Dityrosine Formation in Calmodulin: Cross-Linking and Polymerization Catalyzed by *Arthromyces* Peroxidase†

Dean A. Malencik and Sonia R. Anderson*

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-7305

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ABSTRACT: We employ bovine brain calmodulin, a protein that is subject to photoactivated dityrosine formation [Malencik, D. A., & Anderson, S. R. (1987) *Biochemistry 26*, 695; (1994) *Biochemistry 33*, 13363], as a model for the development of an efficient enzyme-catalyzed protein cross-linking technique. Key steps in the elaboration of the procedure are (1) identification of a peroxidase, from *Arthromyces ramosus*, that catalyzes dityrosine production in proteins that are not acted on by other common peroxidases, (2) monitoring of the intrinsic fluorescence of dityrosine to determine optimum reaction conditions, achieved with calmodulin in solutions containing boric acid−sodium borate (concentration ≥0.2 M), ∼pH 8.3, ∼40 °C, and (3) quenching of the reaction with reduced glutathione. *Arthromyces* peroxidase is the only common peroxidase able to catalyze significant dityrosine production in calmodulin, through a reaction that is largely intermolecular. Gel filtration yields fractions (accounting for ∼40% of the initial calmodulin) that represent differing mobility ranges in $NaDodSO_4$ polyacrylamide gel electrophoresis and contain close to the maximum possible amounts of dityrosine. The various fractions undergo Ca^{2+}-dependent conformational changes detected in sedimentation velocity and/or fluorescence anisotropy measurements. Most of the samples stimulate the Ca^{2+}-dependent activity of smooth muscle myosin light chain kinase. In catalytic assays utilizing the synthetic phosphate acceptor peptide, the average activities range from 50 to 100% of that determined for native calmodulin. However, only the least polymerized fraction and the photogenerated calmodulin dimers significantly enhance the *p*-nitrophenylphosphatase activity of calcineurin. The ability to prepare soluble calmodulin polymers that retain a substantial degree of biological activity and exhibit the intense visible fluorescence of dityrosine illustrates the potential usefulness of *Arthromyces* peroxidase in the zero-length cross-linking of proteins.

The chemical cross-linking of proteins has wide ranging applications in biochemistry, immunology, medicine, biotechnology, and industry. The properties and uses of chemically cross-linked proteins vary from case to case. Reported applications include structural stabilization, proximity determinations, enhancement of antigenicity, attachment to solid supports, and the conjugation of proteins to other molecules, such as enzymes, immunotoxins, and drugs. Homobifunctional and heterobifunctional cross-linking reagents bridge reactive amino acid side chains. Zero-length cross-linking agents induce direct joining in reactions that lead to the loss of atoms from the reactants. Protein cross-linking may be intramolecular and/or intermolecular, capable of generating high molecular weight homopolymers and heteropolymers. Most protein cross-linking techniques target lysyl and/or cysteinyl side chains [cf. comprehensive reviews by Wong (1993) and Hermanson (1996)]. We became interested in the zero-length coupling of tyrosyl side chains (Figure 1) as a useful approach to protein cross-linking in conjunction with work on the photochemistry of calmodulin.

Prolonged 280-nm irradiation of bovine brain calmodulin in the presence of Ca^{2+} leads to the intramolecular coupling of the phenolic side chains of its only two tyrosyl residues, Tyr-99 and Tyr-138 (Malencik & Anderson, 1987).

$$2\ \text{tyrosine} \rightarrow \text{dityrosine} + 2H^+ + 2e^- \qquad (1)$$

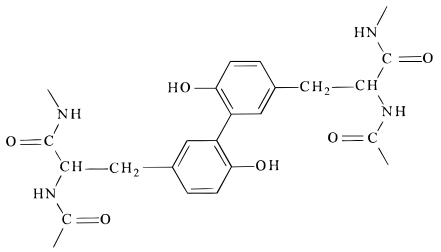

FIGURE 1: Schematic structure of a protein dityrosyl cross-link.

Both tyrosine residues are associated with calcium binding domains (sites III and IV) in the native calmodulin molecule (Babu et al., 1985). Fluorescence measurements, employing the 400 nm emission maximum of the dityrosine chromophore, demonstrated weakened interactions of the purified cross-linked calmodulin molecule with both Ca^{2+} and smooth muscle myosin light chain kinase (Malencik & Anderson, 1987). We subsequently discovered that the presence of superoxide dismutase during the irradiation of calmodulin alters the distribution of 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. The latter reaction mixture yields a pool of dityrosine-containing dimers of calmodulin, whose average interactions with Ca^{2+} and

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* Address correspondence to this author. Telephone: 503-737-4486.

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smooth muscle myosin light chain kinase are nearly the same as those of native calmodulin, and virtually no cross-linked monomer (Malencik & Anderson, 1994). The effects of superoxide dismutase were consistent with the removal of superoxide anion $O^{\bullet -}$, which may be generated when the photoejected electrons react with O_2 (Holler & Hopkins, 1989).

The phenolic coupling of tyrosyl side chains has advantages provided by no other single approach to protein cross-linking. First, the specificity for tyrosine instead of lysine or cysteine significantly extends the range of possibilities for cross-linking. Second, the intense 400−410 nm fluorescence of the dityrosine chromophore facilitates monitoring of the coupling reaction and detection of the cross-linked products, both in fractionation procedures and subsequent applications. In addition, dityrosine is a responsive fluorescent probe, a capacity that we demonstrated in spectroscopic studies of the cross-linked calmodulin monomer (Malencik & Anderson, 1987; Small & Anderson, 1988; Anderson, 1991), a peptide−calmodulin conjugate (Malencik & Anderson, 1988), and the calmodulin dimers (Malencik & Anderson, 1994). Third, the dityrosyl cross-link is stable under mild oxidizing conditions and during acid hydrolysis. Reverse-phase high-performance liquid chromatography of dabsylated protein hydrolysates gives complete amino acid analyses that include dityrosine (Malencik et al., 1990).

In view of the advantages of phenolic coupling, we set a goal of obtaining better yields of cross-linked protein than is possible with ultraviolet irradiation. In our experiments, we recovered only 2.8% of the calmodulin as purified cross-linked monomer (Malencik & Anderson, 1987) and 5−6% as mixed dimers (Malencik & Anderson, 1994). An alternate route, enzyme-catalyzed phenolic coupling, occurs naturally in some organisms, accounting for the presence of dityrosine in specific structural proteins (Andersen, 1964; Deits et al., 1984). The *in vitro* preparation of dityrosine from free tyrosine utilizes a reaction catalyzed by horseradish peroxidase (Amadò et al., 1984).

$$2\ \text{tyrosine} + H_2O_2 \rightarrow \text{dityrosine} + 2H_2O \qquad (2)$$

Application of this reaction to proteins gave conversions of tyrosine to dityrosine ranging from 5.3% with gliadin to 86% with chymotrypsin (Aeschbach et al., 1976). However, reaction time courses were not monitored, and the chymotrypsin was inactivated. No information was given on the optimization of reaction conditions. As discussed later, attempts to apply horseradish peroxidase to the cross-linking of bovine brain calmodulin fail totally. Since the tyrosyl residues of calmodulin undergo photochemical coupling, we suspected that the specificity of the peroxidase is a limiting factor in the enzyme-catalyzed reaction.

The purpose of this article is to develop and refine enzyme-catalyzed phenolic coupling as a tool for the *in vitro* cross-linking of proteins. We chose bovine brain calmodulin as the model for elaboration of the reaction since (1) it represents a relatively simple case, containing two tyrosyl residues in a single 148-residue polypeptide chain, (2) it is an example of a protein that is *not* acted on by most of the common peroxidases, and (3) there is background information on three different dityrosyl calmodulin derivatives produced by photoactivated cross-linking (Malencik & Anderson, 1987, 1988, 1994). The principles established by observations on calmodulin and tyrosine-containing random copolymers should expedite the enzyme-catalyzed cross-linking of other proteins.

MATERIALS AND METHODS

Proteins. Calcium-free bovine brain calmodulin and the photogenerated dimer were prepared according to Malencik and Anderson (1994). The native calmodulin concentrations are based on $E^{1\%}_{280nm} = 2.0$ and a molecular weight of 16 680 (Watterson et al., 1980). Rabbit skeletal muscle troponin C, DTNB myosin light chain, turkey gizzard 20 kDa myosin light chain, 16 kDa myosin light chain, and dogfish parvalbumin were prepared as previously described (Malencik & Anderson, 1987, 1988).

Turkey gizzard myosin light chain kinase containing a single band on $NaDodSO_4$[1] electrophoresis was prepared 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). The catalytic activity of the enzyme was assayed by the coupled, fluorometric method of Malencik and Anderson (1986). The assay medium contains 63 μM synthetic myosin light chain kinase substrate, 0.11 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, 2.5 mM $Mg(CH_3CO_2)_2$, 0.28 mM $CaCl_2$, and 42 mM Mops (K^+) (pH 7.3) at 25 °C.

Porcine brain calcineurin (protein phosphatase 2B) was prepared according to Tallant et al. (1983) and assayed according to the spectrophotometric method of Pallen and Wang (1983). The assay medium contained 0.97 mg/mL *p*-nitrophenyl phosphate, ±17 μg/mL calmodulin (or derivative), 0.5 μM calcineurin, 1.0 mM $MnCl_2$, and 50 mM Mops, pH 7.3, 20 °C.

