Flexibility Involving the Intermolecular Dityrosyl Cross-Links of Enzymatically Polymerized Calmodulin†

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ABSTRACT: The role of dityrosine as a fluorescent crossbridge between adjacent calmodulin molecules within the high molecular mass polymers that are generated by *Arthromyces* peroxidase-catalyzed cross-linking [Malencik, D. A., and Anderson, S. R. (1996) *Biochemistry 35*, 4375] has been examined in frequency domain fluorescence anisotropy studies. Measurements on a polymer fraction possessing a range of molecular masses > 96 000 in $NaDodSO_4$ polyacrylamide gel electrophoresis demonstrate predominating fast local rotations involving the dityrosyl moieties. Normal distribution analyses of the results show peak rotational correlation times of 0.6 ns (zero Ca^{2+}) and 1.2 ns (+Ca^{2+}), values that are smaller than the principal correlation times determined for the global rotation of the free calmodulin monomer in either the presence or absence of Ca^{2+}. The intermolecularly cross-linked segments of the polymers retain a degree of the mobility that is characteristic of the tyrosine-containing sequences of native calmodulin. The half-widths of the normal distribution curves range from 13 ns (zero Ca^{2+}) to ∼90 ns (5 mM Ca^{2+}), thus encompassing varying rates of segmental motion within the polymers. When Ca^{2+} is present, possible contributions from the global rotations of polymer molecules are detected near the operating limits of the method. Experiments with the intramolecularly cross-linked calmodulin monomer give global rotational correlation times of 7.9 ns (zero Ca^{2+}) and 11.4 ns (+Ca^{2+}), which compare to values of 7.2 ns and 9.9 ns found previously in time domain measurements [Small, E. W., and Anderson, S. R. (1988) *Biochemistry 27*, 419]. Rotations of apparent $\phi_2 = 0.2$ to 0.3 ns also are detected, accounting for 31% (−Ca^{2+}) to 23% (+Ca^{2+}) of the anisotropy.

Calmodulin, an evolutionarily conserved protein containing 148 amino acid residues, is a major intracellular receptor for Ca^{2+} in plants and animals. Specific enzymes, involved in a wide variety of physiological processes, typically undergo large increases in catalytic activity upon association with the calmodulin−calcium complex (*1*). X-ray crystallographic studies indicated that the Ca^{2+}-liganded calmodulin molecule consists of two globular lobes connected by an eight-turn α-helix. Each lobe contains two Ca^{2+} ions bound to helix-loop-helix motifs (*2*, *3*). Multidimensional NMR spectroscopy confirmed the crystal structure, with the exception that residues 78−81 are in a loose nonhelical conformation in solution (*4*). The NMR studies also showed that Ca^{2+}-free calmodulin is a flexible dumbbell, possessing stable secondary structure but lacking the hydrophobic cavity necessary for enzyme binding (*5*, *6*).

Malencik and Anderson (*7*) discovered that the UV irradiation of bovine brain calmodulin results in the Ca^{2+}-dependent intramolecular coupling of its only two tyrosyl residues, Tyr^{99} and Tyr^{138}, which reside, respectively, in Ca^{2+}-binding domains III and IV of the native protein (*2*, *8*). The cross-linked calmodulin molecule isolated from UV-irradiated solutions exhibited diminished interactions with both Ca^{2+} and four different enzymes. It was unable to activate either myosin light chain kinase or calcineurin in catalytic assays. These properties contrast to those of the nativelike cross-linked dimers of calmodulin that were obtained later when UV-irradiation was carried out in the presence of superoxide dismutase (*9*).

Dityrosine formation (see Figure 1 for structure) is a recognized posttranslational protein modification, occurring both as a normal physiological process (*10*, *11*) and as a response to environmental agents (*12*−*16*). We expanded our interests in reactions leading to the production of dityrosine since they lead simultaneously to the introduction of a protein cross-link and a fluorescent probe. Fluorescence measurements on dityrosine provide information on the behavior of intermolecular and intramolecular cross-links in proteins and, in appropriate cases, the global hydrodynamic properties of the cross-linked molecules. The singly ionized form of dityrosine exhibits intense 400 nm-range fluorescence (*13*), a quantum yield of ∼85% (*17*), and an excited state lifetime of 4 ns (*18*). These characteristics facilitated steady-state (*7*) and time domain (*18*, *19*) fluorescence anisotropy measurements on the intramolecularly cross-linked calmodulin molecule.

