# Peptide Cross-Linking to Calmodulin: Attachment of [Tyr$^8$]Substance P†

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ABSTRACT: Ultraviolet irradiation of calmodulin in the presence of calcium results in either the intramolecular cross-linking of Tyr$^{99}$ and Tyr$^{138}$ [Malencik, D. A., & Anderson, S. R. (1986) *Biochemistry 25*, 709] or, when [Tyr$^8$]substance P is bound, the generation of peptide–calmodulin adducts. The latter consist of two chromatographically distinct fractions, one of which was purified to homogeneity with phenylagarose, DEAE-Sepharose, and reverse-phase chromatography. Chemical characterization shows that the purified conjugate contains 1 mol/mol of peptide covalently attached to Tyr$^{138}$ of calmodulin. The fluorescence intensity and anisotropy of the dityrosine moiety demonstrate that this novel derivative undergoes interactions with calcium, smooth muscle myosin light chain kinase, and phenylagarose which are similar to those of unmodified calmodulin.

In vitro binding measurements, performed in our laboratory and others, demonstrated efficient calcium-dependent binding of a number of small peptides by calmodulin [cf. Anderson and Malencik (1986, 1988)]. These include adrenocorticotropin and β-endorphin (Weiss et al., 1980; Malencik & Anderson, 1982); glucagon and substance P (Malencik & Anderson, 1982); the dynorphins (Barnette & Weiss, 1983; Malencik & Anderson, 1983a, 1984); secretin, gastric inhibitory polypeptide, and vasoactive intestinal peptide (Malencik & Anderson, 1983a); melittin (Barnette et al., 1983; Comte et al., 1983; Malencik & Anderson, 1984, 1985); the mastoparans (Malencik & Anderson, 1983b, 1984, 1986a; Barnette et al., 1983); crabrolin (Anderson & Malencik, 1986); and helodermin (Malencik & Anderson, 1986b). Competition experiments indicate that the various peptides generally interact with identical or closely overlapping sites which are also related to the binding sites for phenothiazine drugs and calmodulin-dependent enzymes [cf. Anderson and Malencik (1986)]. We hypothesized that sequences similar to those found in the peptides occur in accessible positions on the surfaces of enzymes and other proteins which bind calmodulin and that they are directly involved in the interaction (Malencik & Anderson, 1982). The anticipated structural features—i.e., clusters of basic amino acid residues, associated hydrophobic sequences, and a low incidence of glutamyl residues—were recently identified in high-affinity calmodulin-binding fragments prepared from both skeletal muscle (Blumenthal et al., 1985) and smooth muscle (Lukas et al., 1986; Anderson & Malencik, 1988) myosin light chain kinases.

Although cross-linked complexes of calmodulin with β-endorphin and several of its deletion peptides have been prepared with bis(sulfosuccinimidyl) suberate (Giedroc et al., 1983, 1985), the location of the peptide binding site(s) in calmodulin is still unknown. The possibility of obtaining highly specific cross-linking without an external reagent was suggested by our discovery of *dityrosine* formation during UV irradiation of calcium-containing solutions of bovine brain calmodulin (Malencik & Anderson, 1987). The reactive phenoxy radicals generated in calmodulin are capable of coupling either with each other, as originally demonstrated, or with appropriately located tyrosyl residues of bound peptides. This paper describes the photoactivated cross-linking of calmodulin to [Tyr$^8$]substance P. It includes fractionation of the photoproducts and characterization of the adduct in which [Tyr$^8$]substance P is covalently cross-linked to Tyr$^{138}$ of calmodulin.

## Materials and Methods

Bovine brain calmodulin, prepared according to Schreiber et al. (1981), was subjected to a final purification step by affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormer, 1979). This additional purification removes tryptophan-containing impurities. The calmodulin concentrations are based on $E^{1\%}_{280nm}$ = 2.0 and a $M_r$ of 16 680 (Watterson et al., 1980). Turkey gizzard myosin light chain kinase containing a single band on NaDodSO$_4$[1] electrophoresis was prepared essentially according to the procedure of Sobieszek and Barylko (1984). The enzyme concentrations are based on $E^{1\%}_{280}$ = 10 and on the results of stoichiometric fluorescence titrations with calmodulin (Malencik et al., 1982; Malencik & Anderson, 1986c).

Irradiation of calmodulin was performed with the SLM-Aminco 500 SPF fluorescence spectrophotometer, following the procedure used in the cross-linking of Tyr$^{99}$ and Tyr$^{138}$ (Malencik & Anderson, 1987). The corrected fluorescence spectra and excitation spectra were also obtained with this instrument. Broad excitation bandwidths, usually 20 nm, were used for irradiation. However, narrow excitation bandwidths (2–3 nm) were used in subsequent analytical procedures. Fluorescence measurements on the fractions obtained from the various chromatographic steps were determined with the Hitachi Perkin-Elmer MPF 2A fluorescence spectrophotometer. The fluorescence anisotropies and total intensities reported in the titrations with calcium and myosin light chain kinase were obtained with the SLM 4000 fluorescence polarization spectrophotometer. All fluorometers were connected to circulating constant-temperature water baths. pH titrations

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[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; SP, substance P.

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on samples of the major dityrosine-containing photoproduct were performed with 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 7%), the intensities were determined with the Hitachi Perkin-Elmer MPF 2A fluorometer.

