Dityrosine Formation in Calmodulin†

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Received September 5, 1986; Revised Manuscript Received October 24, 1986

ABSTRACT: Ultraviolet (280-nm) irradiation of bovine brain calmodulin results in calcium-dependent changes in its fluorescence emission spectrum. These consist of a decline in the intrinsic tyrosine fluorescence of the protein and the appearance of a new emission maximum at 400 nm. Chromatography of irradiated calmodulin, using Ultrogel AcA 54 and phenyl-agarose columns, yields several distinctive fractions. One of these, representing 2.8% of the total recovered protein and 53% of the total fluorescence emission at 400 nm, was selected for detailed characterization. Analyses performed on acid hydrolysates reveal the presence of *dityrosine*, a derivative of tyrosine known for its fluorescence near 400 nm, at the level of 0.59–0.89 mol per 16 700 g of protein. Sodium dodecyl sulfate gel electrophoresis experiments demonstrate two components of apparent molecular weights 14 000 (80%) and 16 000 (20%). Observations on the effects of UV irradiation on the thrombic fragments of calmodulin and on related calcium binding proteins (rabbit skeletal muscle troponin C, bovine cardiac troponin C, and parvalbumin) support the interpretation that dityrosine formation in calmodulin results from the intramolecular cross-linking of Tyr-99 and Tyr-138. The dityrosine-containing photoproduct of calmodulin is unable to stimulate the *p*-nitrophenyl phosphatase activity of calcineurin under standard assay conditions. Fluorescence titrations show a generally weakened interaction with calcium ion occurring in two stages. The pK_a of the derivative is considerably higher than that of free dityrosine and is calcium dependent, decreasing from 7.88 to 7.59 on the addition of 3 mM $CaCl_2$. Smooth muscle myosin light chain kinase binds the derivative about 280-fold less effectively than it binds native calmodulin. Of several metal ions tested, only Cd^{2+} approaches Ca^{2+} in its ability to promote the appearance of the 400-nm emission band during UV irradiation of calmodulin. Mn^{2+} and Cu^{2+} appear to inhibit dityrosine formation. Ascorbic acid, dithiothreitol, and glutathione are also inhibitory.

Calmodulin is the principle known mediator of calcium in eucaryotic cells. The binding of calcium stabilizes one or more conformations of the calmodulin molecule recognized by calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase (Cheung, 1967, 1980a,b), adenylate cyclase (Cheung et al., 1975; Brostrom et al., 1975), phosphorylase kinase [Grand et al., 1981; cf. reviews by Malencik & Fischer (1983) and Chan & Graves (1984)], and myosin light chain kinase [cf. reviews by Stull (1980), Small & Sobieszek (1980), Hartshorne & Siemankowski (1981), and Perry et al. (1984)]. Calmodulin also shows calcium-dependent binding of small molecules including specific basic polypeptides [cf. review by Anderson & Malencik (1986)], phenothiazine drugs and other pharmacological agents (Levin & Weiss, 1977; Weiss et al., 1982; Prozialeck & Weiss, 1982), fluorescent dyes (LaPorte et al., 1980; Tanaka & Hidaka, 1980), and naphthalenesulfonamides [cf. review by Asano & Hidaka (1985)].

X-ray crystallographic studies of calmodulin, performed at 3.0-Å resolution, show that the molecule consists of two globular lobes connected by an exposed α-helix containing amino acid residues 65–92. Each lobe binds two calcium ions through helix–loop–helix domains similar to those occurring in other calcium binding proteins (Babu et al., 1985). The changes in conformation which accompany the binding of calcium by calmodulin have been detected in circular dichroism (Klee, 1977; Wolff et al., 1977; Crouch & Klee, 1980), nuclear magnetic resonance [Seamon, 1980; cf. review by Forsén et al. (1986)], fluorescence (Kilhoffer at al., 1981), and ultraviolet difference spectroscopy (Klee, 1977; Crouch & Klee, 1980). The chemical reactivities of specific amino acid residues in calmodulin are markedly affected by calcium binding. Limited tryptic digestion of calmodulin in the presence of calcium produces two fragments containing residues 1–77 and 78–148 while digestion in the absence of calcium results in fragments containing residues 1–106, 1–90, and 107–148 (Andersson et al., 1983; Drabikowski et al., 1977). Acylation of Lys-75 by acetic anhydride occurs 25-fold faster in the presence of saturating concentrations of calcium than in its total absence (Giedroc et al., 1985). Babu et al. (1985) suggested that amino acid side chains in the central helix of calmodulin become exposed upon the binding of calcium. Methionine residues known to be essential for the activation of cyclic nucleotide phosphodiesterase occur at positions 71, 72, and 76 (Walsh & Stevens, 1978).

Mammalian calmodulins contain two tyrosine residues, occurring in the third (Tyr-99) and fourth (Tyr-138) calcium binding domains. Tyr-99 faces into the third calcium binding site and is partially accessible to solvent while Tyr-138 points away from the fourth calcium binding loop—into a hydrophobic pocket, making contact with Phe-89 and Phe-141 (Babu et al., 1985). Tyr-138 has an anomalously high pK_a of 11.9–12, both in the presence and in the absence of calcium, while Tyr-99 has normal pK_a values of 10.1 ($+Ca^{2+}$) to 10.4 ($-Ca^{2+}$) (Klee, 1977). Tyr-138 undergoes nitration with tetranitromethane in the *presence* of calcium—but not in its absence—and acetylation with *N*-acetylimidazole in the *absence* of calcium—but not in its presence (Richman, 1978; Richman & Klee, 1978). In contrast, Tyr-99 reacts with either reagent equally well in the presence and absence of calcium. Neither the acetylation nor the nitration of calmodulin tyrosyl residues affects its ability to activate cyclic nucleotide phosphodiesterase (Walsh & Stevens, 1977; Richman, 1978; Richman & Klee, 1979). Calmodulin is also subject to tyrosine phosphorylation, catalyzed by *src* kinase in the absence of calcium (Fukami et al., 1986). However, the quantity of

†Supported by research grants from the National Institutes of Health (AM13912) and the Muscular Dystrophy Association.

0006-2960/87/0426-0695$01.50/0

^{32}P incorporated and the identity of the affected residue(s) remain undetermined.

The intrinsic fluorescence of calmodulin, which undergoes a 2.5-fold increase in quantum yield on calcium binding, is due entirely to the two tyrosyl residues (Kilhoffer et al., 1981). While recording the fluorescence spectra of calmodulin samples, we have often noted time-dependent changes consisting of a decrease in the characteristic fluorescence at 300 nm and the appearance of a new emission band at 400 nm. The results in this paper show that the fluorescence at 400 nm is due to the calcium-dependent formation of *dityrosine* during UV irradiation of calmodulin. Dityrosine cross-links in proteins, which in some cases are naturally occurring, result from the phenolic coupling of two phenoxy radicals of tyrosine [cf. review by Amadõ et al. (1984)]. Phenolic coupling can be initiated by ·OH [cf. Karam et al. (1984)] and $N_3^{\cdot}$ radicals [cf. Prütz et al. (1983)]. It can also follow the photoejection of electrons accompanying UV irradiation of phenols (Land & Porter, 1963; Dobson & Grossweiner, 1965; Joschek & Miller, 1966). Lehrer and Fasman (1967) showed that the visible blue fluorescence characteristic of poly(L-tyrosine) and copolymers of tyrosine reflects the generation of dityrosine during UV irradiation.

The experiments which follow define the conditions necessary for dityrosine formation in calmodulin. They include fractionation of the reaction mixture and characterization of the major dityrosine-containing photoproduct. Control experiments are performed with the thrombic fragments of calmodulin, with parvalbumin, with cardiac and skeletal muscle troponin C, and with poly(L-tyrosine).