Bovine erythrocyte superoxide dismutase, bovine erythrocyte glutathione peroxidase, horseradish peroxidase (type VIA), soybean peroxidase, bovine milk lactoperoxidase, and *Arthromyces ramosus* peroxidase were purchased from Sigma Chemical Co. Human polymorphonuclear leukocyte myeloperoxidase was obtained from Calbiochem.

Other Reagents. Tris, Mops, and borate buffers were prepared by using distilled water that had been further purified with a Milli-Q reagent water system. After pH adjustment with NaOH or acetic acid, the buffers were treated with Chelex 100 to remove traces of calcium and other metal ions. Reagent-grade NaCl, $CaCl_2$, $Mg(CH_3CO_2)_2$, and $MnCl_2$ were used in the indicated experiments. The best available grades of Tris, Mops, boric acid, glutathione, dithiothreitol, *p*-nitrophenyl phosphate, ATP, NADH, and phosphoenolpyruvate (tricyclohexylammonium salt) were purchased from Sigma Chemical Co. The synthetic smooth muscle myosin light chain kinase substrate [$(Lys)_2$ ArgProGlnArgAlaThrSerAsnValPheSer-NH_2] and mastoparan were supplied by Peninsula Laboratories. Dityrosine was prepared by the enzymatic method of Amadò et al. (1984) and purified by reverse-phase high-performance liquid chromatography. A

[1] 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; $NaDodSO_4$, sodium dodecyl sulfate.

3% solution of H_2O_2 USP was purchased from Fred Meyer and diluted with water just before use.

The dipeptide L-tyrosyl-L-tyrosine and the following random copolymers, poly(Arg, Tyr) 4:1,[2] poly(Lys, Tyr) 4:1, poly(Glu, Tyr) 4:1, poly(Glu, Lys, Tyr) 6:3:1, and poly(Ala, Glu, Lys, Tyr) 6:2:5:1, were obtained from Sigma. Chromatography media, phenylagarose (P-8901), Sephacryl S-200 HR, and Sephacryl S-300 HR, also were supplied by Sigma.

Miscellaneous Analytical Methods. Measurements of total fluorescence intensity ($I_{||} + 2I_{\perp}$) and anisotropy [$(I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$][3] were obtained with either the Perkin-Elmer LS-50 or LS-50B luminescence spectrophotometers, using obey programs written in our laboratory. The fluorescence anisotropy titrations follow the protocol used in previous Ca^{2+} binding studies of dityrosyl calmodulin derivatives (Malencik & Anderson, 1987, 1988, 1994). 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). Protein concentrations were determined by total amino acid analysis and/or by the Coomassie blue method (Bradford, 1976), using native calmodulin as a standard. The results of these two analyses agreed within 10−12%.

Sedimentation velocity and sedimentation equilibrium experiments were carried out with the Beckman Optima XL-A analytical ultracentrifuge. Boundaries usually were monitored at 280 nm. The temperature of the rotor was maintained at 21.1 °C for sedimentation velocity and at 4 °C for sedimentation equilibrium. The rotor speed was fixed at 3600 or 5200 rpm for the sedimentation equilibrium experiments and at 35 000 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 values of $s_{20,w}$ were corrected to the density and viscosity of water at 20 °C. A calculated partial specific volume of 0.728 cm^3/g was employed in the computation of both $s_{20,w}$ and molecular weight for calmodulin (Malencik & Anderson, 1994).

$NaDodSO_4$ polyacrylamide gel electrophoresis was performed in glass tubes, following a variation of the method of Weber and Osborn (1969) that was described by Kerrick et al. (1980). This method proved superior to the Laemmli system in the electrophoresis of polymerized calmodulin samples.

Preparative Scale Cross-Linking of Calmodulin. The following procedure is based on the information given in the first three sections of Results. The reactions to be described were carried out at relatively high concentrations of calmodulin in order to obtain higher degrees of polymerization and to minimize the solution volumes.[4] In the following outline, comments regarding individual preparations are given in parentheses.

(1) The procedure begins with a solution of calmodulin in 0.25 M boric acid−sodium borate buffer, pH 8.4 (38−39 °C). *Arthromyces* peroxidase (50 μg of peroxidase/mg of calmodulin) and superoxide dismutase (50 μg of SOD/mg of calmodulin) are added next. The reaction is initiated by the addition of 2.2−2.4 mol of H_2O_2/mol of calmodulin. The completion of the reaction is established in fluorescence measurements performed on samples removed at various times and diluted 10−50-fold with 0.1 M Na_2CO_3, using excitation and emission wavelengths of 320 and 400 nm, respectively. (Our preparations employed 50 mg of calmodulin at either 9 mg/mL or 50 mg/mL, concentrations too high for direct fluorescence measurement. In both cases, the fluorescence intensity reached a constant level upon 6−8 min of incubation.)

(2) When the fluorescence intensity no longer changes, reduced glutathione (2−4 mol/mol of original H_2O_2) and glutathione peroxidase (1 μg/mg of calmodulin) are added in order to remove free radicals, unreacted H_2O_2, and possibly hydroperoxides [cf. Flohè (1989)]. This mixture is allowed to stand for 20 min.

(3) The reaction mixture is placed on ice, and 20% acetic acid is added slowly, until a flocculent white precipitate appears. This precipitate is collected by centrifugation and dissolved in a minimum volume of either 0.10 M NH_4HCO_3 or 0.10 M ammonium formate, 10 mM Tris, and 1 mM $CaCl_2$ (pH 8.3), depending on which of the following two steps is performed next. Polymerized calmodulin is markedly insoluble in a pH range (3.8−4.0) where native calmodulin and the other proteins are more soluble. [When the reaction was performed at a concentration of 50 mg/mL, the precipitate was washed twice with 0.10 M ammonium formate, pH 3.85 (1 mL of solution/50 mg of calmodulin).]

The next two steps can be undertaken in either order.

(4) The redissolved precipitate from step 3 is applied to a phenylagarose affinity chromatography column (1.7 cm × 11 cm) equilibrated in 0.10 M ammonium formate, 10 mM Tris, and 1 mM $CaCl_2$ (pH 8.3). The column is washed with 100 mL of the equilibration buffer and subsequently eluted with an equivalent solution containing 1 mM EGTA or 1 mM EDTA instead of calcium [cf. Charbonneau and Cormier (1979) and Malencik and Anderson (1994)]. The calmodulin-containing fractions are identified through fluorescence measurements, with excitation and emission wavelengths set to 320 nm and 400 nm, respectively. Individual peaks are pooled separately and concentrated by lyophilization. [Approximately 85−90% of the protein recovered from polymerization at 9 mg/mL eluted upon application of EGTA or EDTA. Phenylagarose chromatography of the sample polymerized at 50 mg/mL resulted in slow equilibration, with bound (65%) and unbound (35%) protein being equally active in myosin light chain kinase assays. Step 4 can be omitted in this case.]

(5) Gel filtration. We began with a Sephacryl S-200 HR sizing column (2.5 cm × 81 cm) equilibrated in 0.10 M NH_4HCO_3. Application of the preparation that had undergone cross-linking at a calmodulin concentration of 9 mg/mL

[2] The numbers refer to the relative proportions of the different amino acid residues present in the random copolymers.

[3] $r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$, 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. In a mixture of components, the observed anisotropy is the sum of the individual anisotropies weighed by the corresponding fractional contributions to the total fluorescence intensity $r_{obs} = \sum f_i r_i$.

[4] The reaction can occur over a wide range of concentrations. We have monitored time courses with reaction mixtures containing 1.0 μM calmodulin.

results in the elution of a single broad peak, which we divided into two pools containing tube numbers 70−85 (designated as 9-S1, 15 mg) and 86−98 (fraction 9-S2, 4 mg). (Each tube contained 2.3−2.4 mL.)

The sample that had been reacted at 50 mg/mL first was applied to the Sephacryl S-200 HR column, with the contents of tube numbers 69−78 (15 mg) and 79−86 (6.6 mg) pooled separately and concentrated by lyophilization. For further resolution of higher molecular material, the former (15 mg) pool was redissolved and applied to a Sephacryl S-300 HR sizing column (2.5 cm × 85 cm). The resulting separation is shown in the inset to Figure 5. Pools corresponding to tube numbers 78−81, 82−84, 85−86, 87−90, 91−94, and 95−102 are referred to as fractions 50-S1 through 50-S6.

(6) About 40% of the original calmodulin is recovered after completion of these steps. The polymerized samples are stable when stored at −80 °C, either as frozen solutions or as lyophilized powders. Excess salts, EDTA, etc. can be removed by repetition of step 3. The lyophilized material is difficult to redissolve unless a buffer, such as 10 mM NH_4HCO_3, is used.

RESULTS

Identification of a Peroxidase Exhibiting Broad Specificity toward Tyrosine-Containing Copolymers. We tested several tyrosine-containing random copolymers as substrates for horseradish peroxidase, soybean peroxidase, lactoperoxidase, myeloperoxidase, and *A. ramosus* peroxidase. Changes in fluorescence intensity, determined with an excitation wavelength of 301 nm and emission wavelength of 377 nm, were recorded as a function of time after initiation of the reaction by addition of H_2O_2. These particular wavelengths were chosen since they correspond to isosbestic and isoemissive points of free dityrosine in borate buffers of varying concentrations, ranging upward from zero (Malencik & Anderson, 1991). The conditions employed in the final analysis are similar to those later shown to be optimal for dityrosine production in calmodulin.