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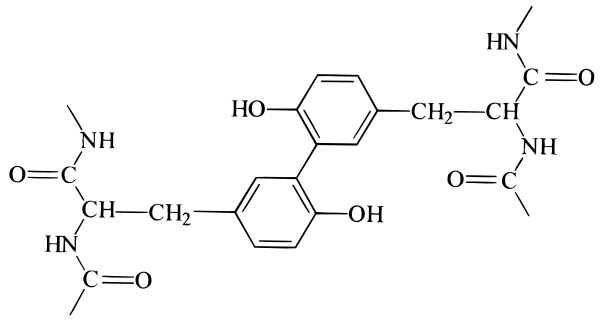

FIGURE 1: Schematic structure of a protein dityrosyl cross-link.

Malencik and Anderson (*20*) developed an efficient enzyme-catalyzed cross-linking procedure leading to high molecular mass calmodulin polymers containing close to the maximum possible amount of dityrosine. Various polymer fractions, obtained through gel filtration and affinity chromatography of the reaction mixtures, activate smooth muscle myosin light chain kinase but not calcineurin. They also display large changes in steady-state fluorescence anisotropy upon Ca^{2+} binding that form the basis of the present frequency domain fluorescence experiments. The enzymatically cross-linked calmodulin preparation provides a unique opportunity for direct observation of the local motions of intermolecular protein cross-links and attached sections of polypeptide chain. The fraction selected for the frequency domain fluorescence anisotropy measurements should exhibit relatively little global rotation on the 4 ns time scale. The results that will be described in this article may contribute to understanding of the pliant nature of calmodulin, of the relative effectiveness of the polymers in the activation of myosin light chain kinase, and of the properties of dityrosine-containing proteins in general.

MATERIALS AND METHODS

Proteins. The cross-linked calmodulin monomer (16.7 kDa) was produced by UV-irradiation of a solution containing 0.5 mg/mL bovine brain calmodulin in 10 mM Tris,[1] 1 mM $CaCl_2$, pH 8.3, as described by Malencik and Anderson (*7*). The purification procedure presented in their report includes gel filtration on an LKB Ultrogel AcA54 sizing column to remove polymers and affinity chromatography on phenylagarose. We confirmed the monomeric state of the product in $NaDodSO_4$ gel electrophoresis (*21*) and in sedimentation velocity experiments performed in the Beckman XL-A analytical ultracentrifuge. The former experiment rules out intermolecular cross-linking, while the latter shows that there is no aggregation of the modified monomer ($\pm Ca^{2+}$). The cross-linked calmodulin polymers were prepared in a reaction catalyzed by *Arthromyces* peroxidase, carried out in a solution containing 30 mg/mL calmodulin in 0.20 M boric acid-sodium borate, pH 8.3, 38 °C, as detailed by Malencik and Anderson (*20*). The fractionation protocol outlined by them includes phenylagarose affinity chromatography and gel filtration on Sephacryl S-300 HR, which has an operating range of 40000−600000 kDa for globular proteins, to produce subfractions of varying degrees of average polymerization. The samples used in the present studies exhibited substantially lower mobilities in $NaDodSO_4$ polyacrylamide gel electrophoresis [performed on a 7.5% gel in glass tubes as described by Weber and Osborn (*21*)] than does the 96 kDa subunit of glycogen phosphorylase (Figure 2). Sedimentation equilibrium measurements on a similar sample were consistent with a limited range of molecular mass species centered near 185 kDa plus a lesser population of higher molecular mass (*20*). The results of these two independent methods thus confirm that the sample contains no low molecular mass components.