Materials and Methods

Bovine brain calmodulin, prepared according to Schreiber et al. (1981), was subjected to a final purification step by using affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). This additional purification removes tryptophan-containing impurities. The calmodulin concentrations are based on $E^{1\%}_{280nm} = 2.0$ and a molecular weight of 16 680 (Watterson et al., 1980). The thrombic fragments of calmodulin were prepared according to the procedure of Wall et al. (1981), following modifications given by Andersson et al. (1983). Rabbit skeletal muscle troponin C and bovine cardiac muscle troponin C were purified according to Potter's procedure (1982) and dogfish parvalbumin according to Pechere et al. (1971).

Calcium-free calmodulin was obtained by trichloroacetic acid (10%) precipitation with solutions containing 5–10 mg/mL protein (0 °C). Following centrifugation, the resulting pellet was dissolved in 1 M tris(hydroxymethyl)aminomethane (Tris),[1] pH 8.0. After this procedure was repeated 1–2 times, the sample was dialyzed against a Chelex 100 treated buffer—usually 50 mM Mops, pH 7.5—and finally against calcium-free water. The resulting preparation contains 0.1 mol of Ca^{2+}/17 000 g, according to atomic absorption analysis. It also retains electrophoretic homogeneity and maximum activity in calcineurin or myosin light chain kinase assays. In the experiments described, the results obtained by using this preparation plus a calcium-free buffer are the same as those found with untreated calmodulin in a comparable buffer containing EDTA or EGTA. In the case of parvalbumin, troponin C, or calmodulin fragments 1–106 and 107–148, the "calcium-free" experiments were performed by using untreated proteins in buffers containing 1 mM EDTA.

Turkey gizzard myosin light chain kinase containing a single band on $NaDodSO_4$ electrophoresis was prepared essentially according to the procedure of Sobieszek and Barylko (1984). The enzyme concentrations are based on $E^{1\%}_{280nm} = 10$ and on the results of stoichiometric fluorescence titrations with calmodulin (Malencik et al., 1982; Malencik & Anderson, 1986). Porcine brain calcineurin was prepared according to Klee et al. (1983). The spectrophotometric assay for calcineurin, described by Pallen and Wang (1983), was used to check the activities of the various fractions of modified calmodulin. The assay medium contained 0.97 mg/mL *p*-nitrophenyl phosphate, ±1.7 μg/mL calmodulin (or derivative), 9.6 μg/mL calcineurin, 1.0 mM $MnCl_2$, and 50 mM Mops, pH 7.3, 25 °C. The basal activity of the enzyme was followed for 1.5 min at 405 nm (1-cm cuvette path length) using the Varian Model 635D spectrophotometer. Calmodulin was then added and the time course of the reaction followed for another 1.5-min interval. The percent stimulation obtained with the various derivatives was compared to that obtained with native calmodulin under the same conditions.

Irradiation of calmodulin was performed with the SLM-Aminco 500 SPF fluorescence spectrophotometer, following the procedure detailed in the text. The corrected fluorescence spectra and excitation spectra were also obtained with this instrument. Fluorescence measurements on the fractions obtained from Ultrogel AcA 54 and phenyl-agarose chromatography were determined with the Hitachi Perkin Elmer MPF 2A fluorescence spectrophotometer. The fluorescence anisotropies and total intensities ($I_{\parallel} + 2I_{\perp}$) reported in the titrations with calcium and/or myosin light chain kinase were obtained with the SLM 4000 fluorescence polarization spectrophotometer. Broad excitation bandwidths, usually 20 nm, were used for irradiation. However, narrow excitation bandwidths (2–3 nm) were used in subsequent analytical procedures. All fluorometers were connected to circulating constant-temperature water baths. pH titrations on samples of the major dityrosine-containing photoproduct were performed by using the Corning 125 pH meter. After pH adjustment (using 0.10 N HCl or 0.10 N NaOH, with a maximum total sample dilution of 3%) and measurement, the intensities were determined with the Hitachi Perkin Elmer MPF 2A fluorometer.

Protein samples were sent to AAA Laboratory (Mercer Island, WA) for amino acid analyses. N-terminal determinations were obtained by using the Applied Biosystems AB 50 gas-phase sequenator maintained at the Oregon State University Gene Research Laboratory. The procedure followed that of Hewick et al. (1981).

$NaDodSO_4$ gels were run on a linear 8–20% gradient mini-gel system (8 cm × 10 cm) using the proper proportion of 30% acrylamide and 0.8% bis(acrylamide). The gel buffers were essentially those of the Laemmli system (1970) except that the separating gel was made 0.75 M in Tris-HCl, pH 8.8, and the running buffer was 50 mM Tris, 60 mM boric acid, and 1 mM EDTA containing 0.1% $NaDodSO_4$. The gels were run at 150 V until the bromphenol blue tracking dye reached the gel bottom (ca. 3 h).

The gels were stained in 50% methanol and 10% acetic acid containing 0.2% Coomassie Blue R-250 for 30 min. The gels were destained in 10% methanol and 10% acetic acid. Extrapolated molecular weights were determined from the fol-

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CaM, calmodulin; MLCK, myosin light chain kinase; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; TnC, troponin C.

lowing standards: phosphorylase (97K), serum albumin (68K), actin (42K), carbonic anhydrase (29K), troponin C (18K), and parvalbumin (12K). Relative staining intensities of unknowns were compared to unmodified calmodulin of known quantity and corrected according to their dye binding ability which was directly proportional to their molecular weights. The Helena Quick Quant gel scanner was used to analyze the gel patterns.

Tris and Mops buffers were prepared by using distilled water that had been further purified with a Milli-Q reagent water system. After pH adjustment with HCl or KOH, the buffers were treated with Chelex 100 to remove traces of calcium and other metal ions. Reagent-grade KCl, $CaCl_2$, $MgCl_2$, $Mg(NO_3)_2$, $ZnCl_2$, $FeCl_3$, $MnCl_2$, and $CuCl_2$ were used in the indicated experiments. The best available grades of Tris, Mops, glutathione, dithiothreitol, ascorbic acid, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. Fatty acid free bovine serum albumin, poly(L-tyrosine) (molecular weight range 80 000), L-tyrosyl-L-tyrosine, and horseradish peroxidase were also obtained from Sigma. Dityrosine was synthesized in a reaction catalyzed by the latter enzyme and purified following the procedure of Amadò et al. (1984). Its identity and purity were confirmed by AAA Laboratory. Phenyl-agarose was purchased from Sigma Chemical Co. and Ultrogel AcA 54 fro LKB, Inc.

Results

Experimental Conditions for Dityrosine Formation in Calmodulin. Our chance discovery of dityrosine formation in calmodulin took place while we were performing repetitive scans of the fluorescence spectrum of a 0.5 mg/mL solution of bovine brain calmodulin in 10 mM Tris-HCl buffer, pH 8.0, containing 0.6 mM $CaCl_2$. After several minutes of irradiation at 280 nm, the fluorescence spectrum of the sample displayed two discrete emission maxima at 305 and 400 nm. The emission band at 305 nm is characteristic of calmodulin, but diminished in intensity when compared to the spectrum recorded at "zero" time. The new emission maximum at 400 nm, which is indicative of the singly ionized species of dityrosine [cf. Amadò et al. (1984)], represented ~30% of the final intensity of the 305-nm peak when the excitation bandpass had been fixed at 10 nm for the period of irradiation.