Briefly, the fluorescence measurements indicate that all five peroxidases catalyze dityrosine formation in L-tyrosyl-L-tyrosine, poly(Lys, Tyr) 4:1,[2] and poly(Arg, Tyr) 4:1. Similar total increases in fluorescence intensity occur with all combinations of enzyme and substrate. However, none of the peroxidases acts on poly(Glu, Tyr) 4:1 or on poly(Glu, Lys, Tyr) 6:3:1. Experiments with poly(Ala, Glu, Lys, Tyr) 6:2:5:1, on the other hand, demonstrate distinct differences among the enzymes. In terms of both the maximum increase in apparent dityrosine fluorescence and the reaction rate, this copolymer is a markedly more effective substrate for *A. ramosus* peroxidase than it is for any of the other enzymes (Table 1).

Detection of Arthromyces Peroxidase-Catalyzed Dityrosine Formation in Calmodulin. The effectiveness of *Arthromyces* peroxidase on the random copolymer poly(Ala, Glu, Lys, Tyr) 6:2:5:1 suggested that it may catalyze the production of dityrosine in proteins that do not react when one of the more familiar peroxidases is employed. Figure 2 shows the time-dependent increases in fluorescence intensity that occur when bovine brain calmodulin (60 μM) is incubated in solutions containing 100 μg/mL *Arthromyces* peroxidase and the indicated concentrations of H_2O_2. The first experiment, performed in 0.22 M boric acid−sodium borate (pH 8.4, 36 °C), shows increases in fluorescence occurring during the first 10 min of reaction. A decline in fluorescence occurring after that time reflects exhaustion of H_2O_2, which was originally present at a concentration of 120 μM. The addition of a 20 μM increment of H_2O_2 reverses this effect. Since the time courses chosen for illustration are based on the results of preliminary determinations, depletion of H_2O_2 occurs late in these particular examples. The quantity of H_2O_2 required compares to that used in the preparation of dityrosine from free tyrosine, 1 mol H_2O_2/mol Tyr (Amadò et al., 1984). (The stoichiometry of peroxidase-catalyzed reactions usually varies from the ideal [cf. Flohé (1989)].) Figure 2 also shows inset gel photos for three different reaction mixtures. These will be discussed in later sections.

Table 1: Effectiveness of Poly(Ala, Glu, Lys, Tyr) 6:2:5:1 as a Substrate for Various Peroxidases[a]

peroxidase	maximum increase in apparent dityrosine fluorescence[b]	time required[c]
Arthromyces	249	20 s
horseradish	77	10 min
soybean	47	10 min
myeloperoxidase	41	1.5 min
lactoperoxidase	16	7 min

[a] Conditions: 10 μg/mL peroxidase, 50 μM poly(Ala, Glu, Lys, Tyr) in terms of tyrosine, 50 μM H_2O_2, 0.20 M sodium borate, pH 8.4, 34 °C. [b] Determined employing an excitation wavelength of 301 nm and an emission wavelength of 377 nm. [c] Time required to reach maximum intensity.

Determination of the calcium dependence of the reaction utilized Tris buffers since calcium borate is insoluble. Under these conditions, which are similar to those used for photoactivated cross-linking (Malencik & Anderson, 1987, 1994), the enzyme-catalyzed reaction occurs only in the absence of Ca^{2+} in the case of calmodulin (Figure 2). However, the resulting fluorescence intensities are lower and the consumption of H_2O_2 are higher[5] than those obtained with the boric acid−sodium borate buffer. Superoxide dismutase appears to stimulate the reaction occurring in both the calcium-free Tris and the boric acid−sodium borate solutions. The reactions of the copolymers are not appreciably affected by Ca^{2+} (not shown).

Effects of Variations in pH, Boric Acid−Sodium Borate Concentration, and Temperature. The effects of differing experimental conditions on the *Arthromyces* peroxidase-catalyzed formation of dityrosine were investigated in order to learn more about the reaction *per se* and to optimize conditions for the preparative scale cross-linking of calmodulin and other proteins. The results in Figure 3 were obtained with solutions containing 55 μM calmodulin, 47 μg/mL *Arthromyces* peroxidase, and concentrations of H_2O_2 incremented to give maximum fluorescence (up to 130 μM). In these experiments, two of the key variables were fixed at close-to-optimal values while the third was systematically varied. We added β-mercaptoethanol (5 mM) to the *reacted* mixtures since we find that the presence of a thiol stabilizes the final fluorescence intensities. The samples within each of the three sets were adjusted to the same final conditions before fluorescence measurements were made.

[5] Similar results were found with *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonate) buffers.

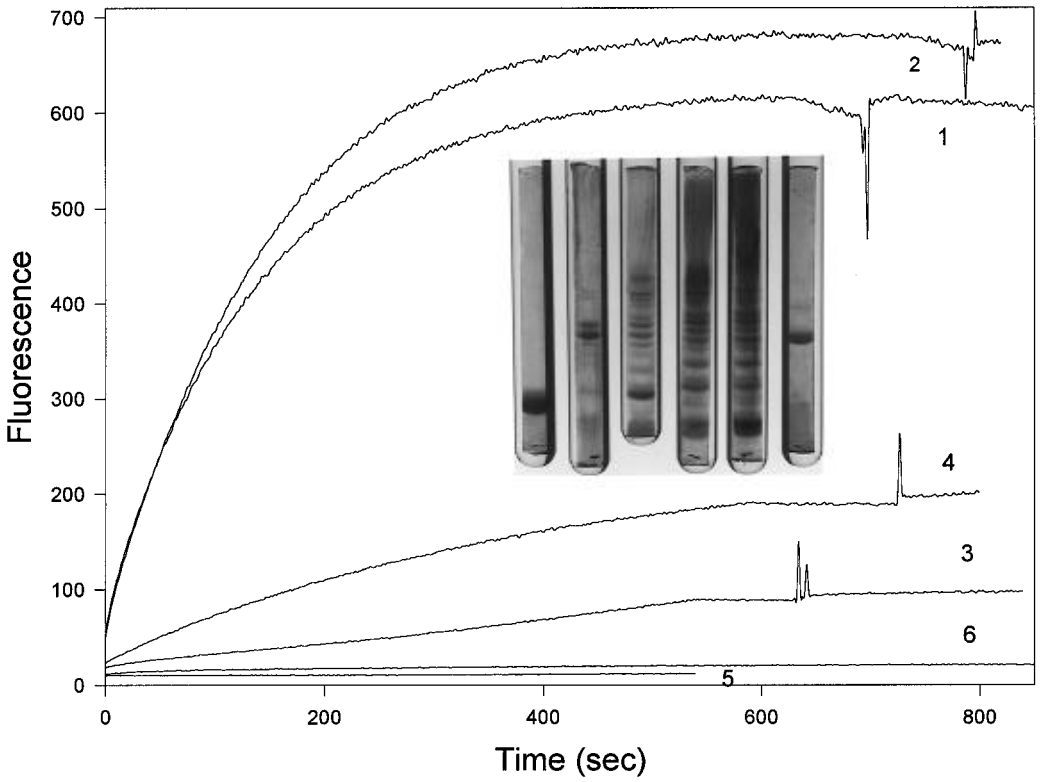

FIGURE 2: Fluorescence changes occurring during the *Arthromyces* peroxidase-catalyzed oxidation of calmodulin. (1) The reaction occurring in a solution containing 60 μM calmodulin, 100 μg/mL peroxidase, 120 μM H_2O_2, and 0.22 M boric acid−sodium borate, pH 8.4 (36 °C). A 20 μM increment of H_2O_2 was added after 11.5 min of incubation. (2) Same as no. 1, with 50 μg/mL superoxide dismutase present from zero time. (3) Results found with 60 μM calmodulin, 100 μg/mL peroxidase, 22 mM Tris acetate, and 200 μM H_2O_2. (4) Same as no. 3, with the addition of 50 μg/mL superoxide dismutase. (5) Same as no. 3, with the addition of 0.4 mM $CaCl_2$. (6) Same as no. 5, with the inclusion of superoxide dismutase. Excitation: 301 nm (3 nm slit width). Emission: 377 nm (3 nm slit). Path: 5 mm. Inset from left to right: results of $NaDodSO_4$ gel (8.75%) electrophoresis performed on umodified calmodulin, on a dimer-containing sample from UV irradiation, on unfractionated reaction mixtures containing 1, 3, and 10 mg/mL calmodulin plus 5% (w/w) peroxidase, and on peroxidase alone.

Assuming that circumstances giving the highest fluorescence intensities favor dityrosine formation over competing reactions, the data in Figure 3 suggested that the preparative scale cross-linking of calmodulin be performed in 0.20−0.25 M boric acid−sodium borate, ~pH 8.3, 36−40 °C. The details of the resulting procedure are described under Materials and Methods. We tested horseradish peroxidase, soybean peroxidase, lactoperoxidase, and myeloperoxidase for possible catalysis of dityrosine formation in calmodulin under these nearly ideal conditions. Of the four enzymes, only myeloperoxidase catalyzes significant reaction, giving extremely slow increases in fluorescence and final intensities that are a small fraction of those obtained with *Arthromyces* peroxidase.