Amino acid analyses were conducted both by AAA Laboratory (Mercer Island, WA) and by us, following the procedure of Knecht and Chang (1986). Microsequencing (Hewick et al., 1981) was performed with the Applied Biosystems AB 50 gas-phase sequenator maintained at the Oregon State University Center for Gene Research and Biotechnology.

$NaDodSO_4$ gels were run on a linear 8–20% gradient minigel 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, with modifications described by us (Malencik & Anderson, 1987). The gels were run at 150 V until the bromphenol blue tracking dye reached the gel bottom (ca. 3 h). Electrophoresis under nondenaturing conditions used a 13.75% gel with a 0.125 M Tris–phosphate, pH 5.5, stacking gel buffer and a 0.175 M Tris–phosphate, pH 7.5, separating gel buffer as described by Williams and Reisfeld (1964). This system was reported to maintain a pH of 8.0 during separation. 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. The Helena Quick Quant gel scanner was used to analyze the gel patterns.

Tris and Mops buffers were prepared with distilled water that had been further purified with a Milli-Q reagent water system and treated with Chelex 100. Reagent-grade or best available grades of chemicals were used throughout. DEAE-Sepharose DCL-6B and phenylagarose were purchased from Sigma Chemical Co., and Ultrogel AcA 54 was purchased from LKB, Inc. [$Tyr^8$]substance P was obtained from Vega Biotechnologies, Inc., and the synthetic smooth muscle myosin light chain kinase substrate ($Lys_2$)-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-$NH_2$ from Peninsula Laboratories. Bovine thrombin was a gift from Earl Davie.

## Results

*Preparation of [$Tyr^8$]SP–Calmodulin Adducts.* Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$) served as a prototype in our search for high-affinity calmodulin-binding peptides (Malencik & Anderson, 1982; Anderson & Malencik, 1986, 1988). The tyrosine-containing analogue of substance P, [$Tyr^8$]SP, offers several advantages for photochemical cross-linking experiments: small size, resistance to cleavage by trypsin or cyanogen bromide, and commercial availability at a moderate cost. Its concise structure ensures that coupling will occur only to tyrosyl residues that reside within or very close to the peptide binding site of calmodulin. Fluorescence titrations, following procedures described by Malencik and Anderson (1982), showed that [$Tyr^8$]SP forms a 1:1 calcium-dependent complex with dansylcalmodulin corresponding to a dissociation constant of 7.1 μM in 0.20 M KCl, containing 50 mM Mops and 1 mM $CaCl_2$, pH 7.3, and of 0.87 μM in 15 mM Tris containing 1 mM $CaCl_2$, pH 8.7.

Solutions (1.6 mL) of calmodulin (30 μM) and [$Tyr^8$]SP (35 μM) were illuminted under conditions (1 mM calcium acetate, 12 mM Tris-HCl, pH 8.7, 15 °C) conducive to intramolecular dityrosine formation. Irradiation was routinely performed in the SLM-Aminco Model SPF-500 fluorescence spectrophotometer, with fixed excitation wavelength and band-pass of 280 nm and 20 nm, respectively. Under these conditions, about 65% of the solution is under illumination at once. Mixing was achieved through occasional stirring. The time course of the reaction, monitored at an emission wavelength of 400 nm, was similar to that obtained with calmodulin in the absence of peptide. That is, the fluorescence increased rapidly—approaching a plateau after about 4 min of reaction (Malencik & Anderson, 1987). However, the final intensity was consistently 25–30% greater than that previously noted. This difference suggested an effect of [$Tyr^8$]SP on the course of the reaction which proved to be profound.[2] The inclusion of 30 μM mastoparan [a high-affinity calmodulin-binding peptide for which $K_d$ = ~0.3 nM (Malencik & Anderson, 1983b)] in the reaction mixture obviated the effect of [$Tyr^8$]SP. No fluorescence changes were detected in comparable solutions containing 1 mM EDTA, with no added calcium, or in solutions of [$Tyr^8$]SP alone plus calcium.

A 36-mg pool of calmodulin which had been irradiated for 5 min at 280 nm in the presence of [$Tyr^8$]SP and calcium was lyophilized, redissolved in 8 mL of water containing 20 mM EDTA, and applied to an LKB Ultrogel AcA 54 column (2.6 cm × 75 cm) previously equilibrated with a 1% $NH_4HCO_3$ solution containing 0.1 mM EDTA. This sizing column facilitates the separation of calmodulin from both unreacted [$Tyr^8$]SP and possible polymerization products of calmodulin (Malencik & Anderson, 1987). The fractions were monitored fluorometrically, with an excitation wavelength of 280 nm and emission wavelengths of 300 and 400 nm. Emission at 300 nm is indicative of tyrosine while that at 400 nm is characteristic of the singly ionized dityrosine chromophore. Under these conditions, ionization occurring in the excited state permits the fluorometric detection of dityrosine in solutions having pH values below the ground-state $pK_a$ (Lehrer & Fasman, 1967). Maximum emission at 400 nm coincided with the calmodulin-containing fractions, which were dialyzed against a solution containing 1% ammonium formate, 1 mM Tris-HCl, and 1 mM calcium acetate, pH 8.0, in preparation for affinity chromatography on a phenylagarose column (1.7 cm × 17 cm).