A range of experimental conditions was examined in order to facilitate the isolation of the major dityrosine-containing photoproduct of calmodulin and to elucidate the mechanism of the reaction. Irradiation was routinely performed in the SLM-Aminco Model 500-SPF fluorescence spectrophotometer, using a fixed excitation wavelength and band-pass of 280 and 20 nm, respectively. Reasonably uniform illumination was obtained by using 0.5–1.0 mg/mL calmodulin solutions (1.6-mL total volume in 1-cm path quartz fluorescence cuvettes). The maximum percent absorbance—at 1 mg/mL—was 37%. The solutions were stirred gently every 20 s or so with small plastic rods. All buffers were air-saturated. Experiments with buffers that had been flushed with oxygen-free nitrogen gave slightly lower yields of the species emitting at 400 nm.

Figure 1A,B demonstrates the remarkable effect of *calcium ion* on the fluorescence intensity changes occurring at both 400 and 308 nm. When calcium is absent—either in calcium-free or in EDTA-containing solutions—very little change in fluorescence takes place at either wavelength. When the calcium concentration is saturating (0.5 mM), there is a rapid increase in intensity at 400 nm which appears to approach a plateau after 3–4 min of illumination. Since prolonged exposure is accompanied by a gradual decline in intensity, the

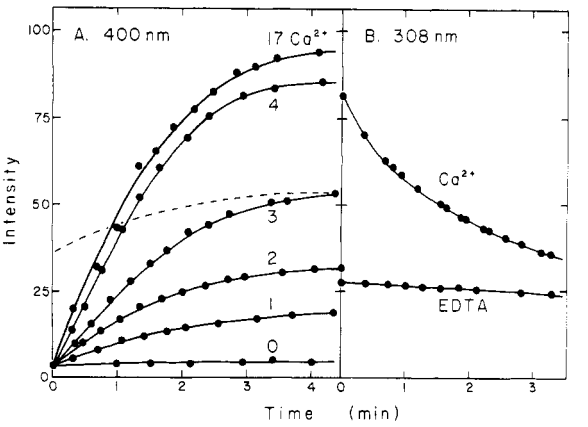

FIGURE 1: Calcium dependence of fluorescence changes occurring during UV irradiation of calmodulin. Panel A shows the fluorescence intensities observed at 400 nm with samples containing 0, 1, 2, 3, 4, and 17 mol of Ca^{2+}/mol of calmodulin. The dashed line illustrates the increase in intensity occurring when additional Ca^{2+} (0.62 mM) is added to the irradiated (5-min) sample containing 2 mol of Ca^{2+}/mol. Panel B shows the intensities determined at 308 nm with samples containing 1 mM $CaCl_2$ or 1 mM EDTA. Conditions: 0.5 mg/mL CaM in 10 mM Tris, pH 8.0 (25 °C). Excitation: 280 nm with a bandwidth of 20 nm. Emission: 400 and 308 nm, with bandwidths of 4 and 2 nm, respectively.

samples are ordinarily removed after 5 min of irradiation. The effects of substoichiometric quantities of calcium—1–3 mol of Ca^{2+}/mol of calmodulin—suggest that the reaction is most effective when all the calcium binding sites are occupied. To determine the extent to which the calcium concentration simply affects the average fluorescence yield of the product, additional calcium (0.62 mM) was added to the irradiated sample originally containing 2 mol of Ca^{2+}/mol of protein. The result is an immediate ~10% increase in intensity followed by gradual changes which fall short of those obtained with samples containing excess calcium from the outset (see dashed line in Figure 1A). This difference probably reflects additional degradation occurring during the 10-min period of irradiation plus the possible formation of alternate photoproducts at low calcium concentrations. The time courses recorded after the addition of 0.5 mM calcium to irradiated calcium-free calmodulin approach those obtained with samples that have not been exposed.

The interpretation of the intensity changes measured at 308 nm (Figure 1B) is complicated since the intrinsic fluorescence of calmodulin is affected by calcium binding—with the occupation of the two high-affinity sites accounting for most of the difference between the calcium-free and calcium-saturated protein (Kilhoffer et al., 1981). Although Figure 1B illustrates only the results obtained at the extremes of calcium concentration, we have observed decreases in intensity at 308 nm occurring over a wide range of nonzero calcium concentrations. Most of the remaining experiments were monitored at 400 nm, a wavelength which is relatively specific for dityrosine and offers the additional advantage of a negligible background.

In order to determine whether the effect of calcium is specific, we examined the influence of several other metal ions (Cd^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+}, and Cu^{2+}) on the radiation-induced fluorescence increase at 400 nm. Diminished fluorescence intensities were obtained with millimolar concentrations of Cd^{2+}, Mg^{2+}, and Zn^{2+}, consistent with the following order of effectiveness.

Ca^{2+}	>	Cd^{2+}	>>	Mg^{2+}	>	Zn^{2+}
1 mM, 100%		1 mM, 76%		2 mM, 22%		1 mM, 14%

No reaction was detected in solutions containing 1 mM Mn^{2+},

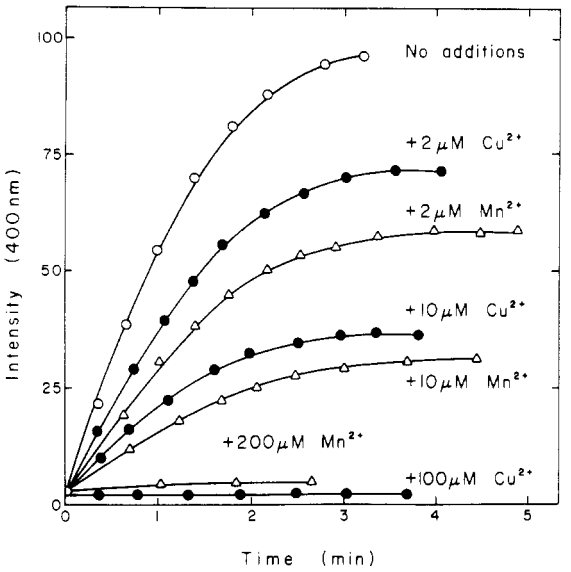

FIGURE 2: Effects of Cu^{2+} and Mn^{2+} on the calcium-stimulated increase in fluorescence at 400 nm. Conditions: 0.5 mg/mL CaM in 10 mM Tris, 1.2 mM $CaCl_2$, and the indicated concentrations of $MnCl_2$ or $CuCl_2$, pH 8.0 (25 °C). Excitation: 280 nm with 20-nm bandwidth. Emission: 400 nm with 4-nm bandwidth.

0.10 mM Fe^{3+}, or 0.10 mM Cu^{2+}. The behavior of the metal ions generally follows their abilities to support the calmodulin-dependent activity of cyclic nucleotide phosphodiesterase. Cadmium resembles calcium in both its ionic radius and its association with calmodulin (Chao et al., 1984; Cheung, 1986). However, the failure of Mn^{2+} to promote the fluorescence change was unexpected since it is efficient in enzyme activation. The addition of none of these cations affects the fluorescence intensities of previously irradiated calcium–calmodulin solutions.

Possible inhibition was investigated in a series of experiments in which calmodulin was irradiated in the presence of calcium (0.62 mM) plus a second divalent cation. Cd^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{3+} have no effect on the calcium-stimulated increase in fluorescence. However, even micromolar concentrations of Mn^{2+} and Cu^{2+} are inhibitory to the reaction detected at 400 nm (Figure 2). The fluorescence changes at 308 nm are less affected, with the rate of decrease in the presence of 1 mM Mn^{2+} being about 50% that observed in its absence. Under the conditions of the experiments, low concentrations of Mn^{2+} and Cu^{2+} are not likely to compete significantly with calcium in specific calmodulin binding. We have noted that Mn^{2+} inhibits dityrosine formation during UV irradiation of L-tyrosyl-L-tyrosine and poly(L-tyrosine).

