

Dityrosine Formation in Calmodulin: Conditions for Intermolecular Cross-Linking[†]

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ABSTRACT: The pattern and extent of photoactivated dityrosine formation in bovine brain calmodulin are strongly affected by the presence of superoxide dismutase during UV irradiation. The addition of the enzyme to Ca^{2+} -containing solutions of calmodulin results in an altered distribution of the dityrosine-containing photoproducts, from a predominance of cross-linked monomer to a mixture of products with inter- and intramolecular cross-linking. When Ca^{2+} is absent, significant dityrosine formation occurs only in the presence of superoxide dismutase. Fractionation of the latter reaction mixture yields a dimer of calmodulin, corresponding to a single component in sedimentation equilibrium, a smaller amount of a more highly polymerized material, and virtually no cross-linked monomer—as was found in the absence of the enzyme [Malencik, D. A., & Anderson, S. R. (1987) *Biochemistry* 26, 695]. Although it is homogeneous in terms of molecular weight, the purified dimer contains three electrophoretic components. Chemical characterization demonstrates intermolecular cross-linking of Tyr-99 to Tyr-138 and probably of Tyr-99 to Tyr-99. On the average, 85–90% of the dimeric calmodulin molecules bind two molecules of smooth muscle myosin light chain kinase. Catalytic activity determinations with this enzyme detect no difference between the dimer and the native protein. Fluorescence anisotropy measurements of Ca^{2+} binding give a Hill coefficient of ≤ 0.73 and a free Ca^{2+} concentration of $\approx 11 \mu\text{M}$ at 50% saturation. The average pK_a of the dityrosine cross-link in the dimeric calmodulin is $8.5\text{--}8.6 (\pm \text{Ca}^{2+})$. Sedimentation velocity experiments show that the average Ca^{2+} -liganded dimer has an elongated structure, with a relative frictional ratio $\sim 30\%$ greater than that of the native monomer. In terms of functional properties, the dimeric calmodulin preparation is more like the native protein than it is like the previously reported cross-linked monomer.

Calmodulin, a strongly acidic protein containing 148 amino acid residues, is the major known Ca^{2+} receptor in eukaryotic cells. Diverse cellular processes are regulated through the interactions of specific enzymes with the calcium–calmodulin complex [cf. reviews by Cheung (1980), Klee (1988), Wylie and Vanaman (1988), and Means (1988)]. X-ray crystallographic studies, recently refined to 1.7 Å, show that the Ca^{2+} -liganded calmodulin molecule consists of two globular lobes connected by an eight-turn α -helix. Each lobe contains two calcium ions bound to helix–loop–helix motifs, similar to those occurring in other Ca^{2+} -binding proteins (Babu *et al.*, 1985, 1988; Chattopadhyaya *et al.*, 1992). There are no X-ray data for Ca^{2+} -free calmodulin. However, the results of a variety of physical and chemical experiments indicate that major structural changes accompany Ca^{2+} binding [cf. reviews by Forsen *et al.* (1986) and Klee (1988)].

The intrinsic fluorescence of mammalian calmodulin is Ca^{2+} -dependent (Kilhoffer *et al.*, 1981) and is entirely due to its two tyrosine residues, Tyr-99 and Tyr-138. Both residues are associated with Ca^{2+} -binding domains. Tyr-99 faces into the third Ca^{2+} -binding loop and is partially accessible to solvent, while Tyr-138 points outward from the fourth Ca^{2+} -binding site into a hydrophobic pocket containing Phe-89 and Phe-141 (Babu *et al.*, 1985). While scanning the emission spectrum of bovine brain calmodulin, we discovered that prolonged UV irradiation carried out in

the presence of Ca^{2+} leads to the *intramolecular* coupling of Tyr-99 and Tyr-138. Steady state fluorescence anisotropy measurements, employing the 400 nm emission maximum of the dityrosine chromophore, demonstrated a generally weakened interaction of the cross-linked calmodulin molecule with Ca^{2+} . Competition experiments with native calmodulin showed that the affinity of the derivative for smooth muscle myosin light chain kinase is also reduced—by a factor of 100–200-fold (Malencik & Anderson, 1987; Anderson & Malencik, 1989).¹ Little dityrosine formed when calmodulin was irradiated in the absence of Ca^{2+} .

Dityrosine cross-links in proteins result from the phenolic coupling of two phenoxy radicals of tyrosine [cf. the review by Amado *et al.* (1984)]. Phenolic coupling can be initiated by $\cdot\text{OH}$ and $\text{N}_3\cdot$ radicals [cf. Karam *et al.* (1984) and Prütz *et al.* (1983)] and by the photoejection of electrons accompanying UV irradiation (Joschek & Miller, 1966). Deducing that O_2 is the most likely acceptor of the ejected electrons, Holler and Hopkins (1989) suggested that the superoxide radical anion ($\text{O}_2^{\cdot-}$) is produced during UV irradiation of phenols. Working on the premise that $\text{O}_2^{\cdot-}$ is a source of electrons for the repair of phenoxy radicals, they showed that the photogeneration of dityrosine from tyrosine occurs most effectively when superoxide dismutase and catalase are both present. Prütz *et al.* (1983) previously

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¹ The cross-linked calmodulin monomer also shows diminished interactions with skeletal muscle myosin light chain kinase, calcineurin, and phosphorylase kinase (D. A. Malencik and S. R. Anderson, unpublished data).

found a similar effect of superoxide dismutase on the production of dityrosine during γ -irradiation.

The studies described in this article explore the patterns of intramolecular and intermolecular dityrosine formation in samples of bovine brain calmodulin that have undergone UV irradiation in the presence of bovine erythrocyte superoxide dismutase. They take into account the effects of several experimental variables: Ca^{2+} concentration, pH, ionic strength, and the addition of a calmodulin-binding peptide. The results lead to the isolation and characterization of a dimeric fraction of calmodulin that contains dityrosine.

MATERIALS AND METHODS

Proteins. 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). The calmodulin concentrations are based on $E_{280\text{nm}}^{1\%} = 2.0$ and a molecular weight of 16 680 (Watterson *et al.*, 1980). 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^2) (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 myosin light chain kinase assays.

Turkey gizzard myosin light chain kinase containing a single band on NaDodSO₄ electrophoresis was prepared essentially according to the procedure of Sobieszek and Barylko (1984). The enzyme concentrations are based on $E_{280\text{nm}}^{1\%} = 10$ and on the results of stoichiometric fluorescence titrations with calmodulin (Malencik *et al.*, 1982; Malencik & Anderson, 1986). The catalytic activity of myosin light chain kinase was assayed by the coupled, fluorometric method of Malencik and Anderson (1986). The assay medium consists of 60 μM synthetic myosin light chain kinase substrate, 0.10 mM ATP, 8.0 μM NADH, 1.0 mM PEP, 0.8 unit/mL lactate dehydrogenase, 4 units/mL pyruvate kinase, specified concentrations of calmodulin and myosin light chain kinase, 2.0 mM MgCl_2 , 0.20 mM CaCl_2 , and 50 mM Mops (K^+) (pH 7.3) at 25 °C. The rate of NADH oxidation was followed fluorometrically in the Perkin-Elmer LS 50 luminescence spectrophotometer, with fixed excitation and emission wavelengths of 348 and 455 nm, respectively.