Weak adsorption of the intramolecularly cross-linked dityrosine derivative of calmodulin to phenylagarose permitted its separation from both native calmodulin and alternate photoproducts (Malencik & Anderson, 1987). However, the species generated in the presence of [$Tyr^8$]SP are avidly bound by phenylagarose. When no significant quantities of protein were detected after the collection of 240 mL of effluent, we applied a linear EGTA gradient—placing 100 mL of 3 mM EGTA in the reservoir and 100 mL of 1 mM calcium acetate in the mixing chamber of the gradient apparatus. (These solutions also contained 1% ammonium formate and 1 mM Tris-HCl, pH 8.0.) Two 400 nm emitting peaks plus overlapping fractions of unreacted calmodulin were subsequently eluted (Figure 1A).

Further purification of the dityrosine-containing components was approached with ion-exchange chromatography on a 1.6 cm × 11 cm column of DEAE-Sepharose DCL-6B. A pH of 6.5 was used in order to minimize the negative charge of dityrosine and to maximize the positive charge of bound peptide. Pools containing phenylagarose fractions 35–40 (I) and 41–46 (II) were adjusted to pH 6.5 and *processed separately*. When a linear gradient of 0.15–0.35 M NaCl (90 mL each) in 20 mM Mops, pH 6.5, failed to elute substantial quantities of the protein, a new gradient of 0.35–0.45 M NaCl (90 mL each) was established. The resulting fractionation of

[2] Irradiation of calmodulin in the presence of substance P results in an increase in fluorescence which equals that obtained in the absence of the peptide.

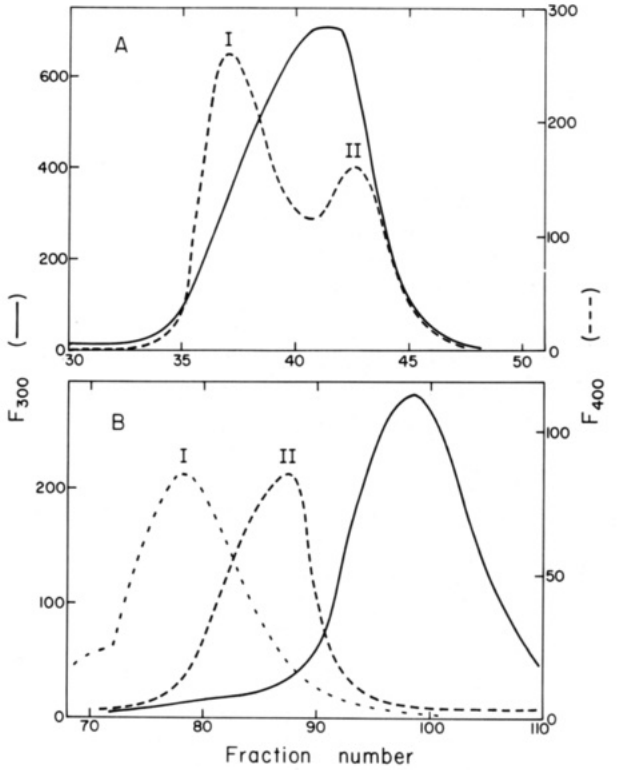


FIGURE 1: Chromatography of calmodulin–[Tyr$^8$]substance P adducts. Panel A shows the fractionation obtained upon application of a 0–3 mM EDTA gradient to the phenylagarose-bound material, as detailed under Results. The fluorescence intensities of the fractions, which contained 2.39 mL each, were determined at 300 (—) and 400 nm (--) with an excitation wavelength of 280 nm. Panel B shows the resolution obtained with DEAE-Sepharose DCL-6B chromatography of combined phenylagarose fractions 41–46 (pool II). Fractions contained 2.25 mL each. A linear NaCl gradient (0.35–0.45 M) was established at fraction 73, as described under Results. [The elution of the 400 nm emitting components of pooled phenylagarose fractions 35–40 (I), *determined in a separate run*, is also illustrated.]

pool II is illustrated in Figure 1B, where a lightly dashed curve has been superimposed to show the corresponding elution of the 400 nm emitting species of pool I. Fractions 82–88 from pool II and 74–84 from pool I were combined and saved. These represented ~550 and ~1300 μg of protein, respectively.[3] Side fractions were also kept. Altogether, 24 mg of essentially native calmodulin was recovered from tubes 92–110.

$NaDodSO_4$ electrophoresis demonstrated that the DEAE-Sepharose-purified pool II fractions are nearly homogeneous, with a major band accounting for 82% of the total protein (as indicated by Coomassie blue staining), while pool I contains equal amounts of two resolvable components (Figure 2A). Since the coupling of [Tyr$^8$]SP to calmodulin would add positive charge, we also ran a normal gel, in which separation occurs at pH 8.0 (Williams & Reisfeld, 1964). Pool II fractions proved to contain 82% or more of a major component which migrates more slowly than calmodulin (Figure 2B). Three bands—representing 37%, 39%, and 24% of the sample (read from top to bottom)—were detected in pool I.