Illumination of calmodulin in calcium-containing solutions of varying pH and ionic strength shows that the reactions detected at 400 nm occur over a wide range of conditions (Table I). Most of the apparent pH dependence is probably due to the low pK_a values of the dityrosine-containing photoproducts, as discussed later. The modest pH dependence and the fact that no detectable changes occur when the solutions are irradiated at 320 nm (using a 20-nm band-pass) indicate that the ground-state tyrosinate ion is not involved in the reactions.

Effects of Free Radical Scavengers. Cysteine and $O_2\cdot^-$ inhibit phenolic coupling by providing electrons for the repair of phenoxy radicals (Prütz et al., 1983). The addition of 1 mM glutathione or dithiothreitol to the calcium–calmodulin solutions almost completely (>90%) prevents the formation of the 400-nm-emitting species. We used Ellman's reagent [cf. Means & Feeney (1971)] to measure oxidation in samples

Table I: Effects of pH Variation on the Fluorescence Intensity Changes Accompanying the Photolysis of Calmodulin[a]

pH	additions	intensity at 400 nm
7.33	0	68
7.65	0	81
7.65	0.20 M KCl	62
8.0	0	100
8.0	0.20 M KCl	73
9.0	0	114
9.7	0	76

[a] The solutions contained 1.0 mg/mL CaM in 10 mM Tris and 0.62 mM $CaCl_2$ (25 °C). Irradiation: 5 min at 280 nm with an excitation band-pass of 20 nm.

Table II: Comparison of the Effects of Ultraviolet Irradiation on Different Proteins[a]

protein	$[Ca^{2+}]$ (mM)	intensity at 400 nm	% change in intensity at 308 nm
CaM	0.62	100	−64
	0	<1	−16
CaM 1–106	0.62	0	
CaM 107–148	0.62	0	
skeletal TnC	0.62	<1	−52
	0	<1	−21
cardiac TnC	0.62	41	
	0	5	
parvalbumin	0.62	<1	
	0	<1	
BSA	0.62	−65	
poly(L-Tyr)	0.62	100[b]	−36
	0	78	−36

[a] The solutions contained 30 μM CaM, TnC, or parvalbumin, 0.3 mg/mL BSA, or 60 μM poly(L-tyrosine) (expressed in terms of the tyrosine monomer) in 10 mM Tris, pH 8.0 (25 °C). Except for poly(L-Tyr), irradiation was performed for 5 min at 280 nm using an excitation band-pass of 20 nm. [b] The band-pass was reduced to 5 nm for poly(L-Tyr).

containing 0.5 mM glutathione, 0.5 mg/mL calmodulin, and either 0.62 mM $CaCl_2$ or zero calcium in 10 mM Tris, pH 8.0. After 5 min of illumination in either the presence or the absence of calcium, the changes in glutathione concentration were −140 ± 11 μM and −61 ± 10 μM, respectively. (The latter range of values coincided with those obtained for protein-free 0.5 mM glutathione controls, with or without calcium.) Evidently, the rate of radical formation is strongly enhanced in the calcium–calmodulin complex. Ascorbic acid is a potent inhibitor at low concentrations. When calcium-containing samples are irradiated in the presence of 5–10 μM ascorbate, no reaction occurs during the first 20 s or so of illumination. After that, the intensity at 400 nm increases rapidly—finally approaching that attained in the absence of ascorbate. The delayed reaction probably results from oxidation and depletion of ascorbate during the lag period.

Several ·OH and $N_3\cdot$ radical scavengers which inhibit dityrosine formation during γ-irradiation of tyrosine and glycyl-L-tyrosine [cf. Boguta & Dancewicz (1981)] have no effect on the fluorescence changes accompanying UV irradiation of calcium–calmodulin solutions. These agents include methanol (tested at concentrations of 10–50 mM), NaSCN (1 mM), and $Mg(NO_3)_2$ (1 mM).

Effects of UV Irradiation on Other Proteins. To further investigate the hypothesis that the 400-nm maximum in the fluorescence spectrum of irradiated calmodulin reflects the calcium-dependent cross-linking of Tyr-99 and Tyr-138, we subjected several other proteins and polypeptides to the same reaction conditions (Table II). Of these, only poly(L-tyrosine) exceeds calmodulin in the rate of fluorescence intensity change at 400 nm. However, the effect of irradiation on this synthetic polymer is only slightly calcium dependent. Of the three

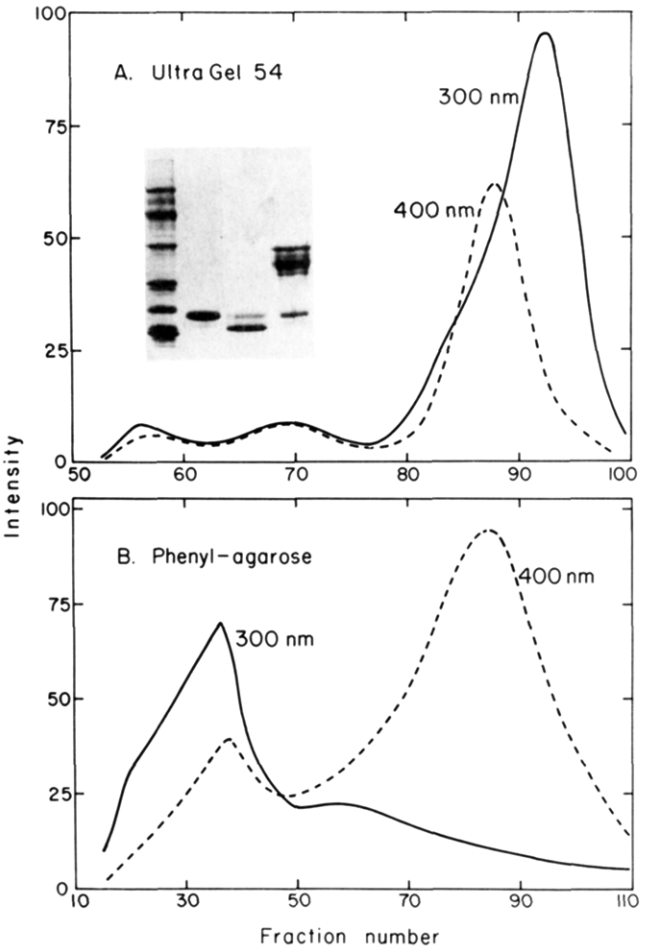

FIGURE 3: Fractionation of UV-irradiated calmodulin. Panel A shows the separation obtained upon the application of a 40-mg sample of irradiated calmodulin to an LKB Ultrogel AcA 54 sizing column (2.6 cm × 75 cm) equilibrated in 1% NH_4HCO_3 and 0.10 mM EDTA. Fractions contained 2.35 mL each. Panel B shows the separation obtained following application of combined Ultrogel AcA 54 fractions 80–98 to a phenyl-agarose column equilibrated in 1% NH_4HCO_3 and 1.0 mM $CaCl_2$. Fractions contained 1.7 mL each. (Native calmodulin elutes later, following application of 2 mM EDTA). The fluorescence intensities of the fractions were measured at 300 and 400 nm. Excitation: 280 nm with 2.5-nm bandwidth. The *inset* shows the results of $NaDodSO_4$ electrophoresis on selected fractions of UV-irradiated calmodulin. Reading from left to right: (1) standards (97K, 68K, 42K, 29K, 18K, and 12K); (2) irradiated CaM recovered from EDTA elution of phenyl-agarose column; (3) phenyl-agarose fractions 80–98; (4) Ultrogel AcA 54 fractions 65–75.