Overview of the Predicted Cross-Linking of Calmodulin. Considering that the photoactivated intramolecular cross-linking of calmodulin occurs only in the presence of Ca^{2+} (Malencik & Anderson, 1987, 1994), we expected the enzyme-catalyzed cross-linking to be primarily intermolecular in the boric acid−sodium borate buffers. If both of the tyrosyl side chains react and the cross-linking efficiency is high, indefinite linear polymerization will take place, with three possible cross-links connecting neighboring calmodulin molecules (Y^{99} to Y^{99}, Y^{138} to Y^{138}, and Y^{99} to Y^{138}). A distribution of species that vary in molecular weight and cross-linking pattern results. To illustrate the latter, we have counted 36 possible linear hexamers, 16 pentamers, 10 tetramers, 4 trimers, and 3 dimers. Closed structures, with all tyrosines cross-linked, also may form.

$NaDodSO_4$ gel electrophoresis (8.75%) performed on three unfractionated samples reacted under optimal conditions at calmodulin concentrations of 1, 3, and 10 mg/mL reveals progressively higher degrees of polymerization, with discrete bands that merge into a continuum in the higher molecular weight range (inset to Figure 2). The lack of resolution in the upper region is inherent in the multiplicity of the polymerization reactions just discussed. Since the average properties of the dimers are already known (Malencik & Anderson, 1994), this article emphasizes the preparation and characterization of higher molecular weight fractions of cross-linked calmodulin.

Chemical and Spectral Characterization of Cross-Linked Calmodulin Samples. We performed amino acid analyses in order to verify the presence of dityrosine in cross-linked calmodulin samples and to detect other modifications, affecting any of the 17 amino acids determined. Table 3 focuses on dityrosine, tyrosine, phenylalanine, lysine, and methionine since these residues are the most likely to become oxidized. The analyses show that the samples generally contain close to the maximum possible amounts of dityrosine, low levels of tyrosine, normal levels of phenylalanine and lysine, and quantities of methionine representing 70−90% of that found with the untreated protein.

The various polymer samples have similar fluorescence properties. Table 4 compares the emission, excitation, and absorption spectra of a free dityrosine standard with the spectra representative of fractions 50-S2 through 50-S6. The ability of dityrosine to associate reversibly with boric acid/

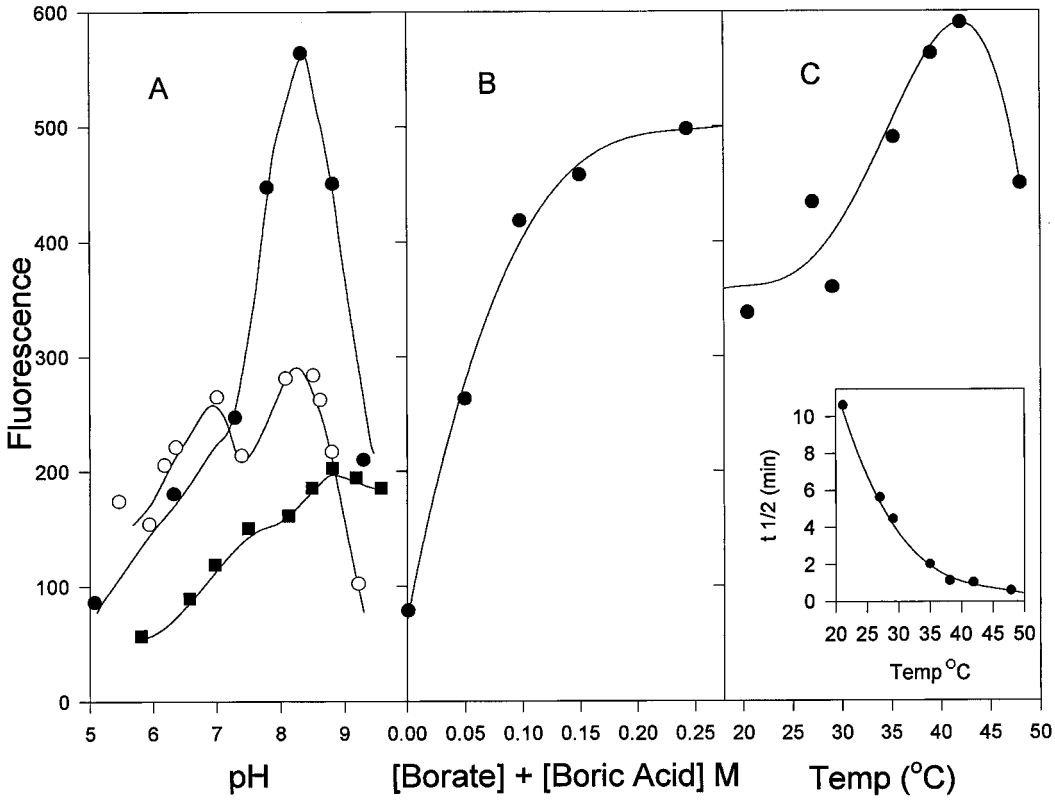

FIGURE 3: Optimization of conditions for the *Arthromyces* peroxidase-catalyzed cross-linking of calmodulin. Panel A shows the final fluorescence intensities (determined at pH 8.5) obtained when 55 μM calmodulin (●), 47 μg/mL *Arthromyces* peroxidase, and concentrations of H_2O_2 incremented to 130 μM are incubated in 0.25 M boric acid−sodium borate buffers of *varying pH values*. Temperature = 34 °C. Results of similar experiments with 25 μM L-tyrosyl-L-tyrosine (■) and 50 μM poly(Ala, Glu, Lys, Tyr) 6:2:5:1 (○) are superimposed. Panel B shows the normalized intensities obtained with calmodulin when the *concentration of the boric acid−sodium borate buffer is varied* while the pH (8.4) and temperature (34 °C) are held constant. The final measurements were performed on diluted (2-fold) samples containing 0.125 M boric acid−sodium borate. Panel C illustrates the effects of *temperature variation* on the final fluorescence intensities of calmodulin solutions containing 0.22 M boric acid−sodium borate, pH 8.4. The inset shows the times required to reach half of the maximum intensity. Excitation: 301 nm (3 nm slit). Emission: 377 nm (3 nm slit). Path: 10 mm.

Table 2: Key to Polymerized Calmodulin Fractions

fraction	concentration during polymerization (mg/mL)	purification procedures	location of gel photo
9-S1	9	phenylagarose; S-200 fractions 70−85	Figure 4
9-S2	9	phenylagarose; S-200 fractions 86−98	Figure 4
50-S1	50	S-300 fractions 78−81[a]	
50-S2	50	S-300 fractions 82−84[a]	Figure 6
50-S3	50	S-300 fractions 85−86[a]	
50-S4	50	S-300 fractions 87−90[a]	Figures 4 and 6
50-S5	50	S-300 fractions 91−94[a]	
50-S6	50	S-300 fractions 95−102[a]	

[a] Refer to Figure 5.

Table 3: Amino Acid Analyses of Polymerized Calmodulin Fractions

fraction	Lys	Met	Phe	Tyr	Dityr
9-S1[a]	7.0	7.2[b,c]	7.1[b,c]	0.52[b,c]	0.85[b,c]
9-S2[a]	6.7	7.0	7.4	0	1.33
50-S1[a]	7.4	6.2	6.7	0	0.89
50-S2[a]	6.7	6.0	8.06	0	0.99
50-S3[a]	7.3	6.9	7.4	0	0.87
50-S4[a]	7.2	7.9	8.1	0.03	1.03
50-S5[a]	6.8	6.5	8.2	0.08	0.94
50-S6[a]	6.9	5.6	8.4	0	0.90
native	7.1	8.7	7.7	2.1	0
ideal native	7.0	9.0	8.0	2.0	0

[a] Refer to Table 2 for a key to the polymerized calmodulin fractions. [b] Nanomoles obtained per 147 nmol of total residues after a 48-h hydrolysis. [c] Average error ±5%.

borate ion (Malencik & Anderson, 1991) is preserved in the cross-linked calmodulin. Fluorescence titrations show that the phenolic pK_a, which is in the range of 7.0−7.1 for free dityrosine (Lehrer & Fasman, 1967; Malencik & Anderson, 1987, 1994), is elevated in the polymers. Note that concentrated solutions of cross-linked calmodulin are colorless, with no significant absorbance at wavelengths above 360 nm.

Sedimentation Velocity Studies. The analysis of van Holde and Weischet (1978) gives a distribution of sedimentation coefficients from the boundaries of *sedimentation velocity* experiments performed with paucidisperse solutes. van Holde−Weischet plots, such as those presented in Figure 4, show the infinite-time extrapolated values of $s_{20,w}$ determined at various relative positions (designated by a fraction ranging from 0 to 1) along the sedimenting boundary.