Final purification of pool II was achieved through reverse-phase chromatography on a Vydac 300-Å $C_{18}$ column. A gradient was formed with water and acetonitrile, each of which contained 0.1% trifluoroacetic acid. The first peak,

[3] The recovery of the internally cross-linked dityrosine derivative of calmodulin was 1 mg per 40 mg of starting material. Key factors determining the yield include the photosensitivity of dityrosine, the accumulation of inhibitory products during prolonged irradiation, and the efficiency of the purification procedures.

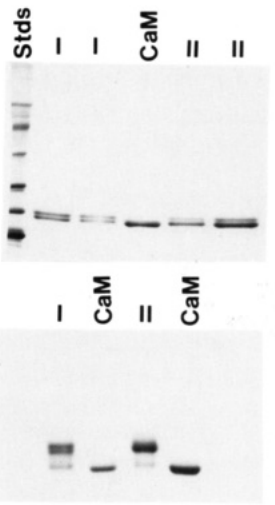


FIGURE 2: Electrophoresis of calmodulin–[Tyr$^8$]substance P adducts. The upper photograph shows the results of $NaDodSO_4$–polyacrylamide gel electrophoresis performed on native calmodulin, on purified pools I (DEAE fractions 74–84) and II (DEAE fractions 82–88), and on molecular weight standards (97K, 68K, 42K, 29K, 18K, and 12K). The lower photo shows the results of electrophoresis under nondenaturing conditions, with a pH of 8.0 maintained during separation. Refer to Materials and Methods for details.

representing 5% of the total absorbance at 280 nm, was eluted at 44% acetonitrile. The second peak, corresponding to 95% of the absorbance, was eluted at 47% acetonitrile. Repetition of the electrophoresis experiments on lyophilized samples of the major peak confirmed the removal of the minor component that had been present after DEAE-Sepharose chromatography. The functional properties of the purified adduct are the same as those of pool II prior to HPLC.

*Chemical Characterization.* The objectives of chemical characterization were to determine whether [Tyr$^8$]SP is covalently bound to calmodulin, to confirm the presence of the dityrosine cross-link, and to identify the affected tyrosine residues of calmodulin.

Microsequencing using the gas-phase sequenator is a sensitive way of detecting covalently bound peptide since the acetylated N-terminus of calmodulin does not react with the Edman reagent. Analyses on 1-nmol samples from both purified pool II and unfractionated pool I were performed on the Applied Biosystems AB 50 gas-phase sequenator, according to standard procedures (Hewick et al., 1981). Identification of the phenylthiohydantoin derivatives obtained in 11 reaction cycles yielded the following sequence in both cases:

Arg Pro Lys Pro Gln Gln Phe --- Gly Leu Met

The complete absence of a detectable phenylthiohydantoin in the eighth reaction cycle confirmed the suspected cross-linking, in which Tyr$^8$ would remain attached to calmodulin.

Table I shows the results of amino acid analyses performed on HPLC-purified pool II. Comparison of these values to the amino acid composition of unmodified calmodulin points to the incorporation of 1 mol of peptide/mol of protein. That is, one tyrosine residue is lost while 1 mol/mol each of dityrosine, arginine, lysine, leucine, and phenylalanine plus two residues of proline is gained. The contribution of the peptide is clearest when the background composition of calmodulin is low, as it is for proline. The results are less definitive in the case of glutamic acid, glycine, or methionine.

In order to determine the distribution of bound peptide between Tyr$^{99}$ and Tyr$^{138}$ of calmodulin, we performed a thrombic digestion on a 150-μg sample of pool II, following the procedure of Andersson et al. (1983). Thrombic cleavage

Table I: Amino Acid Composition of Peptide–Calmodulin Conjugates

| amino acid | untreated CaM[a] | [Tyr[8]]SP–CaM[b] | thrombic frag of [Tyr[8]]SP–CaM[c] |
|---|---|---|---|
| di-Tyr | 0 (0) | 0.88 | 0.86 (0) |
| Tyr | 2.3 (2) | 1.12 | 0 (1) |
| Pro | 2.2 (2) | 4.24 | 1.7 (0) |
| Arg | 5.9 (6) | 6.95 | (1) |
| Lys | 8.4 (7) | 9.4 | 2.3 (1) |
| Phe | 8.3 (8) | 9.21 | 1.9 (1) |
| Leu | 10.1 (9) | 10.9 | 3.2 (2) |
| Gly | 11.4 (11) | 13.0 | 5.1 (3) |
| Met | 9.3 (9) | 8.95 | 4.8 (4) |
| Glx | 25.6 (27) | 24.7 | 12.0 (9) |
| Asx | 22.0 (23) | 23.4 | 7.6 (7) |
| Ser | 4.1 (4.0) | 4.32 | 0.2 (0) |
| Thr | 10.4 (12) | 11.7 | 3.0 (3) |
| Ala | 11.6 (11) | 12.9 | 2.9 (2) |
| Val | 7.1 (7) | 7.34 | 3.1 (4) |
| Ile | 7.6 (8) | 7.91 | 1.6 (2) |
| His | 0.8 (1) | 0.90 | 0.92 (1) |

[a] Nanomoles obtained per 147 nmol of total residues after a 20-h hydrolysis. The values given in parentheses correspond to those of Watterson et al. (1980). [b] Nanomoles obtained per 157 nmol of total residues after a 20-h hydrolysis. [c] Nanomoles obtained per 51 nmol of total residues after a 15-h hydrolysis. The numbers given in parentheses describe the composition of calmodulin sequence 107–148.

of calmodulin in the absence of calcium generates two major fragments containing amino acid residues 1–106 and 107–148 (Walsh et al., 1981). Reverse-phase chromatography of the proteolytic digest, following the procedure used in the preceding section, yielded two major components eluting at 40% and at 46% acetonitrile. The latter contains no dityrosine and is N-terminal in origin. Amino acid analysis of the first peak shows that it contains dityrosine plus the expected amino acid residues of the peptide and calmodulin sequence positions 107–148 (Table I). (The arginine content of this fragment has been omitted from the table since close overlap with the methionine peak prevented its accurate determination.) We conclude that the purified pool II fraction is labeled primarily at $Tyr^{138}$.