proteins which are ancestrally related to calmodulin, skeletal muscle troponin C is considered to resemble it the most. UV irradiation of rabbit skeletal muscle troponin C leads to decreases in the 300-nm emission with no changes detected 400 nm—either in the presence or in the absence of calcium. This is consistent with the fact that a phenylalanine residue occupies the sequence position of skeletal muscle troponin C which is homologous to Tyr-138 in calmodulin [cf. review by Klee & Vanaman (1982)]. Cardiac troponin C, which retains two tyrosine residues homologous to Tyr-99 and Tyr-138, continues to exhibit the distinctive calcium-dependent increase in emission at 400 nm. Irradiation of either dogfish parvalbumin or the two thrombic fragments of calmodulin, containing residues 1–106 and 107–148, results in no measurable change at this wavelength. Dogfish parvalbumin contains two tyrosine residues but has not been sequenced. However, most parvalbumins contain a phenylalanine residue in the sequence position homologous to Tyr-99 in calmodulin (Seamon & Kretsinger, 1983). The fluorescence of a solution of bovine

Table III: Amino Acid Analysis of Calmodulin Photoproducts

amino acid	nmol obtained/147 nmol of total residues with: untreated CaM	phenyl-agarose, 80–98	Ultrogel AcA 54, 54–64	Ultrogel AcA 54, 65–75
Ala	11.4 (11)[a]	11.4	11.6	11.1
Arg	5.88 (6)	5.66	6.5	5.88
Asp	23.2 (23)	22.2	18.7	23.2
Glu	26.3 (27)	27	22.8	26.2
Gly	11.4 (11)	12	12.1	11.7
His	1.02 (1)	1.10	1.83	1.05
Ile	8.72 (8)	8.52	8.50	8.81
Leu	10.2 (9)	9.93	13.0	10.6
Lys	6.80 (7)	6.72	7.56	6.92
Met	6.48 (9)	6.05	4.39	5.95
Phe	8.80 (8)	8.70	7.86	8.44
Pro	1.60 (2)	1.74	3.27	1.73
Ser	3.92 (4)	5.04	7.53	4.50
Thr	12.0 (12)	12.5	9.32	11.2
Tyr	2.15 (2)	0.55	2.67	1.16
Val	7.17 (7)	7.34	9.19	8.49
di-Tyr	0	0.59	0	0

[a] The values given in parentheses correspond to those of Watterson et al. (1980).

serum albumin, which happened to contain an impurity with the *same* intensity at 400 nM as the photoproduct(s) of calmodulin, *decreased* 65% during illumination at 280 nm.

Fractionation and Preliminary Characterization of the Photoproducts of Calmodulin. Heterogeneity of the irradiated calmodulin samples, detected in $NaDodSO_4$ gel electrophoresis, suggested that a sizing column could be useful in the isolation of dityrosine-containing photoproducts. A 40-mg pool of calmodulin that had been irradiated for 5 min at 280 nm in the presence of 0.62 mM $CaCl_2$ was lyophilized, redissolved in 5 mL of water, and dialyzed thoroughly against several changes of 1% NH_4HCO_3 solution containing 0.10 mM EDTA. The sample was then applied to an LKB Ultrogel AcA 54 column (2.6 cm × 75 cm) previously equilibrated against the same buffer. Elution was carried out at 45 mL/h, with 2.35-mL fractions collected. The fluorescence intensity of the effluent was monitored at both 300 and 400 nm, using 280-nm excitation. The elution profile in Figure 3A reveals two minor components plus two major overlapping peaks centered about fractions 88 (400-nm emission) and 92 (300-nm emission). Pools containing fractions 54–64, 65–75, and 80–98 were prepared. Following dialysis and concentration of the samples to 1.0–3.0 mg/mL, several preliminary characterizations were undertaken.

Fractions 54–64 and 65–75 are significant since their elution behavior suggests polymerization, possibly resulting from inter molecular dityrosine formation. $NaDodSO_4$ electrophoresis substantiates intermolecular cross-linking in pooled fractions 65–75 (lane 4 in inset to Figure 3), with a set of bands in the molecular weight range 28 000–40 000 accounting for 67% of the total protein in the sample. Electrophoresis of fractions 54–64 yields several poorly staining bands apparently corresponding to dimers and still higher polymers. Amino acid analyses, however, rule out dityrosine as the direct source of cross-linking in these samples (Table III). The relative yields, apparent molecular weights, fluorescence properties, and enzyme-activating abilities of both these and the fractions later obtained from chromatography on phenyl-agarose are summarized in Table IV.

Activity measurements and $NaDodSO_4$ electrophoresis experiments suggested that fractions 80–98 consist mostly of unmodified calmodulin. A phenyl-agarose affinity column (1.7 cm × 11 cm) was selected as a possible means of separating

Table IV: Characterization of Fractions of Irradiated Calmodulin

fraction	% of total protein[a]	fluorescence maxima (nm)	% of 400-nm emission[b]	activation of calcineurin (%)	mol wt from $NaDodSO_4$ electrophoresis
Ultrogel, 54–64	1.7	330	2.6	0	diffuse bands
Ultrogel, 65–75	5.2	305, 400	8.6	38	28 000–40 000 (67%)[c]
					17 000 (28%)
					11 000 (4%)
phenyl-agarose, 32–40	5.0	305, 400	7.0	0	16 000 (49%)
					14 000 (43%)
					11 000 (8%)
phenyl-agarose, 50–64	2.3		11.7	0	16 000 (42%)
					14 000 (53%)
					11 000 (4%)
phenyl-agarose, 80–98	2.8	400	53	0	16 000 (20%)[c]
					14 000 (80%)
phenyl-agarose (EDTA wash)	83	305	17	100	16 000 (95%)[c]
					14 000 (5%)

[a] Determined by Bradford method and/or amino acid analysis. [b] Measured at 400 nm in 0.02 M Na_2CO_3, pH 9.5 (λ_{ex} = 320 nm). [c] Illustrated in inset to Figure 3.

native calmodulin, which binds avidly to the medium in the presence of calcium (Gopalakrishna & Anderson, 1982), from the fluorescent derivatives. After the addition of 1.0 mM $CaCl_2$, the pooled fractions were applied to the column and washed in with a solution containing 1% NH_4HCO_3 (later changed to NH_4HCO_2) and 1.0 mM calcium. When the fluorescence intensities of the fractions (1.7 mL each) approached the background level (at about 200 mL), the bound calmodulin was eluted through the application of a 2 mM EDTA solution.

Figure 3B shows that a major 400-nm-emitting component is moderately retarded by phenyl-agarose in the presence of calcium, facilitating its separation from both native calmodulin and a lesser peak eluting ahead of it. On the basis of $NaDodSO_4$ electrophoresis and the results of the fluorescence measurements, a pool containing fractions 80–98 was selected for detailed characterization. This brilliantly fluorescent solution represents 2.8% of the total recovered protein and 53% of the total fluorescence emission at 400 nm. (The latter was determined in 0.02 M Na_2CO_3, pH 9.5). $NaDodSO_4$ electrophoresis (lane 3 in inset to Figure 3) revealed that it contains two components of apparent molecular weights of 16 000 and 14 000, accounting for 20% and 80% of the total corrected protein in the sample. No activation of calcineurin was detected (Table IV). The pooled fractions were dialyzed exhaustively against 1% NH_4HCO_3, treated with Chelex 100, lyophilized, and finally dissolved ion a small volume of calcium-free water. This 1.0 mg/mL stock solution was stored at −70 °C. The side fractions contained in tubes 65–79 and 99–110 were saved for future refractionation.

The subsequent elution of the column with EDTA yielded 24 mg of calmodulin—judged to be largely native by its ability to activate calcineurin and by its characteristic fluorescence and absorption spectra. $NaDodSO_4$ electrophoresis (lane 2 in inset to Figure 3) revealed one minor contaminant of apparent molecular weight 14 000. Some residual 400-nm fluorescence was detected in both this and all the other fractions.