Bovine erythrocyte superoxide dismutase, bovine liver catalase, horse radish peroxidase, and thrombin (1500–200 NIH units/mg) were obtained as lyophilized powders from Sigma Chemical Company. Rabbit muscle pyruvate kinase (type VII) was also purchased from Sigma. Chicken heart lactic dehydrogenase was prepared by the method of Pesce *et al.* (1964).

Other Reagents. Tris and Mops buffers were prepared with distilled water that had been purified further with a

Milli-Q reagent water system and treated with Chelex 100. Reagent-grade or the best available grades of chemicals were used throughout.

Mastoparan and the synthetic smooth muscle myosin light chain kinase substrate [(Lys)₂ArgProGlnArgAlaThrSer-AsnValPheSer-NH₂] were purchased from Peninsula Laboratories. ATP (disodium salt, 99–100%), phosphoenolpyruvate (tricyclohexylammonium salt), NADH (disodium salt, grade III), phenylagarose, and scopoletin were obtained from Sigma. Dityrosine was prepared according to the procedure reviewed by Amadò *et al.* (1984).

UV Irradiation. Irradiation of calmodulin was performed with the SLM-Aminco 500 SPF fluorescence spectrophotometer, following the procedure we originally described (Malencik & Anderson, 1987). Broad excitation bandwidths, usually 20 nm, were used for irradiation. Subsequent analytical procedures were performed with the Perkin-Elmer LS 50 luminescence spectrophotometer.³ Both fluorometers were connected to circulating constant-temperature water baths.

Miscellaneous Methods. NaDodSO₄ electrophoresis gels were run on a linear 8–20% gradient minigel system (8 cm \times 10 cm) using the proper proportion of 30% acrylamide and 0.8% bis(acrylamide). The gel buffers were essentially those of the Laemmli system, with the modifications previously described by us (Malencik & Anderson, 1987). The gels were run at 150 V until the bromophenol blue tracking dye reached the gel bottom (ca. 3 h). The gels were then stained in 50% methanol–10% acetic acid containing 0.2% Coomassie Blue R-250 for 30 min and destained in 10% methanol–10% acetic acid.

Amino acid analyses were carried out using reverse phase high-performance liquid chromatography according to our modification (Malencik *et al.*, 1990) of the method of Knecht and Chang (1986).

Sedimentation velocity and sedimentation equilibrium experiments were carried out with the Beckman Optima XL-A analytical ultracentrifuge. Double-sector charcoal-filled Epon cells and quartz windows were used in both types of run. Boundaries usually were monitored at 280 nm; in some cases measurements were also made at 240, 260, and 315 nm. The temperature of the rotor was maintained at 21.1 °C. The rotor speed was fixed at 15 000 rpm for sedimentation equilibrium experiments and at 40 000 rpm for the sedimentation velocity experiments. The results of the sedimentation velocity runs were analyzed by the method of van Holde and Weischet (1978). The equilibrium analysis (carried out at 33 and 48 h) used the nonlinear, least-squares fitting routine of a program developed by Borries Demeler (XL-A Version 2.41) working in concert with Origin (V3.0). The protein had been dialyzed previously against a buffer containing 0.20 M NaCl and 10 mM Mops (pH 7.3). The values of $s_{20,w}$ were corrected to the density and viscosity of water at 20 °C. A calculated partial specific volume (Perkins, 1986) of 0.728 cm³/g was employed in the computation of both $s_{20,w}$ and molecular weight.

² Abbreviations: Mops, 3-morpholinopropanesulfonic 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; NaDodSO₄, sodium dodecyl sulfate.

³ The lamp intensity and position in the SLM-Aminco 500 SPF fluorescence spectrophotometer are conducive to photochemical reactions. No dityrosine formation was detected during use of the Perkin-Elmer LS-50 luminescence spectrophotometer.

Measurements of total fluorescence intensity ($I_{||} + 2I_{\perp}$) and anisotropy $[(I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})]^4$ were obtained with the Perkin-Elmer LS-50 luminescence spectrophotometer, employing an obey program⁵ written in our laboratory by Robert L. Howard.

RESULTS

Development of Experimental Conditions for the Inter-molecular Cross-Linking of Calmodulin. To determine whether the presence of superoxide dismutase has an effect on the pattern and yield of dityrosine formation in calmodulin, we began with conditions known to favor the intramolecular cross-linking of Tyr-99 and Tyr-138 (Malencik & Anderson, 1987). Solutions (1.6 mL) of bovine brain calmodulin (15 μ M) containing 20 mM Tris, 20 mM Mops, and 0.5 mM CaCl_2 (pH 8.4) at 20 °C were irradiated in the SLM-Aminco Model 500-SPF fluorescence spectrophotometer.³ The excitation wavelength and bandpass were fixed at 280 and 20 nm, respectively. The reaction was monitored continuously at an emission wavelength of 400 nm (bandpass = 5 nm). In some cases, such as in the experiments shown in Figure 1A, the samples were removed from the cuvette compartment after the specified periods of illumination. The fluorescence intensities at 400 nm were redetermined with the Perkin-Elmer LS-50 luminescence fluorometer using an excitation wavelength of 320 nm. The change in excitation wavelength is not absolutely necessary; however, it ensures that the observed fluorescence of dityrosine is entirely due to absorption by the species that is ionized in the ground state (Lehrer & Fasman, 1967; Malencik & Anderson, 1991).

Figure 1A confirms the strong Ca^{2+} dependence of dityrosine formation in solutions of bovine brain calmodulin containing no added superoxide dismutase. When 0.5 mM CaCl_2 is present, the emission intensity determined at 400 nm gradually increases until it reaches a maximum value after 8–10 min of irradiation. In the absence of Ca^{2+} , the appearance of the characteristic 400 nm emission is strongly dependent on the addition of superoxide dismutase. Tested at equally effective concentrations of 25 and 50 μ g/mL, the enzyme facilitates increases in the fluorescence intensity of the Ca^{2+} -free samples that approach ~50% of the intensity originally found in the presence of Ca^{2+} . (Identical results were obtained with solutions prepared in Chelex 100-treated buffers, as described in Materials and Methods, and with solutions containing 0.25 mM EDTA.) When Ca^{2+} and superoxide dismutase are both present during irradiation, the intensity values are intermediate between those obtained with zero Ca^{2+} plus enzyme and those obtained with Ca^{2+} alone.

Figure 2 shows the normalized emission spectra that were recorded after 8–10 min of irradiation of two different calmodulin samples. One sample contained 0.5 mM EDTA plus 25 μ g/mL superoxide dismutase, while the other contained 0.5 mM CaCl_2 plus 1 mM EDTA. (The latter EDTA was added after irradiation in order to bring the

