluorescence.* The excitation and emission surfaces of the two tyrosyl residues in t-CaM, shown in Figures 3 and 4 and the representative cross sections of these surfaces shown in Figures 5 and 6, are characterized by the following features: (A) The tyrosine excitation and emission maxima in both apo and holo CaM are 278 and 305 nm, respectively, and are not shifted by variations in pH. The intensities of the tyrosine excitation and emission maxima in apo CaM are almost unaffected by changes of the pH, while in holo CaM, they are significantly reduced at basic pH values. The apparent p$K$ of the quenching of the tyrosine emission at 305 nm is 7.0. (B) Tyrosinate emission, 330–350 nm, is the dominant component of the fluorescence above pH 7.0. The tyrosinate emission spectra are pH dependent and, in both apo and holo CaM, show complex behavior with maxima at pH 7.0 and 8.5. The excitation spectra for the tyrosinate emission show identical pH dependence. The tyrosinate emission and excitation maxima are in the range 330–346 and 280–288 nm, respectively, and vary inside these ranges with pH. (C) A band around 316 nm is observed in apo t-CaM over the entire pH range, but only above pH 7.0 in holo t-CaM. This band is noticeable upon excitation at 288 nm, but not at 278 nm.

*Ca(II) Dependence of Tyrosine–Tyrosinate Fluorescence.* In order to explain the differences in the pH dependencies of the tyrosine fluorescence in apo and holo t-CaM (Figures 5 and 6), we should take into acount the following facts. First, the fluorescence of both the tyrosine and tyrosinate are enhanced by the binding of Ca(II) in a pH-dependent way. Also, the two tyrosyl residues in CaM are in the vicinity of the postulated Ca-binding sites III and IV, both of which contain several carboxylic acid side chains. Carboxylates are known quenchers of tyrosine fluorescence (Rayner et al., 1978). Binding of calcium, which introduces two positive charges, perturbs the electrostatic balance and changes the microenvironment of the Ca(II)-binding sites. A number of studies have shown (La Porte et al., 1980; Ikura et al., 1983b, and references cited therein) that the binding of Ca(II) induces a conformational change in the protein and an associated change in the environment of the tyrosines. Thus, shifts in the p$K_a$ of the charged groups in these sites are expected to result from Ca(II) binding. One possibility is that the binding of calcium shifts the p$K_a$ of the carboxyl groups to a higher value. In apo t-CaM the carboxyl groups are ionized with a normal p$K_a$, i.e., around 4.5, and therefore quench the tyrosine fluorescence over the entire pH range 5.0–10.0. An NMR study of the Ca(II)-binding sites in bovine testes calmodulin (Andersson et al., 1982) revealed a p$K$ value of 4.4 for the competition between $Ca^{2+}$ and $H^+$ ions. This indicates that the average p$K_a$ of the carboxyl groups in the binding sites is 4.4. Ca(II)-binding measurements as a function of pH gave a similar p$K$ value for ram testes CaM (Haeich et al., 1981). We suggest that upon binding of Ca(II) the p$K_a$ of the carboxyl groups in the Ca(II)-binding site is shifted to 7.0. This shift is reflected in the tyrosine fluorescence titration curve of holo t-CaM which, therefore, differs from that of apo t-CaM below pH 8.0. Above pH 8.0, the tyrosine fluorescence is quenched to the same extent by the fully ionized carboxyl groups in both apo and holo t-CaM. The tyrosine fluorescence in histone H1 has a similar pH titration curve with a p$K$ of 3.7. The quenching of the tyrosine fluorescence at higher pH is also attributed to the ionization of adjacent carboxyl group (Jordano et al., 1983). The assignment of the shift in the p$K_a$ to the carboxyl side chains is only one possible explanation. A second possibility is that local conformational changes, induced by the binding of Ca(II), cause a shift in the overall apparent p$K_a$ of the whole tyrosyl microenvironment. The possibility of local changes in the Ca(II)-binding sites and hence in the tyrosyl microenvironment could account for the pH dependence of the tyrosinate fluorescence. The double peak pattern of the tyrosinate excitation and emission spectra as a function of the pH can only be explained by charged groups entering or leaving the vicinity of the tyrosine. This interpretation implies that a localized conformational transition occurs in the Ca(II)-binding sites as a direct result of pH variations.