The samples saved from Ultrogel AcA 54 and phenyl-agarose chromatography account for 29 mg of protein, representing 73% of the starting material.

Chemical Characterization of the Major Dityrosine-Containing Photoproduct of Calmodulin. The fluorescence excitation (Figure 4A) and emission (Figure 4B) spectra of pooled phenyl-agarose fractions 80–98 are close to those obtained with dityrosine under similar conditions. Two 5-nmol samples of the pool were placed in glass tubes containing 1 mL of 6 N HCl, sealed under N_2, and hydrolyzed for 20 h

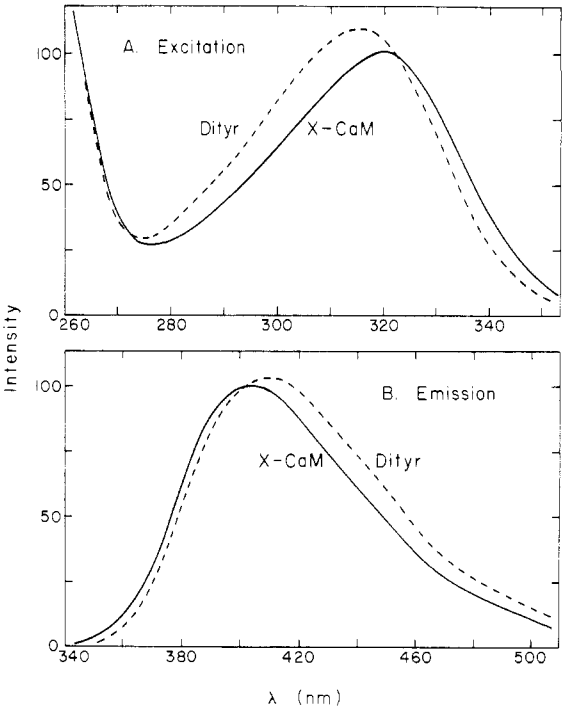

FIGURE 4: Fluorescence spectra of dityrosine and of the dityrosine-containing photoproduct of calmodulin. (Panel A) Excitation spectra (2-nm bandwidth) recorded at a fixed emission wavelength of 400 nm. (Panel B) Emission spectra (5-nm bandwidth) recorded at a fixed excitation wavelength of 320 nm. Conditions: 1.0 μM dityrosine (or derivative) in 24 mM Tris, pH 9.8 (25 °C).

at 115 °C. The hydrolysates were then diluted 8-fold with water, lyophilized, and redissolved in 0.02 M Na_2CO_3 (pH 9.5). After verifying that the excitation and emission spectra of the hydrolyzed fractions coincide with those of dityrosine, we estimated the amount present by assuming that the fluorescence intensities are proportional to concentration. (Very minimal base lines, determined on the hydrolysate of a 5-nmol sample of native calmodulin, were subtracted from both spectra.) The assumption of linearity is reasonable since low, nearly equal (1.3–1.7 μM) concentrations of the dityrosine standard and the samples were compared. The result suggests that the combined fractions contain 0.89 ± 0.03 mol of dityrosine per 17 000 g of protein.

A third 5-nmol sample was sent to AAA Laboratory for hydrolysis and amino acid analysis, which confirmed the presence of dityrosine at the level of 0.59 ± 0.04 mol/17 000 g. Except for the reduced level of tyrosine (0.55 mol/mol), the amino acid composition of this pool is otherwise close to

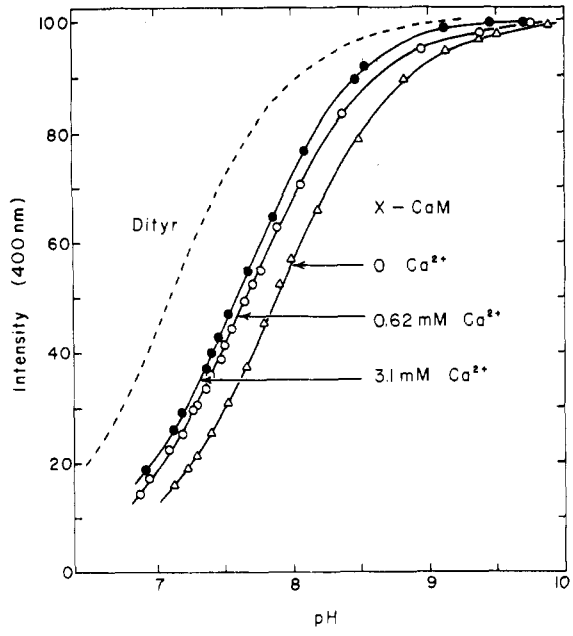

FIGURE 5: pH titrations of dityrosine and of the dityrosine-containing photoproduct of calmodulin. Conditions: 1.4 μM dityrosine (or derivative) in 2.3 mM Mops and 0.184 M KCl (24 °C) plus indicated concentrations of $CaCl_2$. Excitation: 320 nm with 2.5-nm bandwidth. Emission: 400 nm with 5-nm bandwidth.

that of unexposed native calmodulin (Table III). The range of values obtained in the two independent dityrosine determinations may reflect the limitations of the methods used. The fluorescence analysis is subject to overestimation resulting from other fluorescent species. However, the agreement of the excitation and emission spectra with those of dityrosine was excellent. Due to the additional manipulations, on the other hand, the results of amino acid analyses are subject to underestimation.

Possible peptide bond cleavage during UV irradiation of calmodulin is suggested by some of the $NaDodSO_4$ electrophoresis patterns. In order to determine whether the major dityrosine-containing photoproduct has been nicked, we performed N-terminal determinations on a 4-nmol sample using the Applied Biosystems gas-phase sequencer. Since the N-terminal residue of native calmodulin is acetylated, it undergoes no reaction with the Edman reagent. This method is expected to uncover cleavage occurring near the N-terminal end of the molecule or between the two cross-linked tyrosine residues. Considering the apparent molecular weights and the fractionation procedures used, this pool is not likely to contain fragments produced by cleavage within the midsection of calmodulin. The first cycle of reactions suggested *traces* of available amino acid residues. However, the level of the phenylthiohydantoin derivatives dropped to the base line during a second cycle of Edman degradation. Altogether, five cycles were completed. We conclude that this sample does not contain a significant proportion of nicked molecules.

pH Titrations. Low ionization constants, with pK_a values near 7, are characteristic of 2,2′-biphenols (Gross & Sizer, 1959; Lehrer & Fasman, 1967). The singly ionized species is responsible for both the 316-nm absorption and the 410-nm emission maxima of dityrosine. We have monitored pH titrations of both dityrosine and the calmodulin derivative fluorometrically, using fixed excitation and emission wavelengths of 320 and 400 nm, respectively. The results (Figure 5) show that the pK_a of the dityrosine-containing photoproduct is significantly higher than that of free dityrosine, suggesting either that the chromophore is in a nonpolar enviroment or that it undergoes stabilizing interactions with other amino acid side chains in calmodulin. The pK_a value of the calmodulin derivative is also calcium dependent, decreasing from 7.88 to 7.59 on the addition of 3 mM $CaCl_2$. Similar concentrations of calcium have no effect on free dityrosine.

Calcium Binding and Association with Smooth Muscle Myosin Light Chain Kinase. The interaction of calcium with the dityrosine-containing calmodulin derivative was examined in measurements of both fluorescence intensity and anisotropy. With a fixed pH value of 7.50, the intensity measurements are sensitive to changes in both pK_a and the fluorescence yield of the singly ionized dityrosine chromophore. The fluorescence anisotropy, $A = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, is obtained from measurements using linearly polarized exciting light. $I_{\parallel}$ and $I_{\perp}$ are the intensities of the two linearly polarized components of the light emitted at right angles to the excitation direction. The anisotropy of the fluorescent photoproduct of calmodulin should be responsive to both the overall Brownian motion of the molecule and any localized internal rotations [cf. review by Weber (1966)]. The choice of excitation and emission wavelengths excludes any direct contribution of unmodified tyrosine to the measurements. This approach is the same used with fluorescent covalent conjugates of proteins.