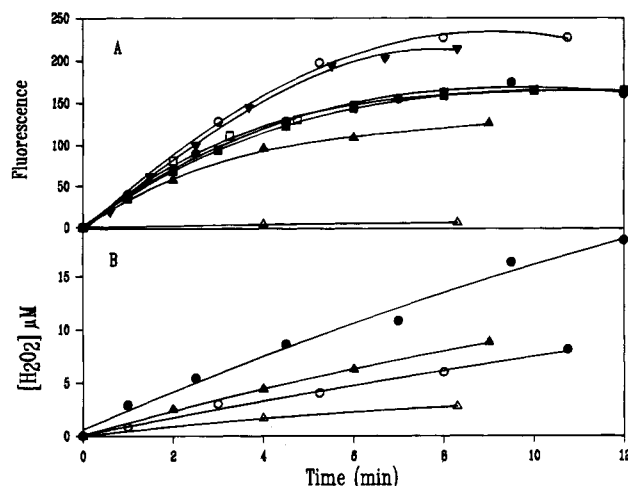


FIGURE 1: Effects of superoxide dismutase and varying Ca^{2+} concentration on dityrosine formation and H_2O_2 production occurring during UV (280 nm) irradiation of calmodulin. Panel A shows the fluorescence intensities observed at 400 nm (excitation: 320 nm) with samples containing 15 μ M calmodulin and 0.25 mM EDTA (Δ), 0.25 mM EDTA plus 25 μ g/mL superoxide dismutase (Δ), 0.5 mM CaCl_2 (\circ), 0.5 mM CaCl_2 plus 25 μ g/mL superoxide dismutase (\bullet), 0.5 mM CaCl_2 plus 10 μ M H_2O_2 added before irradiation (\blacktriangledown), 0.5 mM CaCl_2 , 25 μ g/mL superoxide dismutase, and 10 μ g/mL catalase (\blacksquare), and 0.5 mM CaCl_2 , 25 μ g/mL superoxide dismutase, and 10 μ M added H_2O_2 (\square). Panel B shows the concentrations of H_2O_2 present in the preceding samples. Conditions: 20 mM Tris and 20 mM Mops (pH 8.4) (22 °C).

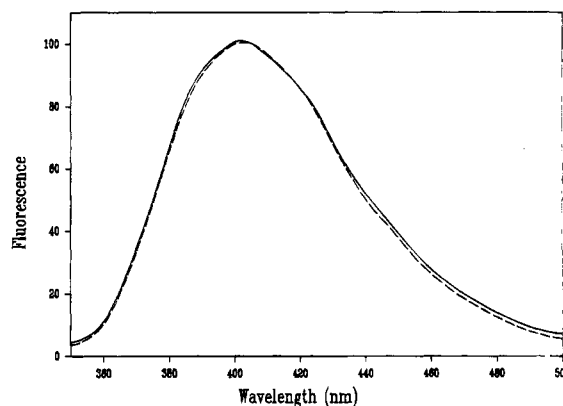


FIGURE 2: Normalized fluorescence spectra of 15 μ M calmodulin solutions that contained either 0.5 mM CaCl_2 (with 1.5 mM EDTA subsequently added) (—) or 1.0 mM EDTA plus 25 μ g/mL superoxide dismutase (---) during 280 nm irradiation. Excitation: 320 nm (3 nm bandwidth). Emission bandwidth: 5 nm. See the legend to Figure 1 for other conditions.

solutions to equivalent final conditions.) The near superposition of the spectra indicates that the same fluorescent chromophore—dityrosine—was present in both cases.

In air-saturated solutions with no competing reactions, the photogeneration of one molecule of dityrosine could be accompanied by the formation of two superoxide anion radicals. These would disproportionate: $2\text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$. We measured the concentrations of H_2O_2 present in solutions of calmodulin immediately after irradiation, employing the horseradish peroxidase-catalyzed oxidation of scopoletin (Foerder *et al.*, 1978). Control experiments, performed in the dark, show that calmodulin (15 μ M) is not a substrate for the peroxidase (10 μ g/mL): that is, no change in the concentration of added H_2O_2 (20 μ M) occurs. The results in Figure 1B demonstrate a significant accumulation of H_2O_2 in irradiated solutions of calmodulin that is

⁴ $\bar{A} = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$, where $I_{||}$ and I_{\perp} are the intensities of the two linearly polarized components of the light emitted at right angles to the excitation direction. $I_{||}$ vibrates in the direction of propagation of the exciting light, and I_{\perp} vibrates normal to the plane corresponding to the directions of excitation and observation.

⁵ Copies of the obey program are available at a cost of \$10 from Robert L. Howard, c/o Sonia R. Anderson, Dept. Biochemistry & Biophysics, Oregon State University, Corvallis, OR 97331.

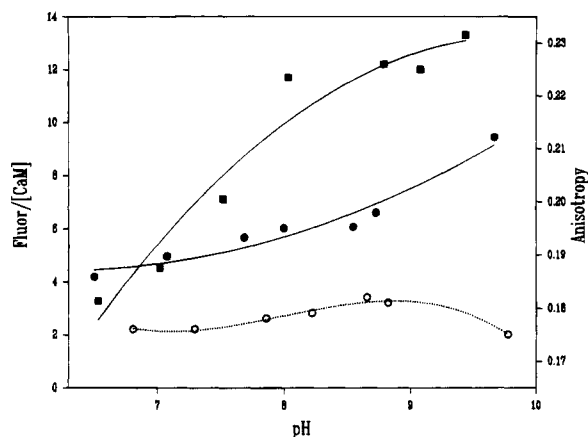


FIGURE 3: Effects of pH variation on the photoactivated generation of dityrosine in calmodulin. Samples containing either 25 μ M calmodulin plus 0.1 mM CaCl_2 (■) or 15.5 μ M calmodulin, 0.5 mM EDTA, and 25 μ g/mL superoxide dismutase (●) were irradiated at 280 nm for 8–10 min. The pH values then were adjusted to 8.3 and the illustrated fluorescence intensities determined. Excitation: 320 nm with a 3 nm bandwidth. Emission: 400 nm with a 5 nm bandwidth. The dotted line shows the results of fluorescence anisotropy measurements performed on the second sample. See the legend to Figure 1 for other conditions.

strongly dependent on the presence of both Ca^{2+} and superoxide dismutase. The interpretation of the concentrations of H_2O_2 formed will be considered in the Discussion section.

The generation of H_2O_2 during UV irradiation could influence the reactions that occur, especially if trace metal ions such as Fe^{2+} are present [cf. review by Liochev and Fridovich (1994)]. We tested the effects of both catalase and externally added H_2O_2 on three of the reaction mixtures. The zero time addition of 10 μ M H_2O_2 has a slight effect on the 400 nm emission obtained with either the solution containing 0.5 mM Ca^{2+} alone or that containing 0.5 mM Ca^{2+} plus 25 μ g/mL superoxide dismutase. The time course remains unchanged when catalase (10 μ g/mL) is added to the latter sample (Figure 1A).

The pH dependence of photoactivated dityrosine formation is distinctly different for the two extreme cases, i.e., solutions of calmodulin either containing EDTA plus superoxide dismutase or containing Ca^{2+} alone. In the latter case, the amount of dityrosine formed appears to increase by a factor of ~ 5 over the pH range 6.5–8.5. In the former case, the corresponding increase is only 1.4–1.5-fold (Figure 3). Samples possessing varying pH values had been irradiated for 8–10 min—until maximum intensity was approached. To compensate for changes in the ionization of dityrosine, all solutions were adjusted to pH 8.3 before obtaining the fluorescence intensities shown. Since different calmodulin concentrations were employed in the two experiments, the fluorescence intensities were normalized.