*Identification of the Source of Tyrosinate Emission.* There is no direct correlation between the quenching of tyrosine

emission at 305 nm and the enhancement of tyrosinate 345-nm emission as the pH is varied (Figures 5 and 6). The enhancement of the fluorescence by Ca(II) as a function of pH is the same for the 305- and 316-nm bands, but is different for the tyrosinate 345-nm emission (Figure 7). The pH titrations of the tyrosinate 345-nm emission in both apo and holo t-CaM are the same and correspond, therefore, to the tyrosine which is less perturbed by the binding of Ca(II). In contrast, the pH titration of the tyrosine 305-nm emission in apo t-CaM differs from that of the holo protein. Thus, we can conclude that Tyr-99 and Tyr-138 have environments which differ in their overall p$K$ and their response to Ca(II) binding. We can also infer that only one tyrosyl residue is ionized at the lower pH values and contributes to the intense tyrosinate 345-nm emission and that only its fluorescence pH titration is unaffected by the binding of Ca(II). The pH titration of the fluorescence of the other tyrosine fluorophore is Ca(II) dependent. Chemical modifications of the tyrosyl residues in bovine brain calmodulin (Klee, 1977; Richman, 1978; Richman & Klee, 1978, 1979) indicate that the reactivity of Tyr-99 is unaffected by Ca(II) binding, whereas the reactivity of Tyr-138 is Ca(II) dependent. Furthermore, they suggest that Tyr-138 becomes more buried as a result of Ca(II) binding while Tyr-99 remained relatively more exposed to the solvent. NMR studies (Krebs & Carafoli, 1982; Ikura et al., 1983a,b) confirmed the above suggestion and also show that Tyr-99 is ionized at lower pH than Tyr-138. On this basis, we assign the intense tyrosinate fluorescence, at 330–350 nm, to Tyr-99. Similarly, the Ca(II)-dependent tyrosine 305-nm band is dominated by emission from Tyr-138. Fluorescence measurements on spinach calmodulin which has only Tyr-138 support this assignment (S. Pundak and R. S. Roche, unpublished results). Selective chemical modifications of Tyr-99 followed by fluorescence measurements are required to confirm the above assignments.

The unusual ground-state ionization of Tyr-99 occurs 3–4.5 pH units below the p$K$ for normal tyrosines. Only the presence of a nucleophilic group(s) in the tyrosyl microenvironment, basic enough to accept the phenolic proton, could cause such ionization. Suitable candidates are carboxylate, histidyl, and lysyl residues. The first two groups could account for the ionization at pH 7.0 and the lysine for the ionization at pH 8.5. Since the sequence and the three-dimensional structure of bovine testes calmodulin are unknown, the identification of the nucleophilic groups is not possible. However, a tentative three-dimensional model for bovine brain calmodulin has been proposed (Kretsinger, 1980). On the basis of the high sequence homology within the CaM family (Sasagawa et al., 1982), we can suggest that there are carboxyl and lysyl residues in the Ca(II)-binding site III of bovine testes calmodulin. The presence of these residues might be the reason for the acidic shift in the p$K_a$ of Tyr-99. Differences in the amidation of the carboxyl side chains in the vicinity of Tyr-99 between bovine testes CaM and other calmodulins could also explain why the unusual tyrosinate fluorescence is observed only in t-CaM and not in the other calmodulins. In the case of bovine adrenodoxin (Kimura et al., 1971, 1972; Lim & Kimura, 1980), Indian cobra cytotoxin (Szabo et al., 1978), and *Ceratitis capitata* histone H1 (Jordano et al., 1983), the tyrosinate emission arises from excited-state proton transfer to ionized carboxylate acting as proton acceptor. The tyrosinate emission in bovine testes CaM, which we have demonstrated arises from direct excitation of ionized tyrosine, is thus a unique case among class A proteins.