*Functional Characterization.* Phenolic coupling enjoys a dual advantage as a tool for protein chemists in that the dityrosine chromophore is a sensitive probe for monitoring the effects of cross-linking. We selected smooth muscle myosin light chain kinase for the characterization of the conjugate since we have experience in its interactions with other derivatives of calmodulin. We used the characteristic absorption and emission properties of the singly ionized dityrosine chromophore to study the binding of both calcium and smooth muscle myosin light chain kinase by the internally cross-linked calmodulin molecule (Malencik & Anderson, 1987). The measurements were performed at an excitation wavelength of 320 nm, a region in which ionized dityrosine absorbs maximally while unionized dityrosine and tyrosine do not absorb (Lehrer & Fasman, 1967). Under these conditions, any residual unreacted calmodulin makes no direct contribution to the fluorescence measurements.

The corrected emission spectra (Figure 3, inset) of pools I and II differ only slightly from those obtained with the intramolecularly cross-linked calmodulin derivative. pH titrations, monitored at 400 nm, were performed in order to identify favorable conditions for binding measurements (Figure 3). The results obtained with purified pool II are consistent with a homogeneous equilibrium over most of the range, corresponding to *ground-state* $pK_a = 8.8$ in both the presence and absence of calcium. As discussed later, this value is considerably higher than the $pK_a$ of either free dityrosine or of

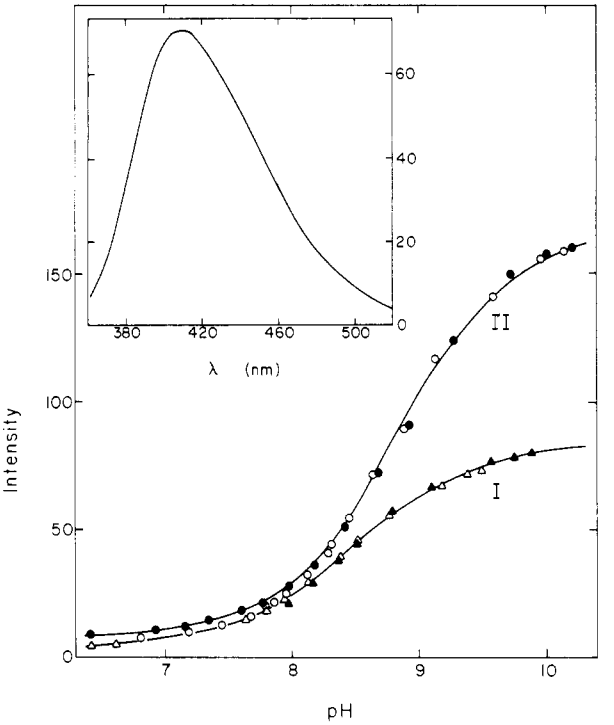


FIGURE 3: pH titrations of calmodulin–[Tyr[8]]substance P adducts. Conditions: 1.0 μM calmodulin derivative in 2.3 mM Mops and 0.184 M KCl (25 °C). Results are shown for both pool I [(△) 1 mM EDTA; (▲) 3 mM calcium acetate] and pool II [(○) 1 mM EDTA; (●) 3 mM calcium acetate] fractions. Excitation: 320 nm with 2.5-nm bandwidth. Emission: 400 nm with 5-nm bandwidth. The inset shows the corrected fluorescence emission spectrum of pool II recorded at pH 8.6.

internally cross-linked calmodulin. Pool I also displays an elevated average $pK_a$ that is independent of calcium. A pH 8.6 buffer was chosen for the following experiments in order to maintain a reasonable level of the fluorescent species on one hand and to avoid extremes of pH on the other.

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 is responsive to both the overall Brownian rotation of macromolecules and local flexibility (Weber, 1966). Fluorescence anisotropy determinations on the internally cross-linked photoproduct of calmodulin revealed a generally weakened interaction with calcium occurring in two stages (Malencik & Anderson, 1987). Observed increases in anisotropy were consistent with a lengthening of the calmodulin molecule upon calcium binding (Small & Anderson, 1987, 1988). The addition of calcium acetate to a Chelex 100 treated solution containing 0.25 μM purified pool II [Tyr[8]]-SP—CaM leads to a gradual change in anisotropy which is more than 90% complete at 0.1 mM calcium (Figure 4A). The midpoint of the transition—$\sim$10 μM $Ca^{2+}$—compares to the average dissociation constant for the native calmodulin–calcium complex determined at pH 7.5 by equilibrium dialysis (Crouch & Klee, 1980). Except for evidence of a high-affinity component detected near 1 μM calcium, titration of pool I gives similar results. Fluorescence intensity changes are negligible (±5%) in both cases.