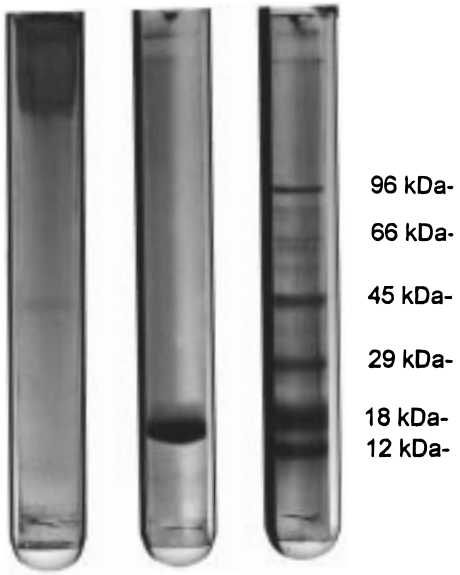

FIGURE 2: Results of $NaDodSO_4$ polyacrylamide gel (7.5%) electrophoresis performed (from left to right) on standards (phosphorylase, 96 kDa; bovine serum albumin (faint), 66 kDa; actin, 42 kDa; carbonic anhydrase, 29 kDa; troponin C, 18 kDa; and parvalbumin, 12 kDa), native unmodified calmodulin, and on the polymer sample.

Fluorescence Measurements. The frequency domain fluorescence measurements were performed at the Laboratory for Fluorescence Dynamics at the University of Illinois, Urbana-Champaign, using a multifrequency phase and modulation fluorometer. The frequency modulation of the excitation source used the harmonic content approach (*22*, *23*). The exciting light was from a Coherent Nd:YAG mode-locked laser pumping a rhodamine dye laser. The dye laser was tuned to 620 nm, which was then frequency doubled to 310 nm. Emission was observed through TB400 and WG335 filters to block exciting light and pass emission above 400 nm. The cross-linked calmodulin derivatives display the characteristically broad excitation and emission bands of singly ionized dityrosine, with corresponding maxima at 320 nm and 400−410 nm (*7*, *20*). The excitation wavelength and emission band-pass employed thus give close to maximum sensitivity while minimizing excitation of unionized dityrosine, which has an absorption maximum at 285 nm.

The exciting light was passed through a polarizer oriented parallel to the laboratory axis, and the emission was observed through a polarizer set at 55 to eliminate polarization artifacts

[1] Abbreviations: Mops, 3-morpholinopropanesulfonic acid; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CaM, calmodulin; MLCK, myosin light chain kinase; r, anisotropy; r_o, limiting or zero time anisotropy; r_1 and r_2, the anisotropies associated with the global and local rotations, respectively; ϕ_1 and ϕ_2, the rotational correlation times associated with the global and local rotations, respectively; t time after excitation; τ, lifetime of the excited state; ϕ_{corr}, peak rotational correlation time in a Gaussian distribution analysis.

Table 1: Frequency-Domain Measurements of the Intensity Decays of Dityrosyl Calmodulin Derivatives[a]

sample	$[Ca^{2+}]$ (mM)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_{av}[c] (ns)	χ^2
CaM monomer	0	4.67 (+0.21,−0.15)[f]	0.792[b]	1.77 (+0.33,−0.29)[g]	0.178[b]	4.01 (3.59)[e]	0.705
CaM monomer	20	4.74 (+0.25,−0.14)[g]	0.843[c]	1.95 (+0.51,−0.14)[g]	0.145[c]	4.28 (3.81)[e]	0.405
poly CaM	0	4.15[f] (±0.02)[g]	0.902	1.15 (±0.05)[g]	0.098	3.86	0.68
poly CaM	0.10	4.15[f] (±0.02)[f]	0.895	1.21 (±0.05)[g]	0.105	3.84	1.24
poly CaM	1.0	4.15[f] (±0.02)[g]	0.845	1.42 (±0.04)[g]	0.155	3.73	0.52
poly CaM	5.0	4.15[f] (±0.02)[g]	0.808	1.61 (±0.04)[g]	0.192	3.66	0.47