*Role of pH in the Biological Function of Calmodulin.* The pH dependence of the fluorescence discussed above serves to emphasize the importance of electrostatic interactions in the conformational flexibility of calmodulin. Calmodulin is a negatively charged protein at physiological pH, 50 aspartyl and glutamyl residues out of 148 amino acids, with a p$I$ of 4.0 (Klee & Vanaman, 1982). The biggest changes in the fluorescence occur between pH 6.0 and pH 8.5 (Figures 5–7). The binding of Ca(II) to calmodulin induces conformational changes which facilitate the interactions between calmodulin and its target proteins (La Porte et al., 1980), and these conformational changes are reflected in the enhancement of the tyrosine fluorescence. Since the Ca(II) enhancement of the tyrosine fluorescence is pH dependent, as we have demonstrated here, we can infer that the transition to the active conformation of CaM is also regulated by pH. We postulate that a combination of the proton and $Ca^{2+}$ ion levels regulates the biological function of calmodulin, via conformational changes. This "coupling" of the effects of the two ions may contribute to the specificity of calmodulin. Such a coupling has been identified in the case of amoeboid cells in which the average pH ranges from 6.3 to 7.4 (Taylor & Fechheimer, 1983). Such changes in the cytoplasmic pH are shown to regulate the contractility of the amoeboid myosin. The authors conclude that pH and pCa are interrelated, and it is possible that changes in pH could at least "fine tune" the regulation of contractility by calcium. Recently, we have found (S. Pundak and R. S. Roche, unpublished results) that the activation of cyclic nucleotide phosphodiesterase (PDE) by bovine brain and testes CaM is regulated by changes in pH. Even with excess amounts of Ca(II)–CaM a 5-fold increase in the activation of PDE was observed when the pH was raised from 6.0 to 7.0.

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**Registry No.** Ca, 7440-70-2; tyrosine, 60-18-4; tyrosinate, 16978-66-8.

## References

Andersson, A., Darkenberg, T., Forsen, S., & Thulin, E. (1982) *Eur. J. Biochem. 126*, 501–505.

Andersson, A., Forsen, S., Thulin, E., & Vogel, H. (1983) *Biochemistry 22*, 2309–2313.

Cheung, W. Y. (1980a) *Science (Washington, D.C.) 207*, 19–27.

Cheung, W. Y., Ed. (1980b) *Calcium and Cell Function*, Vol. 1, Academic Press, New York.

Cowgill, R. W. (1976) in *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 2, pp 442–483, Marcel Dekker, New York.

Graziani, M. T., Finazzi Argo, A., Rotilio, G., Barra, D., & Mondovi, B. (1974) *Biochemistry 13*, 804–809.

Haiech, M., Kilhoffer, M. C., Gerard, D., & Demaille, J. C. (1981) in *Calmodulin and Intracellular $Ca^{2+}$ Receptors* (Kakiuchi, S., Hidaka, H., & Means, A. R., Eds.) pp 55–72, Plenum Press, New York.

Jamieson, G. A., Jr., Bronson, D. D., Shachat, F. H., & Vanaman, T. C. (1980) *Ann. N.Y. Acad. Sci. 336*, 1–13.

Jordano, J., Barbero, J. L., Montero, F., & Franco, L. (1983) *J. Biol. Chem. 258*, 315–320.

Kakiuchi, S., Hidaka, H., & Means, A. R., Eds. (1981) *Calmodulin and Intracellular $Ca^{2+}$ Receptors*, Plenum Press, New York.