Figure 6 contains the results of titrations performed over a range of calcium ion concentrations, from 1 μM to 5 mM. Both the anisotropy (panel A) and intensity (panel B) measurements suggest two stages in calcium binding, a well-known phenomenon in the case of native calmodulin [cf. review by Forsén et al. (1986)]. However, in the latter instance, saturation is complete at 0.10 mM free calcium ion. The changes in anisotropy found for the derivative are consistent with increased internal rigidity and/or overall asymmetry accompanying the binding of calcium. Note that the anisotropy titrations appear to be shifted to lower calcium concentrations. This is primarily a consequence of the averaging, in which the more highly fluorescent components contribute more to the observed anisotropy. Weber (1952) demonstrated that in a mixture of fluorescent species, the average anisotropy equals the sum of the individual anisotropies weighed by the individual fractional contributions to the total fluorescence intensity.

The preceding experiment was repeated in the presence of 2 equiv of smooth muscle myosin light chain kinase, an absolutely calmodulin-dependent enzyme. Interaction of the enzyme with the derivative is evident in the overall increase in anisotropy and in the shift of the binding curve to lower calcium ion concentrations. The previously noted stages in calcium binding seem to merge after the addition of enzyme. Figure 7 illustrates the displacement occurring when a solution containing 1.0 μM derivative plus 2.0 μM myosin light chain kinase is titrated with native calmodulin. The intensity and anisotropy changes indicate that dissociation is ~92% complete on the addition of 2 μM calmodulin. By assuming that all of the enzyme is bound at this point, we estimate that the dissociation constant of its complex with the derivative is ~280-fold larger than that of its complex with native calmodulin. Under somewhat different conditions (0.20 M KCl plus 1.0 mM $CaCl_2$ were present), we determined that the latter value is 2.8 nM (Malencik & Anderson, 1986).

Discussion

Porcine brain calmodulin undergoes calcium-dependent photochemical reactions during ultraviolet (280-nm) irradiation. One particularly prominent reaction results in a new fluorescence emission maximum at 400 nm. Fractionation of the irradiated solutions yields several components including unmodified calmodulin (representing 83% of the recovered

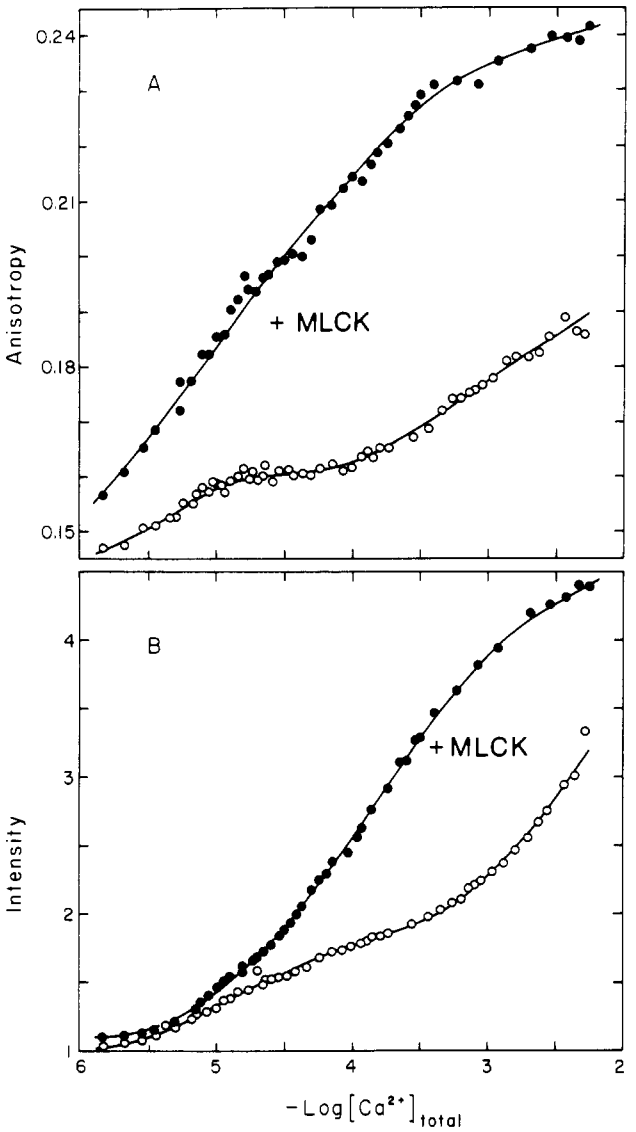

FIGURE 6: Titration of the dityrosine-containing photoproduct of calmodulin with varying concentrations (in molar) of $CaCl_2$. Panel A shows the changes in the fluorescence anisotropy of the dityrosyl chromophore. Panel B shows the changes in fluorescence intensity. Experiments were performed in both the presence (●) and absence (○) of 2 equiv of smooth muscle myosin light chain kinase. Other conditions: 1.0 μM calmodulin photoproduct and 50 mM Mops, pH 7.50 (25 °C). Excitation: 320 nm with 2-nm bandwidth. Emission: Schott KV 380 cutoff filter.

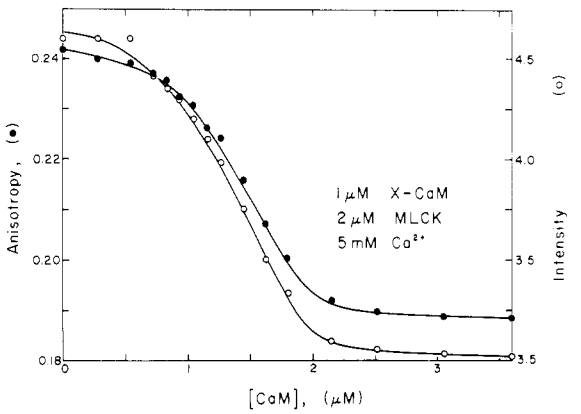

FIGURE 7: Competition between native calmodulin and its dityrosine-containing photoproduct in enzyme binding. A solution containing 1.0 μM photoproduct and 2.0 μM smooth muscle myosin light chain kinase was titrated with varying concentrations of native calmodulin, shown on the x axis. The corresponding fluorescence intensities (○) and anisotropies (●) are shown on the y axis. Conditions: 5.0 mM $CaCl_2$ and 50 mM Mops, pH 7.50 (25 °C). Excitation: 320 nm with 2-nm bandwidth. Emission: Schott KV 380 cutoff filter.

protein), a cross-linked population displaying molecular weights in the range of 28 000–40 000 (3.5%), and a fraction exhibiting 53% of the total fluorescence emission at 400 nm (2.8%). Analyses performed on acid hydrolysates demonstrate that the latter contains 0.59–0.89 mol of dityrosine/17 000 g.