Figure 4 provides the following information on fraction 9-S1 (Table 2), one of the three polymer samples represented in the inset gel photos. (1) *On the average*, the various components in 9-S1 are similarly affected by Ca^{2+} binding, with evident decreases in frictional ratio taking place. (2) The range of values of $s_{20,w}$ rules out the presence of significant amounts of calmodulin monomer ($s_{20,w}$ = 2.14S (0 Ca^{2+}); 2.17S (Ca^{2+}) or calmodulin dimers [$s_{20,w}$ = 2.69S

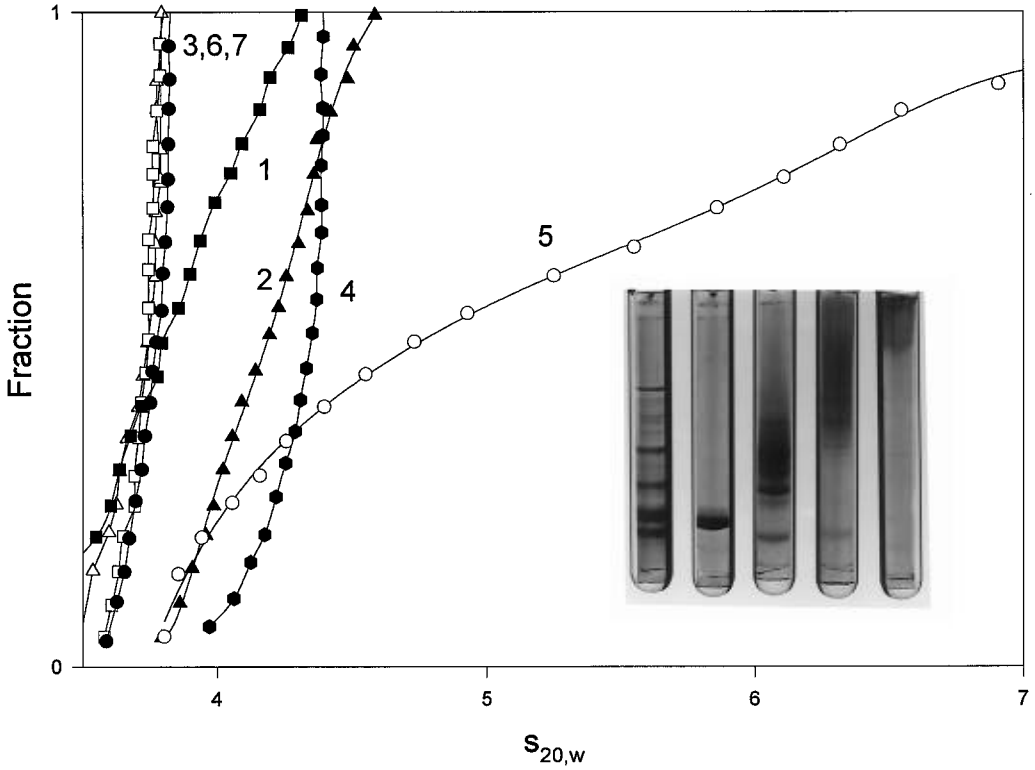

FIGURE 4: van Holde−Weischet plots showing the infinite-time extrapolated values of $s_{20,w}$ corresponding to various positions along the sedimenting boundary of a paucidisperse solute. (1) 0.4 mg/mL polymerized calmodulin, fraction 9-S1 (0.2 mM EDTA). (2) 0.4 mg/mL fraction 9-S1 (0.45 mM $CaCl_2$). (3) 4.3 μM myosin light chain kinase (△). (4) 4.3 μM myosin kinase plus 4.5 μM native calmodulin (0.45 mM $CaCl_2$). (5) 4.3 μM myosin kinase plus 4.9 μM fraction 9-S1 (0.45 mM $CaCl_2$). (6) Same as no. 5 (0.2 mM EDTA) (□). (7) Same as no. 5 plus 40 μM mastoparan (0.45 mM $CaCl_2$) (●). The reproducibility of the $s_{20,w}$ values was generally better than 1%. Other conditions: 0.15 M NaCl, 10 mM Mops, 2.0 mM dithiothreitol, pH 7.3. The inset from left to right shows the results of $NaDodSO_4$ gel (7.5%) electrophoresis performed on standards (phosphorylase, 96 kDa; bovine serum albumin (faint), 66 kDa; actin, 42 kDa; carbonic anhydrase, 29 kDa; troponin C, 18 kDa; and parvalbumin, 12 kDa), on unmodified calmodulin, and on fractions 9-S2, 9-S1, and 50-S4.

Table 4: Spectral Properties of Cross-Linked Calmodulin

	CaM	Dityr std
emission maximum[a]	401 nm	407 nm
excitation maximum[a]	319 nm	315 nm
emission maximum[b] (boric acid/borate)	377 nm	377 nm
excitation maximum[b] (boric acid/borate)	296 nm	294 nm
pK_a[c]	8.0−8.1; 8.2−8.3 (Ca^{2+})	7.0−7.1[f]
absorption maximum[a]	322 nm	315 nm
absorption maximum	284 nm[d]	283 nm[e]

[a] 0.10 M Na_2CO_3, pH 9.5. [b] 0.5 M boric acid−sodium borate, pH 8.4 (25 °C). [c] 0.20 M KCl, 5 mM Tris-HCl, ±1 mM $CaCl_2$ (25 °C). Excitation wavelength: 330 nm. [d] 50 mM Mops, pH 6.6. [e] 20 mM acetic acid, pH 4. [f] Lehrer and Fasman (1967).

(0 Ca^{2+}); 2.62S (Ca^{2+}) (Malencik & Anderson, 1994)]. (3) Smooth muscle myosin light chain kinase, which has a molecular weight of 108 000 and a sedimentation coefficient of 3.74S (Ausio et al., 1994), cosediments with 9-S1. The resulting $s_{20,w}$ values range from 3.8S to 7.7S. The addition of 0.2 mM EDTA or 40 μM mastoparan, a high-affinity calmodulin binding peptide (Malencik & Anderson, 1983, 1986), reverses the association of the polymers with the enzyme. For comparison purposes, Figure 4 includes data obtained with the enzyme alone and with the native calmodulin−enzyme complex. (Note that with all the enzyme-containing samples, less than 6% of the total absorbance at the monitoring wavelength is due to calmodulin.)

Consideration of Molecular Weight Limits Attained. We have applied several methods to the detection of polymerization in samples of cross-linked calmodulin: $NaDodSO_4$ gel electrophoresis, sedimentation velocity, gel filtration, and sedimentation equilibrium. The results of $NaDodSO_4$ gel electrophoresis suggest varying degrees of polymerization that are dependent on both the concentration of calmodulin present during polymerization (Figure 2) and on the fractionation procedures employed (Figures 4 and 6). In order to obtain high molecular weight samples for characterization, we isolated a subset of polymerized calmodulin fractions from the reaction mixture containing 50 mg/mL calmodulin. Successive purifications on Sephacryl S-200 HR and Sephacryl S-300 HR columns (Materials and Methods) yield fractions that are well removed from both the void volume of the latter column and the elution positions of the photogenerated calmodulin dimers and the native monomer (Figure 5). These were divided into pools 50-S1 through 50-S6 (Table 2).

The results of the sedimentation equilibrium and $NaDodSO_4$ polyacrylamide gel (7.5%) electrophoresis experiments complement and reinforce each other since the two methods are subject to different limitations. Electrophoresis of fractions 50-S2 and 50-S4 (inset to Figure 6) reveals broad bands, comprising multiple components with significantly lower mobilities than that of the 96 kDa subunit of glycogen phosphorylase. The diffuse nature of these bands is due in part to the fact that differently cross-linked calmodulin polymers of the same molecular weight may have varying radii of gyration and hence varying mobilities in gel electrophoresis [cf. Nielsen and Reynolds (1978)]. For