Chromatographic Separation of the Dityrosine-Containing Photoproducts of Calmodulin. Five different samples of UV-irradiated calmodulin were chromatographed on the same LKB Ultrogel AcA 54 sizing column (2.6 cm \times 75 cm, equilibrated in a buffer containing 0.10 M ammonium formate and 0.1 mM EDTA) used in the isolation of the dityrosine-containing monomer (Malencik & Anderson, 1987). They were as follows: (1) 10 mg of CaM previously irradiated in a solution containing 10 mM Tris–acetate and 1 mM calcium acetate (pH 8.3); (2) the same as 1 with 50

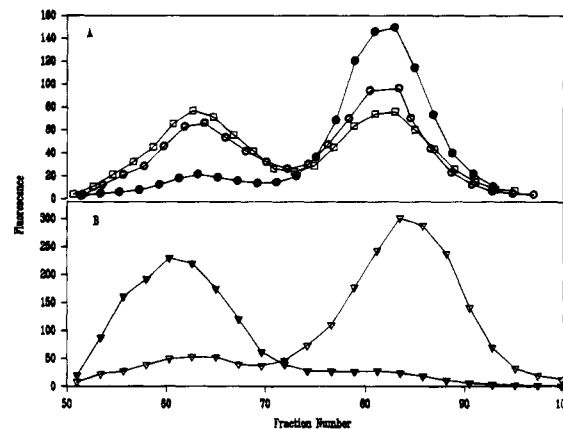


FIGURE 4: Fractionation of UV-irradiated calmodulin on an LKB Ultrogel AcA54 sizing column. Panel A shows the separation obtained upon application of 10 mg samples that had been irradiated in solutions containing 1 mM calcium acetate and 10 mM Tris–acetate (pH 8.3) (●), 1 mM calcium acetate, 10 mM Tris–acetate, and 50 μ g/mL superoxide dismutase (pH 8.3) (○), and 1 mM CaCl_2 , 0.19 M Tris–acetate, and 50 μ g/mL superoxide dismutase (□). (Results identical to the latter were obtained when 1.2 mol of mastoparan/mol of calmodulin was present during irradiation.) The fluorescence intensities were monitored at 400 nm, with an excitation wavelength of 320 nm. Fractions contained 2.4 mL each. Panel B shows the fractionation of a 27 mg sample that had been irradiated in a solution containing 20 mM Tris, 20 mM Mops, 0.5 mM EDTA, and 50 μ g/mL superoxide dismutase (pH 8.6). Fluorescence intensities were monitored at both 300 (▽) and 400 nm (▼), with excitation wavelengths of 280 and 320 nm, respectively.

μ g/mL superoxide dismutase present during illumination; (3) 10 mg of CaM from a solution containing 0.19 M Tris–acetate, 1 mM CaCl_2 , and 50 μ g/mL superoxide dismutase (pH 8.3); (4) the same as 3 plus 1.2 mol of mastoparan/mol of CaM; (5) 27 mg of CaM from a solution containing 20 mM Tris, 20 mM Mops, 0.5 mM EDTA, and 50 μ g/mL superoxide dismutase (pH 8.6). The calmodulin concentrations (present before a 10-fold reduction in solution volume by lyophilization) ranged from 29 μ M for the first four mixtures to 58 μ M for the fifth.

The elution profiles obtained with the Ca^{2+} -containing samples, monitored at 400 nm, are depicted in Figure 4A. The first sample, containing no superoxide dismutase, yields major and minor components centered about fraction numbers 81 and 62, respectively. The major component corresponds to the previously reported cross-linked monomer, which elutes slightly ahead of native calmodulin (Malencik & Anderson, 1987). The presence of superoxide dismutase during irradiation leads to a redistribution of the two components, with similar peak intensities resulting. Increasing the ionic strength of the reaction mixtures produces a small additional shift in favor of the apparent polymer. The binding of mastoparan, a 14-residue peptide with high affinity for calmodulin (Malencik & Anderson, 1983; Anderson & Malencik, 1986), has no further effect. (For simplicity, this result was not illustrated.)

Ultrogel AcA 54 chromatography of the sample that had been irradiated in the presence of EDTA and superoxide dismutase primarily yields the polymer with very little dityrosine detected in the later fractions (Figure 4B). Three different pools containing fractions 51–57, 58–67, and 75–91 were selected for further purification on phenylagarose. Each was lyophilized and redissolved in a minimum volume of buffer containing 0.1 M ammonium formate and 10 mM

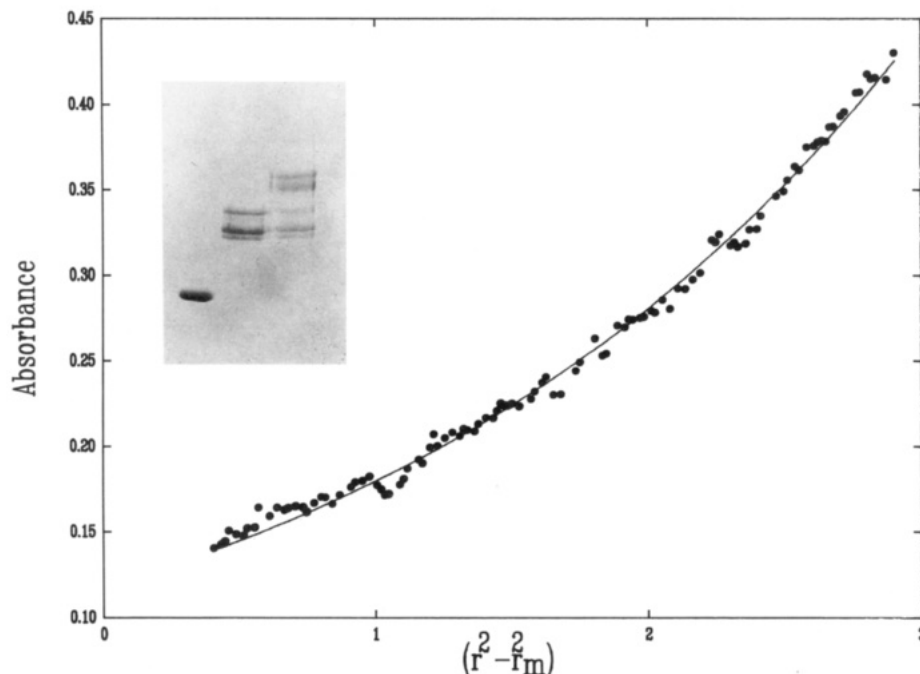


FIGURE 5: Sedimentation equilibrium of the dimeric calmodulin preparation. The absorbance monitored at 280 nm is plotted as a function of radial position, where r^2 is the square of the distance from the axis of rotation (cm^2) and r_m^2 is the square of the distance of the meniscus from the axis (cm^2). The smooth curve was calculated for a single component of molecular weight $33\,750 \pm 180$ and a baseline of 0.016. Rotor speed: 15 000 rpm. Conditions: 0.7 mg/mL calmodulin in 10 mM Mops, 0.20 M NaCl, and 0.10 mM EDTA (pH 7.3) (21.1°C). The inset shows the results of NaDodSO₄ electrophoresis performed (from left to right) on samples of native calmodulin, the purified dimer preparation, and a more polymerized side fraction (see text).

Tris Cl (pH 8.0). Sufficient CaCl_2 was added from a 0.2 M stock solution to ensure a 1.0 mM excess of CaCl_2 over the estimated residual concentration of EDTA.