[a] Conditions: 3 μM CaM, 50 mM Mops, pH 7.4, 20 °C for monomer; 6 μM CaM, 10 mM Tris, 0.10 M KCl, pH 8.3, 20 °C for polymers. [b] The fit to the data included an apparent third component (which accounts for scattered light) with $\tau_3 = 0$, $\alpha_3 = 0.03$. [c] $\tau_3 = 0$, $\alpha_3 = 0.012$. [d] The weighted average lifetime, $\tau_{av} = \Sigma\alpha_i\tau_i/\Sigma\alpha_i$. [e] Data from Small and Anderson (*18*). [f] The value of τ_1 was fixed at 4.15 ns. [g] In parentheses, errors are reported as correlated 67% confidence limits of the reduced χ^2.

(*24*). Phase and modulation values for both lifetime and dynamic polarization data were obtained as previously described (*25*−*27*). The lifetime data were analyzed either by assuming a sum of discrete exponentials (*27*) or using a continuous distribution model which assumes either Lorentzian or Gaussian distributions (*28*−*30*). For the cross-linked polymers, a Gaussian distribution of rotational rates was used as a model instead of two discrete rotational correlation times. Analyses were performed using a constant, frequency-independent standard deviation of 0.2° for the phase angle and 0.004 for the modulation ratio. Nonlinear least-squares fits were performed with the Globals Unlimited software (Urbana, IL). Correlated error analyses were performed on the lifetimes and rotational correlation times, and the rigorous 67% confidence limits of the reduced χ^2 are reported.

Measurement Conditions. Lyophilized protein samples were dissolved in buffers prepared from distilled, deionized water immediately before the experiments were performed. The buffer consisted of 50 mM Mops, pH 7.4, in the case of the monomer and of 20 mM Tris, 0.10 KCl, pH 8.3, in the case of the polymers. The protein concentrations (3−6 μM in terms of equivalent monomer units)[2] corresponded to absorbance values at 310 nm that were less than 0.1, thus avoiding inner filter effects. The signal was well above background. The pH values employed correspond to the midrange of the ground-state ionizations characteristic of the monomer and the polymers (*7*, *20*). Constant temperature was maintained at 20.0 ± 0.1 °C during the measurements.

RESULTS

Excited-State Lifetime Determinations. Frequency-domain data for the intensity decays of both the cross-linked calmodulin monomer, which contains the intramolecular cross-link produced by the coupling of Tyr^{99} and Tyr^{138}, and the polymer sample correspond to two excited-state lifetimes plus a third component of 0 ns lifetime to account for scattered light in the case of the monomer. There is a predominant component (with a pre-exponential factor of 0.80 or higher) corresponding $\tau_1 = 4.15$ ns (polymers) or $\tau_1 = 4.7$ ns (monomer) and a lesser component with $\tau_2 = 1-2$ ns (Table 1). The measurements that were performed in the presence and absence of Ca^{2+} gave similar lifetimes and distributions. The 4 ns range of τ is ideally suited for observations on the global rotations of proteins the size of calmodulin, which as a spherical molecule containing 0.2 g H_2O/g of protein would exhibit a single rotational correlation time of 6.5 ns (20 °C) (*18*), and on the fast local rotations that may occur within protein molecules of any size. Note that the existence of more than one value of τ does not affect the determinations of rotational correlation time unless the τ values are associated with specific components (*31*).

Table 2: Distribution Analyses of the Anisotropy Decays of Dityrosyl-Linked Calmodulin Polymers[a]

$[Ca^{2+}]$ (mM)	ϕ_{corr} (ns)	width (ns)	r_o	χ^2
0	0.57 (+0.18,−0.17)[c]	12.6 (±0.7)[c]	0.322 (+0.01,−0.008)	1.45
0.01	0.88 (+0.28,−0.15)[c]	22 (+1.5,−4.5)[c]	0.313 (+0.021,−0.007)	1.29
0.03	2.0[b] (+0.34,−0.28)[c]	35 (+3.6,−3.4)[c]	0.308 (+0.003,−0.004)	0.95
0.10	1.0 (+0.46,−0.90)[c]	41 (+6.1,−25)[c]	0.330 (+0.045,−0.011)	2.37
1.0	1.2 (+0.36,−0.01)[c]	72 (+13,−37)[c]	0.329 (+0.009,−0.007)	1.58
5.0	1.2 (+0.3,−0.28)[c]	92 (+18,−74)[c]	0.330 (+0.008,−0.006)	1.15