The association of calmodulin ($M_r$ 16 800) with turkey gizzard myosin light chain kinase ($M_r$ 125 000) is detectable in anisotropy measurements on either the intrinsic tryptophan fluorescence of the enzyme or the fluorescence of calmodulin conjugates (e.g., dansylcalmodulin) (Malencik et al., 1982;

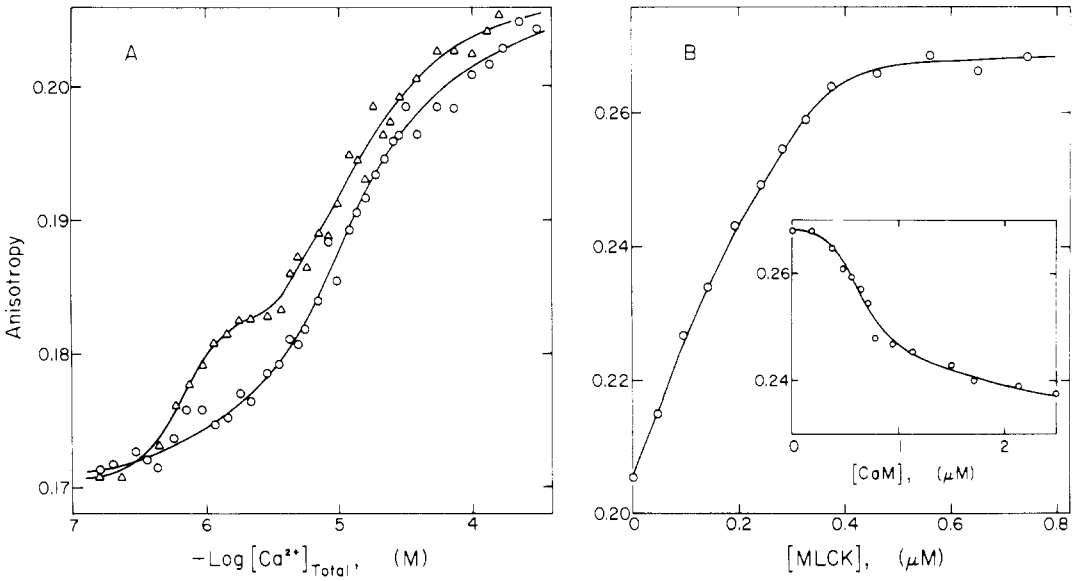


FIGURE 4: Fluorescence anisotropy titrations of calmodulin–[Tyr$^8$]substance P adducts. Panel A shows the effects of varying concentrations of *calcium* acetate on solutions containing 0.5 $\mu$M pool I (△) or 0.25 $\mu$M pool II (○). Conditions: 0.15 NaCl, 50 mM Tris, pH 8.6 (25 °C). Panel B shows the changes occurring upon the addition of turkey gizzard *myosin light chain kinase* to a solution of 0.5 $\mu$M pool II. The inset portrays a back-titration, in which *native* calmodulin is added to the solution containing 0.5 $\mu$M pool II plus 0.74 $\mu$M myosin kinase. Conditions: 50 mM Tris, 1 mM $CaCl_2$, pH 8.6 (25 °C). Excitation: 320 nm with 2-nm bandwidth. Emission: Schott KV 380 cutoff filter.

Anderson & Malencik, 1986). The addition of varying concentrations of myosin kinase, from 0 to 0.74 $\mu$M, to a solution containing 0.5 $\mu$M of the purified pool II fractions plus 1 mM $Ca^{2+}$ causes an increase in anisotropy which is essentially complete at 0.4 $\mu$M enzyme (Figure 4B). Similar results were obtained in both the presence and absence of 0.1 M KCl. A competitive displacement experiment—depicted by the following equilibria—was performed by adding unmodified calmodulin to the solution corresponding to the last point on the titration:

$$\text{MLCK–[Tyr}^8\text{]SP–CaM} \rightleftarrows \text{MLCK} + \text{[Tyr}^8\text{]SP–CaM}$$

$$\text{MLCK} + \text{CaM} \rightleftarrows \text{MLCK–CaM}$$

The inset to Figure 4B shows an initial lag, explainable by the association of native calmodulin with excess enzyme, followed by a decline in anisotropy. Depending on the interpretation of the nonlinearity in the original stoichiometric titration, 55–64% of the derivative is dissociated upon the addition of 2 $\mu$M unmodified calmodulin. By assuming that all of the myosin light chain kinase is bound, we calculate that the dissociation constant for the complex of the enzyme with the derivative is of the same magnitude as that obtained with native calmodulin. (The calculations give $K_d$ values that are 56–83% of the latter.) This result is in marked contrast to that obtained with the internally cross-linked derivative, for which the $K_d$ was estimated to be 280-fold greater than that of unmodified calmodulin (Malencik & Anderson, 1987). In order to learn whether the covalently bound peptide is available for interaction with native calmodulin, we performed a control experiment in which calmodulin was added to a solution containing 0.5 $\mu$M derivative plus 1.0 mM $Ca^{2+}$. No changes were detected at added calmodulin concentrations up to 3 $\mu$M. Pool I fractions showed similar interactions with myosin light chain kinase. As in the experiments with calcium, the fluorescence intensity did not change appreciably in any of these measurements.