Phenylagarose affinity chromatography separated the weakly interacting cross-linked monomer of calmodulin from both the native protein and nonbinding contaminants (Malencik & Anderson, 1987). An EDTA/EGTA gradient was used to elute the present column ($1.7\text{ cm} \times 11\text{ cm}$), with the mixing chamber of the gradient apparatus initially containing 1.0 mM CaCl_2 , 10 mM Tris Cl, and 0.10 M ammonium formate (pH 8.0) (68 mL) and the reservoir containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM Tris Cl, and 0.10 M ammonium formate (pH 8.0) (75 mL). Fractions containing 2.0 mL were collected. A breakthrough peak containing inactive calmodulin and varying amounts of superoxide dismutase was discarded in each case. The major dityrosine-containing component from each of the first two pools eluted from phenylagarose at the same point as native calmodulin i.e., when $[\text{EDTA}] + [\text{EGTA}] \approx [\text{Ca}^{2+}]$. The most concentrated fractions were dialyzed against several changes of 0.10 M ammonium formate and finally against water. They were then lyophilized, redissolved in a small volume of H_2O , and stored at -80°C . We recovered ~ 0.4 , 1.5, and 22 mg of protein, corresponding to Ultrogel AcA 54 fractions 51–57, 58–67, and 75–91 (cf. Figure 4B). The middle pool (58–67) is the principal subject of the characterizations that follow. The material purified from fractions 75–91 consisted largely of native calmodulin.

Analytical Ultracentrifugation and Electrophoresis. These experiments consider the homogeneity and hydrodynamic properties of the dityrosine-containing photoproduct of calmodulin that was generated in solutions containing superoxide dismutase and zero Ca^{2+} . We began by determining the molecular weight distribution, expecting to find multiples of 16 680, the molecular weight of calmodulin

(Watterson *et al.*, 1980). Sedimentation equilibrium data (Figure 5), in which the absorbance of the solution at points ranging from the meniscus to near cell bottom was monitored at 280 nm, reveal a single-component system corresponding to a molecular weight of $33\,750 \pm 180$ (reduced $\chi^2 = 0.997$). Analysis of the measurements obtained at 260 and 315 nm yields the same result. Evidently the protein that was purified from Ultrogel AcA 54 fractions 58–67 consists of one or more cross-linked dimers of calmodulin.

Sedimentation velocity measurements on the dimer give values for $s_{20,w}$ of 2.62S and 2.69S obtained with solutions that include 0.5 mM CaCl_2 and 0.5 mM EDTA, respectively. Parallel experiments with native calmodulin in the presence of 0.5 mM CaCl_2 give $s_{20,w} = 2.14\text{S}$ and 2.17S, determined at protein concentrations of 1.7 and 0.11 mg/mL, respectively. Note that differences in the values assumed for the partial specific volume (0.728 versus $0.707\text{ cm}^3/\text{g}$) largely account for differences between our sedimentation coefficients and those previously reported [cf. Klee (1980)].

We have estimated the conformational properties of the calmodulin dimer through the application of known relationships involving molecular weight, sedimentation coefficient, relative frictional ratio (f/f_0), partial specific volume, and hydration [cf. Schachman (1959) and Tanford (1961)]. When the latter two values are the same for monomer and dimer,

$$\frac{(f/f_0)_2}{(f/f_0)_1} = \frac{(s_{20,w})_1 (M_2)^{2/3}}{(s_{20,w})_2 (M_1)}$$

where $(f/f_0)_2$, $(s_{20,w})_2$, and M_2 and $(f/f_0)_1$, $(s_{20,w})_1$, and M_1 represent the relative frictional ratio, sedimentation coefficient, and molecular weight, respectively, of the dimer and monomer, respectively. The values of $s_{20,w}$ (2.62S and 2.14S)

Table 1: Amino Acid Analyses of Calmodulin Dimer and Its Cross-Linked Thrombic Fragments

amino acid	dimer ^a	cross-linked (1-106)(107-148) ^a	possible cross-linked (78-106) ^b
Asp	20.5 (23)	20.8	8.2 (5)
Glu	26.1 (27)	29.2	4.6 (5)
Ser	2.8 (4)	3.8	1.4 (2)
Thr	11.1 (12)	10.9	1.6 (1)
Gly	12.2 (11)	12.5	2.9 (2)
Ala	12.1 (11)	13.3	2.5 (3)
Pro	2.7 (2)	1.8	0.2 (0)
Val	7.5 (7)	7.0	0.6 (1)
Arg	7.4 (6)	6.8	1.4 (3)
Met	7.7 (9)	7.1	0.3 (0)
Ile	7.2 (8)	7.2	1.1 (1)
Leu	9.9 (9)	8.9	1.3 (1)
Phe	7.4 (8)	7.7	0.8 (2)
Lys	11.1 (8)	8.0	1.0 (1)
His	1.4 (1)	1.1	0 (0)
Tyr	0.43 (1)	0	0
DiY	0.50 (0.5)	0.78 (1)	0.6 (0.5)

^a nmol obtained/148 nmol of total residues. Ideal values are given in parentheses. ^b nmol obtained/29 nmol of total residues. Ideal values (Watterson *et al.*, 1980) are given in parentheses.

show that the relative frictional ratio of the calcium-liganded dimer is about 30% greater than that of the native monomer.

Although homogeneous in molecular weight, the dimeric fraction of calmodulin contains three electrophoretically distinct species corresponding to 23%, 52%, and 25% of the Coomassie Blue staining intensity (inset to Figure 5). All migrate more slowly than native calmodulin. The material purified from Ultrogel AcA 54 fractions 51-57 contains additional components of still lower electrophoretic mobility and presumably higher molecular weight.

Chemical Characterization of the Dityrosine-Containing Dimer(s) of Calmodulin. The formation of a single dityrosine cross-link between calmodulin molecules may occur in one of three ways: Tyr-99 to Tyr-99 (with 25% probability, assuming random reaction), Tyr-99 to Tyr-138 (50% probability), and Tyr-138 to Tyr-138 (25% probability). The ideal dimer would contain 1 mol of tyrosine and 0.5 mol of dityrosine per 16 680 g. The amino acid analysis carried out on a 10 μ g sample of hydrolyzed dimer confirms the presence of 0.5 mol of dityrosine, 0.42 mol of tyrosine (suggesting a loss due to alternate photochemical reactions), and usually normal quantities of the other amino acids (Table 1).

Thrombic cleavage of calmodulin in the presence of EGTA, which occurs primarily at the Arg-106-His-107 bond (Wall *et al.*, 1981), helps to establish the pattern of intermolecular cross-linking.⁶ Table 2 summarizes the cleavage products ideally expected for each of the three singly cross-linked dimers of calmodulin. We prepared a thrombic digest of a 1 mg sample of the purified dimer, following the procedure of Andersson *et al.* (1983). Application of this digest to the Ultrogel AcA 54 column previously described yielded four sets of pooled fractions: (1) tubes 71-83 encompassing a 400 nm emitting peak centered at fraction 75 that contains \sim 250 μ g of protein (determined by the Bradford assay with calmodulin as a standard); (2) tubes 59-70 containing \sim 140 μ g of protein, including undigested dimer and overlapping fractions from

Table 2: Ideal Thrombic Cleavage Products Expected for the Various Cross-Linked Dimers of Calmodulin^a

fragment	quantity of fragment expected for each type of cross-link		
	Y ⁹⁹ -Y ⁹⁹	Y ⁹⁹ -Y ¹³⁸	Y ¹³⁸ -Y ¹³⁸
(1-106)	0	1	2
(107-148)	2	1	0
(1-106) _{2X}	1	0	0
(107-148) _{2X}	0	0	1
(1-106) X (107-148)	0	1	0

^a The table shows the moles of each cleavage product expected per mole dimer. (1-105)_{2X}: represents a pair of fragments linked at Y⁹⁹. (107-148)_{2X}: fragments linked at Y¹³⁸. (1-106) X (107-148): complementary fragments linked between Y⁹⁹ and Y¹³⁸.

the adjacent peak; (3) tubes 84-101 including a 300 nm emitting peak centered at fraction 95 that contains \sim 210 μ g of protein (estimated from a fragments 1-105 standard); (4) tubes 102-119 including a 300 nm emitting peak centered at fraction 109 that contains \sim 80 μ g of protein (estimated from a fragments 106-148 standard). The latter two pools exhibited little to no dityrosine fluorescence and were assumed to consist largely of fragments 1-106 and 107-148 (confirmed by subsequent amino acid analyses).