Kilhoffer, M. C., Demaille, J. C., & Gerard, D. (1981) *Biochemistry 20*, 4407–4414.
Kimura, T., & Ting, J. J. (1971) *Biochem. Biophys. Res. Commun. 45*, 1227–1231.
Kimura, T., Ting, J. J., & Huang, J. J. (1972) *J. Biol. Chem. 247*, 4476–4479.
Klee, C. B. (1977) *Biochemistry 16*, 1017–1024.
Klee, C. B., & Vanaman, T. C. (1982) *Adv. Protein Chem. 35*, 213–321.
Krebs, J., & Carafoli, E. (1982) *Eur. J. Biochem. 124*, 619–624.
Kretsinger, R. H. (1980) *Ann. N.Y. Acad. Sci. 356*, 14–19.
Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M., & Yagi, K. (1983a) *Biochemistry 22*, 2568–2572.
Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M., & Yagi, K. (1983b) *Biochemistry 22*, 2573–2579.
Lim, B. C., & Kimura, T. (1980) *J. Biol. Chem. 255*, 2440–2444.
Longworth, J. W. (1981) *Ann. N.Y. Acad. Sci. 366*, 237–245.
Longworth, J. W., & Rahn, R. O. (1967) *Biochim. Biophys. Acta 147*, 526–535.
Porath, J. (1974) *Methods Enzymol. 34*, 13–20.
Pundak, S., & Roche, R. S. (1983) *Biophys. J. 41*, 220a.
Rayner, D. M., Krajcarski, D. T., & Szabo, A. G. (1978) *Can. J. Chem. 56*, 1238–1245.
Richman, P. G. (1978) *Biochemistry 17*, 3001–3005.
Richman, P. G., & Klee, C. B. (1978) *Biochemistry 17*, 928–935.
Richman, P. G., & Klee, C. B. (1979) *J. Biol. Chem. 254*, 5372–5376.
Sasagawa, T., Ericsson, L. W., Walsh, K. A., Schreiber, W. E., Fisher, E. H., & Titani, K. (1982) *Biochemistry 21*, 2565–2569.
Szabo, A. G., Lynn, K. R., Krajcorski, D. T., & Rayner, D. M. (1978) *FEBS Lett. 94*, 249–252.
Szabo, A. G., Lynn, K. R., Krajcorski, D. T., & Rayner, D. M. (1979) *J. Lumin. 18*, 582–585.
Taylor, D. L., & Fechheimer, M. (1981) in *Calmodulin and Intracellular $Ca^{2+}$ Receptors* (Kakiuchi, S., Hidaka, H., & Means, A. R., Eds.) pp 349–372, Plenum Press, New York.
Vincenzi, F. F., Hinds, T. R., & Raess, B. U. (1980) *Ann. N.Y. Acad. Sci. 356*, 233–244.

# The Rate-Limiting Step in the Actomyosin Adenosinetriphosphatase Cycle†

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ABSTRACT: We have previously shown that myosin does not have to detach from actin during each cycle of ATP hydrolysis. In the present study, using the A-1 isoenzyme of myosin subfragment 1, we have investigated the nature of the rate-limiting steps in the ATPase cycle. Our results show that, at 15 °C, at very low ionic strength, $K_{ATPase}$ determined from the double-reciprocal plot of ATPase activity vs. actin concentration is more than 6-fold stronger than $K_{BINDING}$ determined by directly measuring the binding of A-1 myosin subfragment 1 to actin during steady-state ATP hydrolysis. Computer modeling shows that this large difference between $K_{ATPase}$ and $K_{BINDING}$ is not compatible with $P_i$ release being the rate-limiting step in the ATPase cycle. If $P_i$ release is not rate limiting, it is possible that the ATP hydrolysis step, itself, is rate limiting. However, this predicts that, at high actin concentration, the value of the initial $P_i$ burst should be close to zero. Therefore, we measured the magnitude of the initial $P_i$ burst in the presence of actin, using both direct measurement and measurement of relative fluorescence magnitude. Our results suggest that the magnitude of the initial $P_i$ burst in the presence of actin is considerably higher than would be expected if the ATP hydrolysis step were the rate-limiting step in the ATPase cycle. However, we can explain all of our data if there is a special rate-limiting step in the cycle that follows the ATP hydrolysis step, precedes the $P_i$ release step, and occurs at about the same rate whether myosin subfragment 1 is bound to or dissociated from actin. This rate-limiting step may play an important role in determining the velocity of muscle contraction.

It is now generally accepted that muscle contraction is driven by a cyclic interaction of the two muscle proteins, actin and myosin, coupled to the hydrolysis of ATP. Considerable information is now available about the mechanism of the actomyosin ATPase activity in vitro (Adelstein & Eisenberg, 1980). Most of this information has been obtained in steady-state and pre-steady-state kinetic studies with the soluble fragments of myosin, heavy meromyosin (HMM), and subfragment 1 (S-1). However, one major question remains unresolved, the nature of the rate-limiting step in the ATPase cycle. This question is particularly important because, for a wide variety of different muscles, the rate of the actomyosin ATPase activity correlates with the speed of muscle contraction (Barany, 1967).

Until recently, it was assumed that S-1 had to detach from actin during each cycle of ATP hydrolysis (Lymn & Taylor, 1971; Eisenberg & Keilley, 1972); ATP binding was thought to irreversibly dissociate the S-1 from actin, and the ATP hydrolysis step was thought to occur only after S-1 dissociated from actin. However, recent work (Stein et al., 1979, 1981; Mornet et al., 1981) has clearly demonstrated that ATP does

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