To determine whether the purified pool II derivative activates the phosphotransferase activity of smooth muscle myosin light chain kinase, we applied the continuous fluorometric assay in which ADP production is coupled to NADH oxidation via pyruvate kinase and lactate dehydrogenase (Malencik & Anderson, 1986; Malencik et al., 1986). In addition to the coupling enzymes and their substrates, the reaction mixtures contained 70 $\mu$M of the synthetic myosin light chain analogue, 0.10 mM ATP, 2.0 mM magnesium acetate, 0.20 mM calcium acetate, 50 mM Mops, pH 7.3, 10 nM myosin kinase, and 10 nM calmodulin. The rates of NADH oxidation were −0.97 $\mu$M/min for unreacted calmodulin and −0.76 $\mu$M/min for the conjugate labeled at Tyr$^{138}$. The addition of 56 $\mu$M [Tyr$^8$]-substance P to the mixture reduced the rates obtained with native calmodulin to −0.64 $\mu$M/min but had neglibible effect on the reaction rate obtained with the derivative. The reproducibility of these measurements was ±5%.

## Discussion

Ultraviolet (280 nm) irradiation of bovine brain calmodulin in the presence of calcium results either in the intramolecular cross-linking of Tyr$^{99}$ and Tyr$^{138}$ (Malencik & Anderson, 1987) or, when [Tyr$^8$]substance P is bound, in the generation of peptide–calmodulin adducts. The reaction with the peptide competes effectively with intramolecular cross-bridging. In fact, phenylagarose chromatography failed to detect significant quantities of the internally cross-linked derivative in solutions of calmodulin that had been irradiated in the presence of [Tyr$^8$]SP.

The fraction in which [Tyr$^8$]SP is coupled primarily to Tyr$^{138}$ (pool II) was purified to homogeneity. Its abilities to adsorb to phenylagarose, to bind calcium, and to associate with smooth muscle myosin light chain kinase are close to those of native calmodulin. The conjugate of calmodulin labeled at Lys$^{148}$ with POS-TP [10-[[(succinimidooxy)carbonyl]-ethyl]-2-(trifluoromethyl)phenothiazine] (Faust et al., 1987) binds and activates cyclic nucleotide phosphodiesterase while that labeled at Lys$^{75}$ with norchlorpromazine isothiocyanate is a potent antagonist of both phosphodiesterase and myosin kinase (Newton et al., 1983, 1985). Faust et al. suggested that the phenothiazine ring of POS-TP lies in a hydrophobic pocket of calmodulin located near Lys$^{148}$. The X-ray data indeed revealed a potential cavity—lined with Phe$^{89}$, Phe$^{141}$, Phe$^{92}$, Tyr$^{138}$, Ile$^{85}$, Ile$^{125}$, Val$^{142}$, Val$^{136}$, Met$^{145}$, Met$^{144}$, Met$^{124}$, and Met$^{109}$ (Faust et al., 1987; Babu et al., 1985). Possibly the

C-terminal pentapeptide of [Tyr$^8$]substance P (-Phe-Tyr-Gly-Leu-Met-$NH_2$)—in either an $\alpha$-helical or extended form (Anderson & Malencik, 1986, 1988)—can also occupy this site. The p$K_a$ of the Tyr$^{138}$ adduct, 8.8, is substantially higher than that of either free dityrosine, 7 (Lehrer & Fasman, 1967), or that of the internally cross-linked dityrosine derivative of calmodulin, 7.59–7.88 (Malencik & Anderson, 1987). The hydrophobic environment of this pocket could account for the anomalously high p$K_a$ values of both the adduct and Tyr$^{138}$. [The latter is in the range of 11.9–12 (Klee, 1977).]

Neither of two cross-linked complexes of calmodulin with deletion peptides of $\beta$-endorphin—containing 1 and 2 mol of bound peptide per mole of calmodulin—was able to activate or inhibit cyclic nucleotide phosphodiesterase (Giedroc et al., 1985). In all, our fraction II derivative is more like the conjugate of calmodulin labeled at Lys$^{148}$ by POS-TP than it is like the conjugates of calmodulin labeled at as yet *unidentified positions* by $\beta$-endorphin. The differences between the two types of peptide–calmodulin adduct may be explainable by the cross-linking procedures used and/or variations in the subsites occupied by $\beta$-endorphin and [Tyr$^8$]substance P. Since the coupling of $\beta$-endorphin requires a bulky external reagent [bis(sulfosuccinimidyl) suberate], the reaction is most likely to occur at solvent-accessible side chains of calmodulin. In contrast, the attachment of [Tyr$^8$]SP to calmodulin is direct and may occur at solvent-inaccessible residues. Considering that it is specific for tyrosine, the latter reaction is also likely to be more homogeneous than that obtained with bis(sulfosuccinimidyl) suberate.