The primary pool (tubes 71-83) was lyophilized and redissolved in H₂O, giving a solution containing 0.42 mg/mL protein. Further purification was obtained by reverse phase chromatography on a Vydac 300 \AA C₁₈ column, with monitoring at 215 nm. A gradient was formed with water and acetonitrile, each of which contained 0.1% trifluoroacetic acid. The rate of increase was 1% acetonitrile/min across the range 40-50%. Application of a 70 μ L aliquot of the primary pool resulted in peaks eluting at 40-41% acetonitrile (\sim 2 μ g), 43-44% (\sim 3 μ g), 45-46% (\sim 14 μ g), and 46-47% (\sim 5 μ g).

Amino acid analyses were performed on all four fractions. The peak eluting at 45-46% acetonitrile accounts for \sim 60% of the recovered protein and has the amino acid composition expected for a pair of cross-linked fragments containing residues 1-106 and 107-148 of calmodulin (Table 1). This result, together with the detection of fragments 1-106 and 107-148, demonstrates the presence of the intermolecular Tyr-99-Tyr-138 cross-link in the purified dimer preparation. In our experience, the variability in these analyses is greatest with the acidic residues and with arginine, due to adsorption to glass and to a reduced color yield, respectively (Malencik *et al.*, 1990).

The analyses of the other three fractions are not as clear-cut. All contain little methionine or proline, suggesting that the calmodulin sequences preceding Lys-77 or following Val-108 are absent. The amount of histidine varies, with the second most prevalent component (eluting at 46-47% acetonitrile) having none. Since calmodulin has only one histidine residue, His-107, we compared the amino acid composition of this fraction to that of fragments containing residues 1-77, 78-106, 78-148, 1-106, and 107-148 [cf. Watterson *et al.* (1980)]. The inclusion of the three additional sequences is based on the susceptibility of the Lys-77-Asp-78 bond of calmodulin to tryptic cleavage (Walsh *et al.*, 1977). Table 1 demonstrates a fair fit of sequence 78-106 to the data. In any case, the analyses for methionine, histidine, serine, and valine indicate that an intermolecular cross-link connecting Tyr-99 to Tyr-99 is more likely than

⁶ We considered CNBr digestion, but found that it destroys dityrosine.

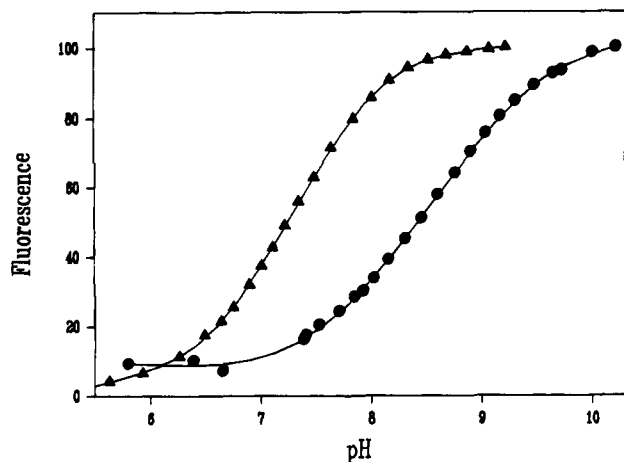


FIGURE 6: pH titrations of dityrosine (\blacktriangle) and of the dityrosine-containing calmodulin dimer preparation (\bullet). Conditions: 12 μ g/mL dimer or 0.9 μ M dityrosine in 2.2 mM Tris and 0.184 M KCl (22 $^{\circ}$ C). Excitation: 330 nm with a 3 nm bandwidth. Emission: 400 nm with a 5 nm bandwidth (see text for details).

either of the other two combinations. To save space, the amino acid analyses of the other fractions—including the non-dityrosine-containing fragments 1–106 and 107–148—are not given.

pH Titrations. Determination of the average pK_a of the dityrosine cross-links helps to establish conditions for the Ca^{2+} -binding measurements described in the next section. The results are also interesting for comparative purposes. The singly ionized species of dityrosine is responsible for both the 316 nm absorption and 410 nm emission maxima of free dityrosine (Lehrer & Fasman, 1967). We monitored pH titrations of both dityrosine and the dimer, using fixed excitation and emission wavelengths of 330 and 400 nm, respectively. Figure 6 shows that the average pK_a of the dityrosine-containing dimer is substantially higher than that of free dityrosine: 8.5–8.6 versus 7.2. In addition, the titration of the dimer suggests heterogeneity: the middle 80% of the total fluorescence change corresponds to a span of 2.2–2.3 pH units. In the case of free dityrosine, the span of 1.99 pH units is closer to the ideal value of 1.91. With the dimer, identical results were obtained in the presence and absence of Ca^{2+} . The fact that the average pK_a value is higher than that of the cross-linked monomer—7.9 (no Ca^{2+}) and 7.6 (with 3 mM $CaCl_2$) (Malencik & Anderson, 1987)—largely accounts for the differences in fluorescence intensity seen in the reaction time courses (Figure 1A).

Calcium Binding by the Cross-Linked Dimer(s) of Calmodulin. When the rotational correlation times and the excited state lifetime are similar in magnitude, the fluorescence anisotropy⁴ is sensitive to both the overall Brownian rotation and the local flexibility of macromolecules [cf. Weber (1953) and Lakowicz (1983)]. Steady state (Malencik & Anderson, 1987) and time-resolved (Small & Anderson, 1988) anisotropy measurements demonstrated the elongation and loss of segmental flexibility that accompany the binding of Ca^{2+} by the dityrosine-containing monomer of calmodulin.⁷ The addition of Ca^{2+} to a calcium-free solution of the purified dimer (13 μ g/mL) results in a gradual increase in

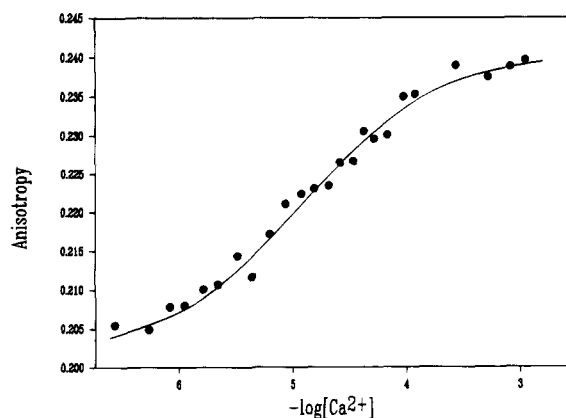


FIGURE 7: Fluorescence anisotropy of the calmodulin dimer preparation determined as a function of total $CaCl_2$ concentration (M). Conditions: 13 μ g/mL dimer in 50 mM Tris and 0.15 M NaCl (pH 8.6) (25 $^{\circ}$ C). The smooth curve was calculated as described in the text. Excitation: 320 nm (5 nm bandwidth). Emission: 400 nm (10 nm bandwidth).

anisotropy (Figure 7), showing that the measurements are still responsive to the conformational changes that occur. Since the average pK_a of the dityrosyl moiety is independent of Ca^{2+} binding, the total intensity ($I_{||} + 2I_{\perp}$) does not change.