The generation of dityrosine during ultraviolet irradiation of poly(L-tyrosine), copolymers, and the dipeptide Tyr-Tyr was demonstrated by Lehrer and Fasman (1967). Marked calcium dependence is an unusual feature of the reaction occurring in calmodulin. Of several cations examined, only Cd^{2+} approaches Ca^{2+} in its ability to promote increases in fluorescence at 400 nm—the emission maximum of the dityrosine-containing photoproduct of calmodulin. The results of amino acid analysis and $NaDodSO_4$ electrophoresis are consistent with the formation of an intramolecular cross-link between Tyr-99 and Tyr-138. Observations on thrombic fragments of calmodulin (containing residues 1–106 and 107–148) and on related calcium binding proteins reinforce the conclusion that the reaction depends on the availability of a pair of tyrosine residues homologous to Tyr-99 and Tyr-138. No dityrosine formation was detected with the fragments, parvalbumin, or rabbit skeletal muscle troponin C. In each of the latter proteins, one of the two tyrosine residues has been replaced with phenylalanine [cf. Klee & Vanaman (1981) and Seamon & Kretsinger (1983)]. Cardiac troponin C, on the other hand, retains the implicated pair of tyrosine residues and undergoes calcium-dependent changes in fluorescence similar to those found with calmodulin. Cross-linking of Tyr-99 and Tyr-138 is consistent with the X-ray crystallographic studies of calmodulin, which showed that the loop regions of calcium binding sites III and IV lie side by side in an antiparallel arrangement that includes hydrogen-bond formation between Ile-100 and Val-136 (Babu et al., 1985). The strong calcium dependence of dityrosine formation in calmodulin implies conformational changes accompanying calcium binding which affect the reactivity, accessibility, and/or proximity of Tyr-99 and Tyr-138.

Free radical reagents which are known to repair phenoxy radicals—dithiothreitol and glutathione—ascorbic acid strongly inhibit the changes in fluorescence detected at 400 nm. Apparent inhibition also occurs with Mn^{2+}, which is proficient in the activation of calmodulin-dependent enzymes (Chao et al., 1984; Cheung, 1984), and Cu^{2+}. These metal ions may catalyze the formation of alternate photoproducts or possibly participate in repair processes.

$NaDodSO_4$ electrophoresis on the combined peak fractions of the dityrosine-containing photoproduct reveal two components of apparent molecular weight 14 000 (80%) and 16 000 (20%). The results of amino acid analysis and N-terminal determination carried out with the gas-phase sequenator indicate that peptide bond cleavage was probably not involved in the generation of the 14K species. The mobilities of calcium binding proteins during $NaDodSO_4$ gel electrophoresis are known to depend on factors in addition to molecular weight [cf. review by Dedman & Kaetzel (1983)]. Possibly, the increased mobility reflects incomplete unfolding of the cross-linked calmodulin molecule by $NaDodSO_4$. The 400-nm-emitting components had actually migrated ahead of native calmodulin during gel filtration on LKB Ultrogel AcA 54.

Following standard procedure used in studies of fluorescent protein conjugates, we utilized the characteristic absorption and emission properties of the singly ionized dityrosyl moiety

to monitor the binding of both calcium and smooth muscle myosin light chain kinase by the internally cross-linked calmodulin molecule. The excitation and emission wavelengths used exclude the fluorescence of any residual tyrosine in the preparation. The calcium titrations reveal a generally weakened interaction occurring in two stages. Although this could reflect heterogeneity within the population, native calmodulin is known for similar behavior. ^{1}H NMR and other measurements demonstrated that calcium binding occurs first at two high-affinity sites—probably III and IV, in the C-terminal half of calmodulin—and second at two low-affinity sites—presumably I and II in the N-terminal half (Seamon, 1980; Ikura et al., 1983; Forsén et al., 1986). Perhaps the first stage in calcium binding by the photoproduct takes place at sites I and II and the second stage at cross-linked sites III and IV.

The addition of smooth muscle myosin light chain kinase shifts the calcium binding equilibrium of dityrosine-containing calmodulin to lower concentrations. Large increases in fluorescence anisotropy—expected from the rise in molecular weight accompanying the binding of the enzyme—also occur. These changes were used in competition experiments, demonstrating that the binding of cross-linked calmodulin by myosin light chain kinase is ~280-fold less effective than the binding of native calmodulin. The introduction of a cross-link between Tyr-99 and Tyr-138 may stabilize a conformation of calmodulin which is unfavorable for the binding of either calcium or the enzyme.

The pK_a of the cross-linked calmodulin derivative is considerably higher than that of free dityrosine, which has a value of 7.1. It also changes on calcium binding, decreasing from 7.88 to 7.59 on the addition of 3 mM $CaCl_2$. This suggests that the dityrosine chromophore is located in a nonpolar environment and/or undergoes interactions with other amino acid side chains affected by calcium binding.

Amino acid analysis of the intermolecularly cross-linked calmodulin fractions showed that very little dityrosine is present and that, on the average, 1 equiv of tyrosine remains. This suggests that intermolecular cross-linking is the product of a competing photochemical reaction, possibly similar to the ones involved in melanin production.

ACKNOWLEDGMENTS

We thank Reginald McParland for performing N-terminal analyses and Enoch Small for helpful discussions.

Registry No. Dityrosine, 980-21-2; cadmium, 7440-43-9; calcium, 7440-70-2; manganese, 7439-96-5; copper, 7440-50-8; ascorbic acid, 50-81-7; dithiothreitol, 3483-12-3; glutathione, 70-18-8; myosin light chain kinase, 51845-53-5.

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Hamster Female Protein, a Pentameric Oligomer Capable of Reassociation and Hybrid Formation

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Received July 21, 1986; Revised Manuscript Received October 21, 1986

ABSTRACT: Syrian hamster female protein (SFP), a serum oligomer composed of five identical subunits, was reassociated in vitro from monomer subunits. The reconstituted pentamer was genuine by morphologic, antigenic, and structural criteria. Another female protein (FP), a homologue from Armenian hamsters (AFP), also reassociated into a pentamer after dissociation with 5 M guanidine hydrochloride. These two FP's hybridized when a mixture of them was dissociated and then reassociated. Differences between the parent FP's were used to show that the recombinant pentamer contained monomer subunits from both SFP and AFP. Reassociation of both FP's was enhanced by increasing FP concentration and also by adding Ca^{2+} during reassembly. The two FP's differed in their reassociation profile in that SFP was especially efficient in reassembly, whereas AFP was more dependent upon Ca^{2+}. Female protein is a homologue of C-reactive protein and amyloid P component, and all of these proteins (pentraxins) share a similar structure. The in vitro dissociation–reassociation of female protein described herein may reflect an in vivo dissociation–reassociation which is functionally important and a common metabolic feature within this family of proteins.

C-Reactive protein (CRP)[1] is an acute-phase protein that was discovered in humans more than 50 years ago (Tillett & Francis, 1930; Abernethy & Avery, 1941). CRP represents a gene that evolved millions of years ago and has been maintained in many species with little change during evolution (Baltz et al., 1982). CRP and a homologous protein, serum amyloid P component (SAP), have been found in most animals and are known to participate in a variety of biological processes [for a review, see Kushner et al. (1982)]. Such conservation of structure and ubiquitous expression suggests that these proteins have an important function; however, a unique role has not been ascribed to them. This family of proteins (pentraxins) shares a common oligomeric structure in which five subunits (≃25 000–30 000 daltons each) are noncovalently assembled into a symmetrical cyclic pentamer (125 000–150 000 daltons) with a characteristic morphology detectable by electron microscopy. Hamster female protein (FP), a recently described member of this family, is a sex-limited homologue that shares many structural and functional features with CRP and SAP (Coe, 1983; Coe & Ross, 1985).

[1] Abbreviations: cpm, counts per minute; CRP, C-reactive protein; FP, female protein; AFP, Armenian FP; SFP, Syrian FP; [A + S]FP, mixture of AFP and SFP homologous pentamers; A*, ^{125}I-AFP; S*, ^{125}I-SFP; (A/S)FP, recombinant heterologous pentamer containing both AFP and SFP monomer subunits; HEA, hen egg albumin; PC, phosphorylcholine; PAGE, polyacrylamide gel electrophoresis; RGG, rabbit γ-globulin; SAP, serum amyloid P component; SDS–PAGE, sodium dodecyl sulfate–PAGE; SDG, sucrose density gradient; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.