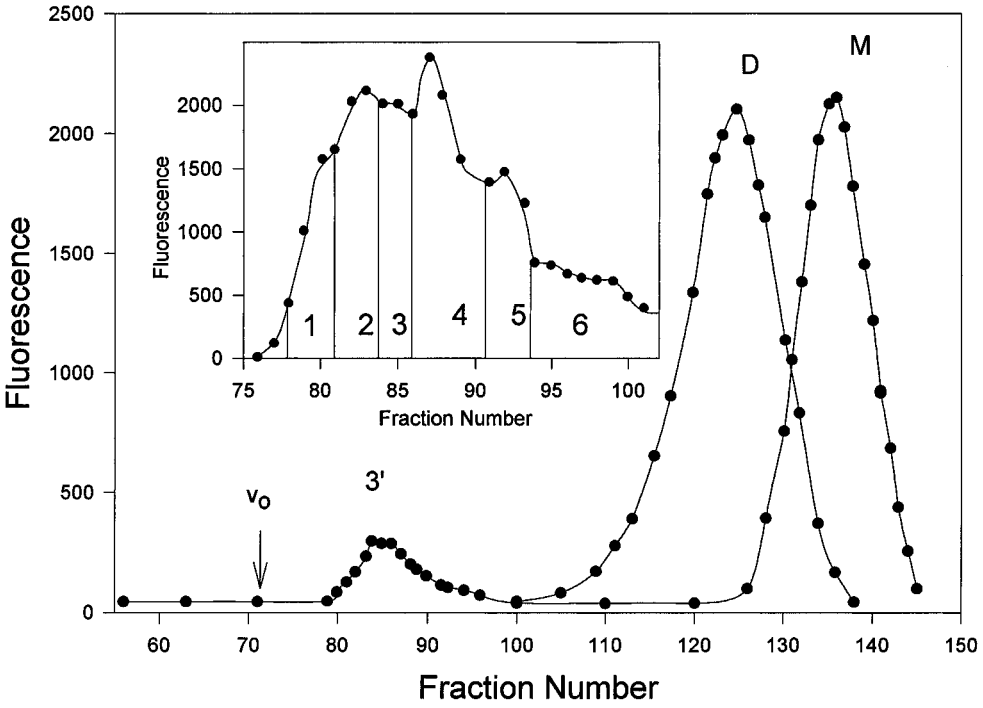

FIGURE 5: Fractionation of cross-linked calmodulin on a Sephacryl S-300 HR sizing column (inset). This sample (15.5 mg) had been polymerized at a protein concentration of 50 mg/mL and subjected to preliminary fractionation on Sephacryl S-200 HR (Materials and Methods). The pools correspond to fractions 50-S1 through 50-S6. The outer figure shows the separations obtained in separate runs with the native calmodulin monomer (M), the photogenerated dimers (D), and refractionated 50-S3 (3'). Tubes contained 2.36 mL. Excitation: 320 nm. Emission: 400 nm. (Slits widths normalized to 5 nm.) Path: 1 cm.

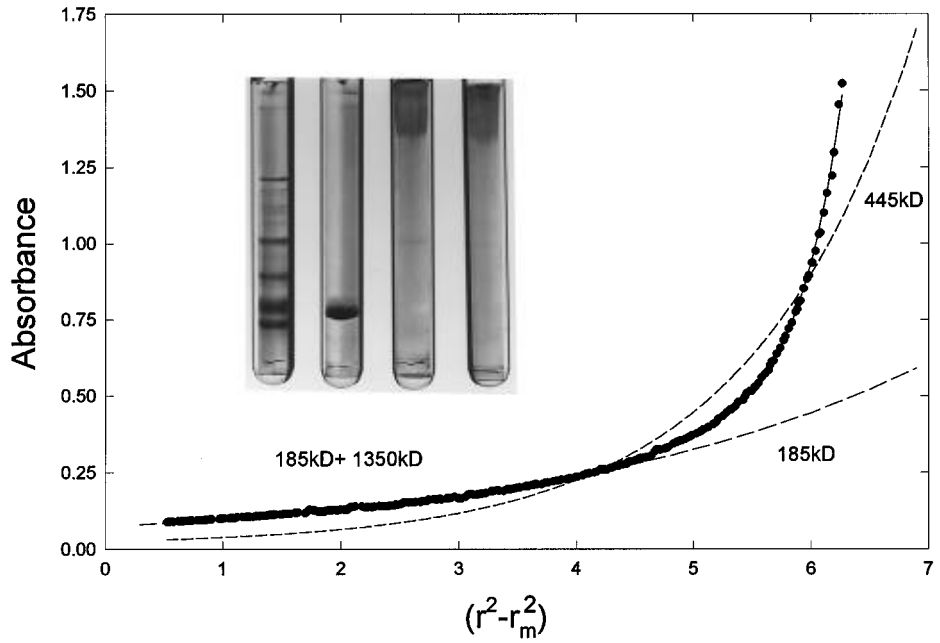

FIGURE 6: Sedimentation equilibrium of a highly polymerized calmodulin sample. The absorbance monitored at 280 nm is plotted as a function of radial position, where r^2 is the square of the distance from the axis of rotation (cm^2) and r_m^2, is the square of the distance of the meniscus from the axis (cm^2). Smooth curves were calculated for a single 445 kDa component, for a single 185 kDa component, and for two components of 185 (80%) and 1350 kDa (20%). Rotor speed: 5200 rpm. Conditions: 0.6 mg/mL fraction 50-S4, 10 mM Mops, 0.15 M NaCl (pH 7.3) (4 °C). The inset shows the results of $NaDodSO_4$ gel (7.5%) electrophoresis performed (from left to right) on standards (phosphorylase, 96 kDa; bovine serum albumin (faint), 66 kDa; actin, 42 kDa; carbonic anhydrase, 29 kDa; troponin C, 18 kDa; and parvalbumin, 12 kDa), on unmodified calmodulin, and on fractions 50-S4 and 50-S2.

example, the cross-linked calmodulin dimers migrate as three major components in the Laemmli system (Malencik & Anderson, 1994) (two predominant bands are visible in the gel photo shown in Figure 2), and the cross-linked calmodulin monomer migrates faster than the unmodified monomer (Malencik & Anderson, 1987). The multiplicity possible for large polymers is very high (see overview).

Sedimentation equilibrium determinations on three of the pools (50-S2, 50-S4, 50-S5) give qualitatively similar results. Figure 6 compares the sedimentation equilibrium data obtained with pool 50-S4 to three different theoretical curves: calculated for single components of either 185 or 445 kDa and for a mixture of two components of 185 (80% of absorbance) and 1350 kDa (20% of absorbance). No one

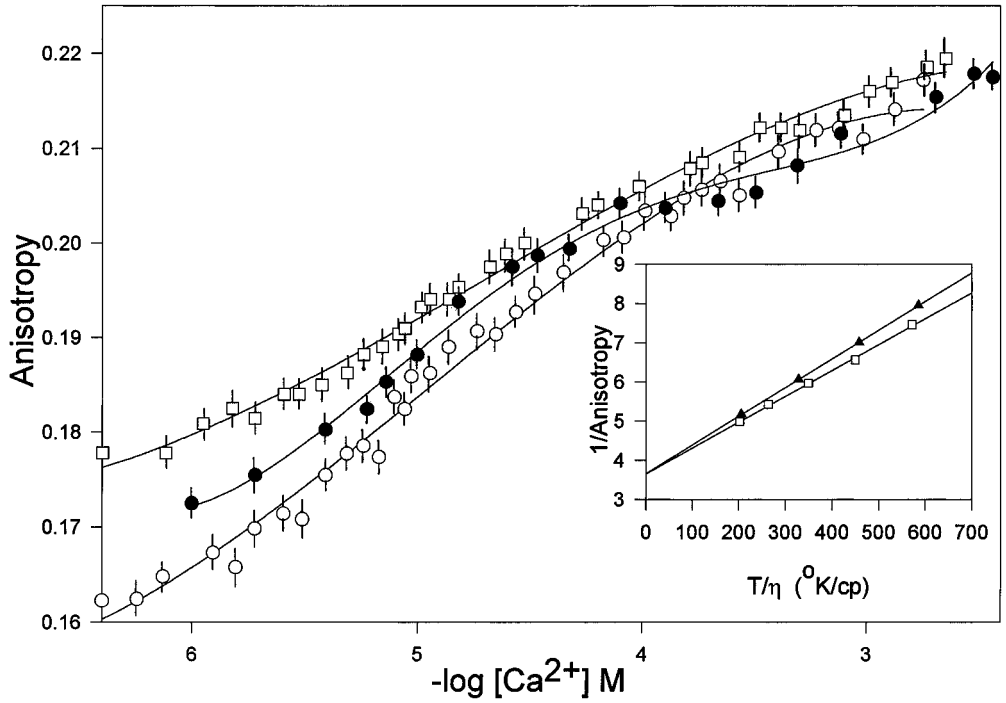

FIGURE 7: Observed fluorescence anisotropy values of polymerized calmodulin fractions determined as a function of total $CaCl_2$ concentration (M). (○) Fraction 9-S2 (see Table 2). (●) Fraction 50-S5. (□) Fraction 50-S2. (The smooth curves are not theoretical. Fractions 9-S1, 50-S4, and 50-S6 gave results similar to those shown.) The reproducibility of the anisotropy values was typically ±0.001. Conditions: 16 μg/mL polymer in 50 mM Tris, 0.15 M NaCl, pH 8.5 (25 °C). Excitation: 320 nm (5 nm bandwidth). Emission: 400 nm (10 nm bandwidth). Inset: Perrin plots for (□) fraction 50-S2 and (▲) fraction 9-S1. The straight lines correspond to $r_0 = 0.274$ and ϕ/τ 20 °C = 1.89 (□) and to $r_0 = 0.274$ and ϕ/τ 20 °C = 1.70 (▲). Conditions: 14 μg/mL polymer in 50 mM Tris, 0.15 M NaCl, 50 μM EDTA, pH 8.5.

single component accounts for the observed dependence of the absorbance on radial position: the nearest overall approach is attained with 445 kDa. On the other hand, the theoretical curve for the two components closely describes the behavior of 50-S4. (Treated as a fit to the data, these values give $\chi^2 = 1.6 \times 10^{-5}$.) The agreement between the calculated and observed curves should not be taken as a literal reflection of the composition of the sample, however.

We envision that pool 50-S4 contains a population of different molecular weight species that is centered near 185 kDa plus a second population of much higher molecular weight. This range, which is not affected by the shapes of the polymers [cf. van Holde (1985)], is in general agreement with that suggested by the $NaDodSO_4$ polyacrylamide gel experiments. Programs for determining three and higher-component fits are not immediately available. However, the results of such analyses could not differ greatly from the close approach obtained with the two mathematical components.