The facts that mastoparan apparently blocks the coupling of [Tyr$^8$]substance P to calmodulin and that the activation of myosin light chain kinase by the derivative is unaffected by the presence of free [Tyr$^8$]SP in the assay medium indicate that covalent attachment occurs at a point either within or very close to the original peptide association site. The interactions of the conjugate with myosin light chain kinase suggest that Tyr$^{138}$ is either on the periphery or completely outside of the corresponding enzyme binding site. The possibility that the bound peptide is close to both Tyr$^{99}$ and Tyr$^{138}$ was suggested by $^1$H NMR studies on mastoparan (Muchmore et al., 1986) and a peptide derived from skeletal muscle myosin light chain kinase (Klevit et al., 1985) and by energy-transfer measurements on melittin (Steiner et al., 1986). Preliminary characterization of the incompletely purified pool I fraction shows that it contains a mixture of species labeled at Tyr$^{138}$ and Tyr$^{99}$ (unpublished results). Both pool I and pool II appear to be functionally different from the photoproduct in which Tyr$^{99}$ and Tyr$^{138}$ are cross-linked to each other. The latter is distinguished by strongly diminished interactions with phenyl-agarose, myosin light chain kinase, and calcium (Malencik & Anderson, 1987).

Coupling with tyrosine-containing peptides has advantages that may find other applications. These include specificity, the avoidance of an intermediary reagent, and the formation of a fluorescent species whose properties can be used to monitor both the course of the reaction and the macromolecular associations of the product. The procedures described here may be extended to tyrosine-containing analogues of high-affinity calmodulin-binding peptides such as the mastoparans and melittin.

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# Differential Scanning Calorimetry of Cu,Zn-Superoxide Dismutase, the Apoprotein, and Its Zinc-Substituted Derivatives†

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ABSTRACT: We have employed differential scanning calorimetry (DSC) to investigate the thermally induced unfolding of native Cu,Zn-superoxide dismutase (SOD), the apoprotein derived from native SOD, and the zinc-substituted derivatives of the apoprotein. We observe two overlapping melting transitions for native bovine SOD with heat capacity maxima at temperatures ($T_m$) of 89 and 96 °C when a scanning rate of 0.82 deg/min is employed. By contrast, the dithionite-reduced native SOD (which contains $Cu^+$ rather than $Cu^{2+}$) exhibits only a single transition at 96 °C. Significantly, we find that the concentration of $O_2$ present in native SOD samples influences the relative magnitudes of the 89 and 96 °C peaks. Specifically, the lower temperature transition becomes less pronounced as the concentration of $O_2$ in the sample decreases. On the basis of these observations, we propose that the lower temperature peak corresponds to the melting of the oxidized native protein, while the higher temperature peak reflects the melting of the reduced native protein, which forms spontaneously during the heating process. Our interpretation profoundly differs from that of Lepock et al. [Lepock, J. R., Arnold, L. D., Torrie, B. H., Andrews, B., & Kruuv, J. (1985) *Arch. Biochem. Biophys. 241*, 243–251], who have proposed that the low-temperature transition corresponds to the reduced form of the protein. We present evidence that suggests that their experiments were complicated by the presence of potassium ferrocyanide, which, in addition to reducing the cupric center, also perturbs the protein. In contrast to native SOD, we observe that apo-SOD melts monophasically with a $T_m$ of only 57 °C at the identical scan rate used for melting the native protein. This result demonstrates that metal ions play a significant role in enhancing the thermal stability of native SOD. A series of DSC melts on apo-SOD as a function of added $Zn^{2+}$ reveals that binding of the first 2 equiv of $Zn^{2+}$ ions induces most of the overall thermal stabilization observed for the binding of a total of four $Zn^{2+}$ ions to the SOD protein dimer. While not altering the peak area, addition of the third and fourth equivalents of $Zn^{2+}$ does cause the melting transition to sharpen and to exhibit a small increase in $T_m$. We also find that the DSC melting profiles for SOD exhibit a strong dependence on scan rate. Such a scan rate dependence can occur when the overall process is kinetically limited and/or irreversible. Consequently, we have considered these possibilities in our interpretation of the calorimetric data.

Native bovine copper–zinc superoxide dismutase ($Cu^{II}_2$-$Zn_2$SOD)[1] is a dimer of identical subunits, each of which contains one $Cu^{2+}$ and one $Zn^{2+}$ ion and has an approximate $M_r$ of 16000 (Valentine et al., 1981). The X-ray crystal structure at 2-Å resolution (Tainer et al., 1982) shows a globular protein with each subunit comprised of eight antiparallel β-strands, one disulfide linkage, and a metal binding region in which the $Cu^{2+}$ and $Zn^{2+}$ ions are bridged by a histidyl imidazolate which holds them 6.3 Å apart. In addition to the bridging histidyl imidazolate, the $Cu^{2+}$ ion is coordinated to three histidyl ligands and a water molecule while the $Zn^{2+}$ ion is coordinated to an aspartyl carboxylate group and two histidyl imidazoles (Figure 1). The subunits are strongly

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[1] Abbreviations: Cu,Zn-SOD, native form of cuprozinc superoxide dismutase as isolated from bovine liver. In general, $X_2Y_2$SOD signifies those derivatives of the native protein in which the metal ions X and Y have been substituted for $Cu^{II}$ and $Zn^{II}$, respectively (X and Y may be the same). All metal ion oxidation states are assumed to be 2+ unless noted otherwise. $E_2Zn_2$SOD denotes that protein derivative which is free of metal ions in the copper sites (E = empty).

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