The smooth curve drawn through the data points was calculated by the application of the principle of additivity of anisotropies (Weber, 1952) to the empirical Hill equation. It is based on the assumptions that virtually all of the Ca^{2+} is free and that ϕ , the average fractional saturation of the protein with Ca^{2+} , can be calculated from the observed anisotropy (A_{obs}) and the anisotropies of the Ca^{2+} -free dimer (A_0) and the Ca^{2+} -saturated dimer (A_c):

$$\phi = (A_{obs} - A_0) / (A_c - A_0)$$

The parameters used to fit the data were $A_0 = 0.202$, $A_c = 0.241$, n (Hill coefficient) = 0.727, and $c_{1/2}$ (concentration of free Ca^{2+} when $\phi = 0.5$) = 12.3 μ M. Estimation of the concentration of bound Ca^{2+} gives a value for $c_{1/2}$ of ≈ 11 μ M free Ca^{2+} .

Binding and Activation of Smooth Muscle Myosin Light Chain Kinase by the Cross-Linked Calmodulin Dimer(s). Smooth muscle myosin light chain kinase contains an interaction site for the fluorescent dye 9-anthroylcholine that may correspond to the ATP-binding site (Malencik *et al.*, 1982; Malencik & Anderson, 1986). Association of the enzyme with calmodulin increases its affinity for the dye, with the dissociation constant decreasing from 20 to 6.4 μ M. The resulting changes in fluorescence are useful in stoichiometric titrations of myosin light chain kinase with calmodulin.

Figure 8A illustrates the changes in dye fluorescence occurring when a solution containing 0.49 μ M enzyme, 1.0 mM $CaCl_2$, and 5.0 μ M 9-anthroylcholine is titrated with varying concentrations of native calmodulin. F_0 is the fluorescence of free dye alone. ΔF is the difference between the fluorescence intensity obtained with the enzyme–calmodulin solution and that obtained with a corresponding solution of calmodulin alone. The latter value reflects a relatively weak interaction between calmodulin and 9-anthroylcholine, corresponding to $K \sim 440$ μ M (LaPorte *et al.*, 1980). Since the dissociation constant of the enzyme–calmodulin complex is ca. 1.8 nM (Malencik & Anderson,

⁷ The cross-linked monomer has a rotational correlation time of 9.8 ns (20 $^{\circ}$ C) and an average excited state lifetime of 3.8 ns when excess Ca^{2+} is present (Small & Anderson, 1988).

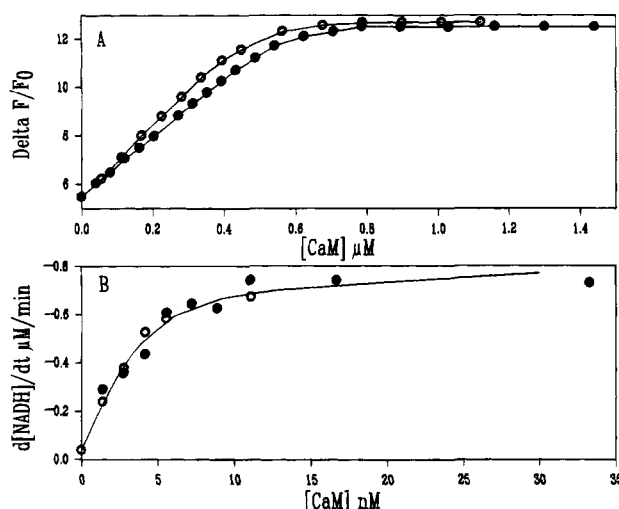


FIGURE 8: Binding and activation of smooth muscle myosin light chain kinase by the calmodulin dimer preparation. Panel A shows the stoichiometric fluorescence titration of the MLCK-9-anthroylcholine complex with native calmodulin (O) and with the calmodulin dimer preparation (●). (In each case, 1 mole is defined as 16 680 g.) Excitation: 360 nm with a 3 nm bandwidth. Emission: 460 nm with a 5 nm bandwidth. Conditions: 0.49 μM MLCK, 5.0 μM 9-anthroylcholine, 0.10 M KCl, 47 mM Mops, and 0.5 mM CaCl₂ (pH 7.3) (25 °C). Panel B shows the MLCK phosphotransferase activity determined with varying concentrations of native calmodulin (O) and with the dimer preparation (●). The smooth curve was calculated for [MLCK] = 4 nM, K_m = 1 nM calmodulin, and V_{max} = -0.775 μM NADH (or -0.775 μM ATP)/min. Details of the coupled assay system are described in the Materials and Methods.

1986), almost total binding occurs up to the addition of 1 mol of calmodulin/mol of enzyme. Repetition of this experiment with the cross-linked dimer preparation shows that 10–15% higher concentrations (with 1 mol still defined as 16 680 g) are required to reach the same fraction of the total change in fluorescence. The direct contribution of dityrosine to these measurements is minimal at the excitation (360 nm) and emission wavelengths (460 nm) employed.

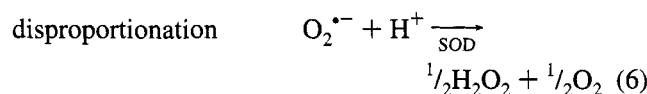
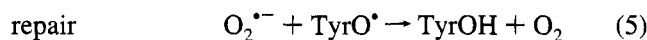
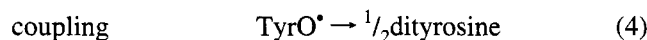
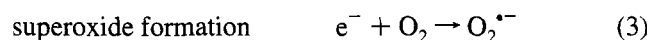
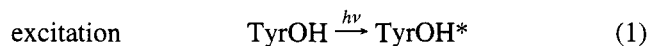
Figure 8B shows the dependence of the rate of phosphorylation of the synthetic myosin light chain kinase substrate, measured in terms of NADH oxidation in the coupled assay, on the concentrations of both native calmodulin and the dimer. Note that the two samples are virtually indistinguishable in terms of maximum activation and concentration dependence. The curve through the data points was calculated for K_{CaM} = 1 nM, [MLCK] = 4 nM, and V_{max} = -0.775 μM NADH/min.

DISCUSSION

The presence of superoxide dismutase during UV irradiation affects the extent and pattern of dityrosine formation in bovine brain calmodulin. Since mammalian calmodulin contains only two tyrosines, the number of possible dityrosine-containing products is limited. Intramolecular cross-linking of Tyr-99 and Tyr-138 occurs during 280 nm irradiation of the calcium-calmodulin complex, with almost no dityrosine formed in the absence of Ca²⁺ (Malencik & Anderson, 1987). The addition of superoxide dismutase to the solutions facilitates intermolecular cross-linking in both the presence and absence of Ca²⁺. In the latter case, the dityrosine-containing products are largely dimeric, with little cross-linked monomer detected.