Fluorescence Anisotropy Studies of Calcium Binding by Samples of Polymerized Calmodulin. Anisotropy measurements employing the fluorescence of the dityrosine chromophore demonstrated the range of Ca^{2+} concentrations over which conformational changes (elongation and loss of flexibility) take place in the cross-linked calmodulin monomer (Malencik & Anderson, 1987; Small & Anderson, 1988), in a conjugate of [Tyr^8]substance P with calmodulin (Malencik & Anderson, 1988), and in the cross-linked calmodulin dimers (Malencik & Anderson, 1994). In the latter two cases, the concentration of Ca^{2+} producing half of the maximum increase in anisotropy (11−12 μM) compares to that expected from the average dissociation constant of the native calmodulin−Ca^{2+} complexes. The stepwise addition of Ca^{2+} to calcium-free samples of polymerized calmodulin (9-S1, 9-S2, 50-S2, 50-S4, 50-S5, and 50-S6) (Table 2) demonstrates increases in the average anisotropy[3] taking place over total Ca^{2+} concentrations of ∼1 μM to ∼1 mM or higher (Figure 7). With ∼70% or more of the total anisotropy change reached at 100 μM Ca^{2+}, these titration curves are more like that of the calmodulin dimers than they are like that of the cross-linked monomer. However, residual increases in average anisotropy detected at [Ca^{2+}] > 1 mM suggest the presence of weaker binding components in the polymers.

Overall decreases in flexibility accompanying Ca^{2+} binding largely account for the increases in average anisotropy. The *global* rotational correlation time, which is directly related to molecular volume, is 6.8 ns (20 °C) in the case of the Ca^{2+}-free calmodulin monomer (Small & Anderson, 1988). Accordingly, the larger calmodulin polymers should undergo little overall rotational diffusion during the 3.9 ns excited state lifetime (τ) of dityrosine. The influence of faster rotations (such as those due to local segmental flexibility) in Ca^{2+}-free samples of 9-S1 and 50-S2 (Table 2) is evident in calculations of the *average* rotational correlation time (ϕ) from the observed anisotropy (r) and the anisotropy extrapolated to infinite viscosity (r_0).

$$\phi/\tau = r/(r_0 - r) \qquad (3)$$

The Perrin plots [cf. Weber (1952) and Lakowicz (1983)] shown in the inset to Figure 7 correspond to average values of ϕ of 6.6 and 7.4 ns for these two disparate samples.

Activation of Smooth Muscle Myosin Light Chain Kinase and Calcineurin by Fractions of Polymerized Calmodulin. Table 5 shows that the photogenerated dimer and polymer-

Table 5: Activation of Smooth Muscle Myosin Light Chain Kinase by Fractions of Polymerized Calmodulin[a]

	reaction rate (μM NADH/min)[b] obtained at indicated concentrations of sample[c]		
fraction	10.5 nM[c]	21 nM[c]	10.5 nM[c] plus 10.5 nM native CaM
native CaM	1.15	1.14[d]	
CaM dimer	1.13		
9-S1[b]	1.15		
9-S2[b]	1.19		
50-S1[b]	0.19	0.35[d]	1.26
50-S2[b]	0.55	0.91[d]	1.10
50-S3[b]	0.64	1.06[d]	1.13
50-S4[b]	0.62	1.10[d]	1.20
50-S5[b]	0.68	1.03[d]	1.10
50-S6[b]	0.74	1.06[d]	

[a] Conditions: 63 μM synthetic myosin kinase substrate, 0.11 mM ATP, 2.5 mM Mg $(CH_3CO_2)_2$, 0.28 mM $CaCl_2$, 2 mM dithiothreitol, 10.5 nM myosin kinase, 42 mM Mops, pH 7.3, 25 °C (see Materials and Methods for coupling system). [b] Reproducibility was generally ±4%. [c] Concentration based on 1 mol = 16 700 g. [d] The addition of 2 mM EGTA to the assay reduced the rate of NADH oxidation to −0.07 ± 0.01 μM/min. [e] Refer to Table 2 for key to polymerized calmodulin fractions.

Table 6: Activation of Calcineurin by Fractions of Polymerized Calmodulin

	relative activities[a] determined with	
fraction	1 μM sample[b]	1 μM sample plus 1 μM native CaM
native CaM	2.6[d]	
CaM dimer	2.5	
9-S1[c]	1.15	1.66
9-S2[c]	1.70	2.1
50-S1[c]	0.75	1.85
50-S2[c]	0.80	1.79
50-S3[c]	0.86	1.58
50-S4[c]	0.80	1.67
50-S5[c]	0.83	1.73
50-S6[c]	0.96	1.73

[a] Ratio of the rate of hydrolysis obtained with the enzyme in the presence of the indicated CaM sample to the rate obtained with the enzyme alone. Conditions: ~0.5 μM calcineurin, 0.97 mg/mL *p*-nitrophenyl phosphate, 50 mM Mops, 1 mM dithiothreitol, 1 mM $MnCl_2$, pH 7.3, 20 °C. [b] One mole is defined as 16 680 g. [c] Refer to Table 2 for key to polymerized calmodulin fractions. [d] The error was typically ±0.1 in all the assays.

ized fractions 9-S2 and 9-S1 (check Figure 4) activate myosin light chain kinase as effectively as does native calmodulin. The polymers resulting from the reaction carried out at 50 mg/mL also activate the enzyme, but on the average less completely than did the preceding derivatives. Increasing the concentration of polymer in the assay from 10.5 to 21 nM results in further stimulation in these cases. Maximum enhancement occurs in all the assays to which 10.5 nM native calmodulin was added. Since the K_m of the enzyme for calmodulin is ~1 nM (Malencik & Anderson, 1986), the reaction rate obtained with 10.5 nM each of the two native proteins is close to V_{max}. The low rate of NADH oxidation determined in assay mixtures containing no Ca^{2+} (2 mM EGTA) reflects the background ATPase activities of the enzymes.

Native calmodulin and the photochemically cross-linked dimer stimulate the *p*-nitrophenyl phosphatase activity of calcineurin 2.5−2.6 fold (Table 6). However, in contrast to the results obtained with myosin light chain kinase, only the least polymerized fraction in the present study (9-S2) significantly activates calcineurin. The more highly polymerized material tested inhibits the basal activity of the enzyme and apparently competes with native calmodulin.

DISCUSSION

The value of *Arthromyces* peroxidase as a tool for the zero-length cross-linking of proteins is strongly supported by our observations on cross-linked bovine brain calmodulin. First, the cross-linking may take place with a high degree of efficiency. Samples of cross-linked calmodulin recovered after fractionation account for ~40% of the original protein. Second, intermolecular cross-linking catalyzed by *Arthromyces* peroxidase may result in products that are both highly polymerized and soluble. Third, the reaction may result in the coupling of most or all of the reactive tyrosyl side chains, with minimal concomitant modification of other amino acid residues. Fractions of cross-linked calmodulin contain close to the maximum possible amounts of dityrosine. The quantities of phenylalanine and lysine are normal while those of methionine correspond to 70−90% of the value expected for the untreated protein. The residual amounts of tyrosine are very low, beyond the limits of detection in several cases. Some of the tyrosyl residues that remain at the unreacted ends of the polymers may take part in alternate reactions or undergo intrapolymer coupling.

In addition, enzyme-catalyzed cross-linking, carried out under carefully controlled and monitored conditions, may preserve some or all of the biological activity of the affected protein. The various polymerized fractions of calmodulin undergo Ca^{2+}-dependent conformational changes detected in sedimentation velocity or fluorescence anisotropy measurements. All of the samples of polymerized calmodulin activate smooth muscle myosin light chain kinase. The *average* activities determined in catalytic assays employing the synthetic phosphate acceptor peptide as substrate correspond to 50−100% of the activity obtained with native calmodulin. The higher molecular weight fractions tested do not significantly stimulate the *p*-nitrophenyl phosphatase activity of calcineurin. However, they apparently compete with native calmodulin in the binding of the enzyme.

The *Arthromyces* peroxidase-catalyzed cross-linking of calmodulin occurs most effectively in Ca^{2+}-free solutions that contain boric acid−sodium borate (concentration ≥0.2 M). There are well defined pH and temperature optima near 8.3 and 40 °C, respectively. The distinctive pH dependence of the yields obtained with calmodulin and poly(Ala, Glu, Lys, Tyr) (Figure 3A) may relate to the effect of boric acid/sodium borate on the reaction (Figure 3B) and to the ionizations of lysyl and tyrosyl side chains. Free dityrosine undergoes a reversible association with boric acid/monoborate ion that is optimal near pH 8 (Malencik & Anderson, 1991).[6] Fluorescence spectra of the calmodulin polymers in buffers containing these components (Table 4) reveal interactions that may exert a stabilizing influence on protein dityrosine or relate to complexed reaction intermediates. Since poly(Glu, Tyr) 4:1 is not a substrate for any of the

[6] The optimum pH is a function of the pK_a. For a dityrosyl moiety with a pK_a of 8.0 to 8.1, maximum association with boric acid/monoborate will occur at pH 8.6−8.7 [cf. Malencik and Anderson (1991)].

Table 7: Calcium-Binding and Related Proteins as Peroxidase Substrates

protein	peroxidase-induced dityrosine fluorescence[b]	
	Arthromyces	horseradish
calmodulin	100%	0%
skeletal muscle troponin C	82.6%	0%
20 kDa myosin light chain	96.5%	0%
16 kDa myosin light chain	39.2%	6.3%
DTNB myosin light chain	6.6%	0
parvalbumin	19.8%	

[a] Conditions: 50 μg/mL peroxidase, 50 μg/mL superoxide dismutase, 120 μM H_2O_2, protein concentrations adjusted to give 110 μM total tyrosine, 0.25 M boric acid−sodium borate, pH 8.4, 39 °C. [b] Determined with an excitation wavelength of 301 nm and an emission wavelength of 377 nm.

peroxidases, maintenance of positively charged lysyl side chains may be important in calmodulin and poly(Ala, Glu, Lys, Tyr). This would contribute to the abrupt decline in yields found at pH values greater than 8.3. The dipeptide L-tyrosyl-L-tyrosine displays a different reaction profile, with maximum dityrosine formation detected near pH 8.9 (Figure 3A).

Several precautionary measures helped to ensure the good yields obtained. First, fluorometric monitoring establishes the minumum amount of H_2O_2 required, avoiding exposure of the protein to large excesses of the peroxide. Second, the addition of reduced glutathione and glutathione peroxidase stops the gradual decline in dityrosine fluorescence that occurs upon prolonged standing of the reaction mixtures. Third, the presence of superoxide dismutase results in moderate enhancement of the reaction (Figure 2). This may reflect the removal of superoxide anion or some other activity of the enzyme. No reaction is detected when calmodulin is incubated with H_2O_2 and superoxide dismutase alone.

The enzyme-catalyzed cross-linking described by Aeschbach et al. (1976) has several disadvantages when compared to our procedure. (1) Large excesses of H_2O_2 were employed. (2) There was no monitoring. (3) Reactions were carried out at pH 9.5, a range that is highly unfavorable for either poly(Ala, Glu, Lys, Tyr) or calmodulin. (4) Nothing was done to remove unreacted H_2O_2, free radicals, or possible hydroperoxides. (5) The peroxidase used has a more limited range of action than that of *Arthromyces* peroxidase. A separate attempt at horseradish peroxidase-catalyzed protein cross-linking, performed in the presence of catechol, resulted in the oxidation of lysine (Stahmann & Spencer, 1977).

The broad specificity exhibited by *Arthromyces* peroxidase significantly extends the range of proteins that may be cross-linked by phenolic coupling. Experiments with random copolymers suggest that exposed tyrosyl residues located within sequences or environments that include neutral or basic residues are possible targets for all of the common peroxidases. None of the five enzymes tested catalyzes dityrosine formation in the acidic copolymer poly(Glu, Tyr) 4:1.[3] *Arthromyces* peroxidase stands out for its ability to catalyze cross-linking reactions in the copolymer poly(Ala, Glu, Lys, Tyr) 6:2:5:1 and in the acidic protein calmodulin. Preliminary experiments with proteins that are evolutionarily related to calmodulin reveal additional cases of cross-linking catalyzed by *Arthromyces* peroxidase but not by horseradish peroxidase (Table 7).

We have elaborated on the intermolecular cross-linking of a protein containing two reactive tyrosyl residues. However, the conditions and principles established extend to other cases, including intramolecular cross-linking (for possible conformational stabilization) and the generation of heteropolymers or of other homopolymers. The coupling reaction may be directed through adjustments to the protein concentrations and the addition or removal of reactive tyrosyl residues by genetic engineering. The photoactivated conjugation of [Tyr^8]substance P to calmodulin (Malencik & Anderson, 1987) demonstrated the feasibility of preparing heterodimers by phenolic coupling.

The *Arthromyces* peroxidase-catalyzed reaction also may be used to attach proteins to solid supports or to couple antigens to carriers for use in antibody production. Zero-length protein cross-linking often is used for the latter purpose since the incorporation of homobifunctional or heterobifunctional reagents sometimes results in antibodies that are specific for the reagent [cf. Hermanson (1996)]. The zero-length cross-linking of tyrosyl side chains rather than of carboxylate groups and/or lysyl side chains (the usual targets of zero-length cross-linking) has several possible advantages. Lysyl, glutamyl, and aspartyl side chains that occur in antigenic sites would not be affected directly by the reaction. Since tyrosine has a more limited average rate of occurrence in proteins than lysine (Klapper, 1977) and the enzyme has a degree of specificity, the average number of cross-links introduced per molecule may be less and the likelihood of obtaining a soluble product correspondingly increased.

Enzyme-catalyzed phenolic coupling may be useful in the detection of protein conformational changes that affect the reactivities, accessibilities, and/or local environments of protein tyrosyl residues. As an example of this possibility, we find that the peroxidase-catalyzed cross-linking of calmodulin (but not of the copolymers) occurs only in the absence of Ca^{2+}. Finally, the intense visible fluorescence characteristic of dityrosine-containing proteins would be an advantage in all applications of protein cross-linking. No other *in vitro* protein cross-linking technique results in visibly fluorescent products.

REFERENCES

Aeschbach, R., Amadò, R., & Neukom, H. (1976) *Biochim. Biophys. Acta 439*, 292−301.

Amadò, R., Aeschbach, R., & Neukom, H. (1984) *Methods Enzymol. 107*, 377−388.

Andersen, S. O. (1964) *Biochim. Biophys. Acta 93*, 213−215.

Anderson, S. R. (1991) *J. Biol. Chem. 266*, 11,405−11408.

Ausio, J., Malencik, D. A., & Anderson, S. R. (1992) *Biophys. J. 61*, 1656−1663.

Babu, Y. S., Sack, J. S., Greenhough, T. G., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) *Nature 315*, 37−40.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Charbonneau, H., & Cormier, J. J. (1979) *Biochem. Biophys. Res. Commun. 90*, 1039−1047.

Deits, T., Farrance, M., Kay, E. S., Medill, L., Turner, E. E., Weidman, P. J., & Shapiro, B. M. (1984) *J. Biol. Chem. 259*, 13525−13533.

Flohé, L. (1989) in *Glutathione* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) pp 643−731, John Wiley & Sons, New York.

Hermanson, G. T. (1996) *Bioconjugate Techniques*, Academic Press, Inc, San Diego.

Holler, T. P., & Hopkins, P. B. (1989) *Anal. Biochem. 180*, 326−330.

Kerrick, W. G. L., Malencik, D. A., Hoar, P. E., Potter, J. D., Coby, R. L., Pocinwong, S., & Fischer, E. H. (1980) *Pflügers Arch. 386*, 207−213.
Klapper, M. H. (1977) *Biochem. Biophys. Res. Commun. 78*, 1018−1024.
Knecht, R., & Chang, J. Y. (1986) *Anal. Chem. 58*, 2375−2379.
Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, (Lakowicz, J. R., Ed.) pp 111−153, Plenum Press, New York.
Lehrer, S. S., & Fasman, G. D. (1967) *Biochemistry 6*, 757−767.
Malencik, D. A., & Anderson, S. R. (1986) *Biochemistry 25*, 709−721.
Malencik, D. A., & Anderson, S. R. (1987) *Biochemistry 26*, 695−704.
Malencik, D. A., & Anderson, S. R. (1988a) *Biochemistry 27*, 944−950.
Malencik, D. A., & Anderson, S. R. (1988b) *Biochemistry 27*, 1941−1949.
Malencik, D. A., & Anderson, S. R. (1991) *Biochem. Biophys. Res. Commun. 178*, 60−67.
Malencik, D. A., & Anderson, S. R. (1994) *Biochemistry 33*, 13363−13372.
Malencik, D. A., Anderson, S. R., Bohnert, J. L., & Shalitin, Y. (1982) *Biochemistry 21*, 4031−4039.
Malencik, D. A., & Anderson, S. R. (1983) *Biochem. Biophys. Res. Commun. 114*, 50−56.
Malencik, D. A., & Zhao, Z., & Anderson, S. R. (1990) *Anal. Biochem. 184*, 353−359.
Nielsen, T. B., & Reynolds, J. A. (1978) *Methods Enzymol. 48*, 3−10.
Pallen, C., & Wang, J. H. (1983) *J. Biol. Chem. 258*, 8550−8553.
Small, E. W., & Anderson, S. R. (1988) *Biochemistry 27*, 419−428.
Sobieszek, A., & Barylko, B. (1984)in *Smooth Muscle Contraction* (Stephens, N. L., Ed.) pp 283−316, Marcel Dekker, New York.
Stahmann, M. A., & Spencer, A. K. (1977) *Biopolymers 16*, 1299−1306.
Tallant, E. A., Wallace, R. W., & Cheung, W. Y. (1983) *Methods Enzymol. 102*, 244−256.
van Holde, K. E. (1985) in *Physical Biochemistry* (van Holde, K. E., Ed.) pp 124−129, Prentice Hall, Englewood Cliffs, NJ.
van Holde, K. E., & Weischet, W. (1978) *Biopolymers 17*, 1387−1403.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962−975.
Weber, G. (1952) *Biochem. J. 21*, 145−155.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406−4412.
Wong, S. S. (1993) in *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press, Inc, Boca Raton, FL.

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