Sedimentation equilibrium measurements show that the purified dimer is homogeneous in terms of molecular weight. NaDodSO₄ gel electrophoresis, on the other hand, reveals the presence of three components. Chemical characterization points to a dimer population containing primarily two kinds of intermolecular dityrosine cross-link: Tyr-99 to Tyr-138 and probably Tyr-99 to Tyr-99. The third electrophoretic component could be a product of undetected coupling of Tyr-138 to Tyr-138 or some other type of cross-linking. In all, our results indicate that the electrophoretic heterogeneity is more likely due to covalent modification rather than to differences in the binding of NaDodSO₄ [cf. Andrews (1986)] or calcium [cf. Burgess *et al.* (1980)]. The average pK_a of the dityrosine cross-link in the dimer, 8.5–8.6, is independent of Ca²⁺ and is significantly higher than the pK_a values obtained with the monomer. [The latter values were 7.9 (zero Ca²⁺) and 7.6 (3 mM CaCl₂ present) (Malencik & Anderson, 1987).] The extended pH range over which the ionization of the dimer occurs is consistent with the presence of more than one kind of dityrosine cross-link.

As summarized by Holler and Hopkins (1989), the photooxidation of tyrosine to dityrosine involves the following reactions.



Rapid removal of the superoxide radical anion in the reaction catalyzed by superoxide dismutase (eq 6) inhibits the repair process taking place in reaction 5, thus prolonging the lifetime of the tyrosyl radical (TyrO[•]) and facilitating the intermolecular coupling of calmodulin molecules (eq 4). Measurements of the production of H₂O₂ demonstrated that the disproportionation of O₂^{•−} occurs through both the enzyme-catalyzed and spontaneous reactions. Figure 1B shows that the presence of both Ca²⁺ and superoxide dismutase results in maximum H₂O₂ production. In all cases, the amount of H₂O₂ generated is in excess over the quantity of dityrosine formed. UV irradiation in the presence of Ca²⁺ alone results in ~6% conversion of calmodulin tyrosyl residues to dityrosine, corresponding to only ~0.9 μM in the present case (Malencik *et al.*, 1990). The excess of H₂O₂ produced indicates that the tyrosyl radicals participate in alternate reactions, such as other types of cross-linking and disproportionation (Prütz *et al.*, 1983). This may account for the reduced level of tyrosine characteristic of the dimer preparation (Table 1). Control experiments with catalase or added H₂O₂ indicate that the presence of the latter has little effect on dityrosine production over the 8–10 min time period covered.

The rate of increase in dityrosine formation with increasing pH is much less for solutions of calmodulin containing

EDTA and superoxide dismutase during UV irradiation than it is for solutions containing calcium and no enzyme. This distinction is greatest over the neutral pH range 6.5–8.0. It may reflect the role of H^+ in the disproportionation of $O_2^{\cdot-}$ (eq 6) and/or the ionization of tyrosyl radical species occurring during an extended lifetime. Differences in the ionization of tyrosine *per se* do not account for these effects since the fluorescence spectrum of bovine brain calmodulin ($\pm Ca^{2+}$) is independent of pH in this range (data not shown).

The strong Ca^{2+} dependence of the intramolecular coupling of Tyr-99 and Tyr-138 persists in the presence of superoxide dismutase. The near failure of these side chains to undergo intramolecular cross-linking in the absence of Ca^{2+} suggests limitations on their relative separation, spatial orientations, and/or mobilities. Their participation in the intermolecular reaction when superoxide dismutase is present confirms their inherent reactivities. Energy transfer experiments detected no effects of Ca^{2+} binding on the spatial relationship of Tyr-99 and Tyr-138 (Steiner & Montevalli-Alibadi, 1984). Nonetheless, conditions allowing the two side chains to come within reacting distance during the lifetime of the tyrosyl radical [which may be on the order of 50 μs (Grossweiner & Mulac, 1959)] are clearly evident only in the calcium–calmodulin complex. The anisotropy measurements in Figure 3 show that variations in the pH of the irradiated Ca^{2+} -free mixtures have little effect on the relative amounts of cross-linked monomer and dimer formed.

In terms of functional properties, the dimeric calmodulin preparation is more like the native protein than it is like the internally cross-linked monomer. Calcium binding, monitored in fluorescence anisotropy measurements on solutions containing 0.15 M NaCl and 50 mM Tris (pH 8.6), shows that 50% saturation occurs when $[Ca^{2+}]_{free} \approx 11 \mu M$. This midpoint compares to the average dissociation constant for the native calmodulin–calcium complex determined at pH 7.5 by equilibrium dialysis (Crouch & Klee, 1980). However, Ca^{2+} binding by the dimer occurs over an extended range of concentrations, corresponding to an upper limit of 0.73 for the Hill coefficient. This result suggests the existence of either binding heterogeneity, possibly due to the presence of more than one kind of cross-link in the population, or true negative cooperativity. Calcium binding by native calmodulin has features of both binding site heterogeneity and positive cooperativity. The dityrosine-containing calmodulin monomer showed a generally weakened interaction with Ca^{2+} occurring in two stages, with saturation still unattained when $[Ca^{2+}] = 10 \text{ mM}$ (Malencik & Anderson, 1987).

Fluorescence titrations demonstrate that, on the average, 85–90% of the dimeric calmodulin molecules bind two molecules of smooth muscle myosin light chain kinase. Catalytic activity measurements on the enzyme, which is absolutely calmodulin-dependent [cf. Hartshorne (1987)], detect no differences between the dimer and native calmodulin. The ability of a calmodulin dimer to bind two molecules of the enzyme is reasonable in view of existing structural information on the proteins.

Gizzard myosin light chain kinase contains 972 amino acid residues, with the calmodulin-binding domain encompassing residues 796–815 (Olson *et al.*, 1990). It also is an elongated molecule, with the hydrodynamic properties of a prolate ellipsoid measuring $26 \text{ \AA} \times 500 \text{ \AA}$ (Ausio *et al.*, 1992). X-ray crystallographic studies on the complex

containing a model peptide corresponding to the enzyme's calmodulin-binding domain demonstrate a compact structure, in which the N- and C-terminal domains of calmodulin engulf the α -helical form of the peptide. All eight helices of calmodulin are in close proximity to the bound ligand (Meador *et al.*, 1992). However, Tyr-99 and Tyr-138 are not among the amino acid side chains making contact with it. Our Ultrogel AcA 54 fractionation of reaction mixtures containing both Ca^{2+} and superoxide dismutase show that the presence of mastoparan [a high-affinity calmodulin-binding peptide (Malencik & Anderson, 1983; Anderson & Malencik, 1986)] has no effect on the quantities and distribution of cross-linked monomer and polymer produced during UV irradiation of calmodulin.

The highly elongated structure of myosin light chain kinase may contribute to the accessibility of the surface of bound calmodulin, leaving room for a second calmodulin molecule and the reversible binding of a second enzyme molecule. Sedimentation velocity experiments reveal that the average calmodulin dimer also has an extended structure, with a relative frictional ratio $\sim 30\%$ greater than that of the native monomer. This could represent an increase in the axial ratio from 4 to 10 [cf. Schachman (1959)].

The three-dimensional structures of the N- and C-terminal domains of calmodulin are unperturbed by peptide binding (Meador *et al.*, 1992). The relatively normal Ca^{2+} - and enzyme-binding properties of the calmodulin dimers are in marked contrast to those of the cross-linked monomer, in which Tyr-99 is coupled intramolecularly to Tyr-138. The introduction of an intramolecular cross-link may stabilize an unfavorable conformation of the C-terminal domain of calmodulin. However, the intermolecular cross-linking of these residues apparently has little effect on critical internal structure or the availability of binding surfaces.

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