[a] Figure 4 contains the six profiles of amplitude versus rotational correlation time. [b] This measurement was performed on a separate polymer preparation. [c] In parentheses, errors are reported as correlated 67% confidence limits of the reduced χ^2.

Frequency Domain Anisotropy Studies. We carried out the frequency domain anisotropy measurements shown in Figure 3 in order to learn whether there are Ca^{2+}-dependent rotations taking place on the 4 ns time scale that are sensed by the dityrosine chromophores of the enzymatically polymerized calmodulin sample. The differences between the phase angles of the parallel and perpendicular components of the emitted light ("phase", as given in Figure 3) and of the percent modulation (not shown for simplicity) as a function of modulation frequency demonstrate a suitable correlation between the 4 ns excited-state lifetime and the rates of Ca^{2+}-dependent rotations. We applied a normal or Gaussian distribution analysis to the data instead of the customary two- or three-component fit since polymerization is an inherently heterogeneous process (*32*), with the sample under study comprising a selected population exhibiting apparent molecular masses greater than 96 000 in $NaDodSO_4$

[2] Polymer concentrations are expressed in terms of monomer units, with 1 mol = 16.7 kDa.

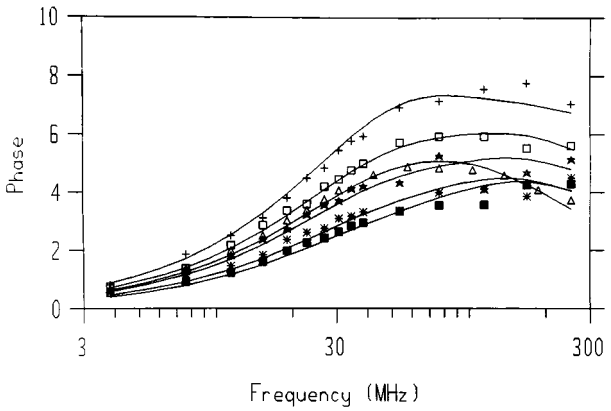

FIGURE 3: Frequency domain anisotropy data for a mixed population of high molecular mass calmodulin polymers. Differential polarized phase angle as a function of modulation frequency is shown for samples (from top to bottom) containing 0.1 mM EDTA (+), 10 μM $CaCl_2$ (□), 30 μM $CaCl_2$ (△), 100 μM $CaCl_2$ (☆), 1 mM $CaCl_2$ (*), and 5 mM $CaCl_2$ (■). The smooth curves were calculated for the normal distributions summarized in Table 3. Conditions: 6.0 μM calmodulin (1 mol = 16.7 kDa) in 20 mM Tris, 0.10 M KCl, pH 8.3, 20 °C. Excitation: 310 nm. Emission: 420 nm.

gel electrophoresis (Figure 2). The results of the Gausian distribution analysis are presented in a plot of relative amplitude versus the range of rotational correlation times that are consistent with the data. The profiles obtained with a solution containing 6 μM of polymerized calmodulin[2] and six different calcium concentrations (0, 0.01, 0.03, 0.10, 1.0, and 5.0 mM) reveal short rotational correlation times centered at a peak ($\phi_{corr} = \mu$), with the remaining larger values of ϕ distributed along the right half of a normal distribution curve (Figure 4, Table 2).

The values of ϕ_{corr} (0.6−1.2 ns) obtained with the polymerized calmodulin are much smaller than the global rotational correlation times reported by Small and Anderson (*18*) for the free monomer: 0.6 ns versus 7.2 ns (zero Ca^{2+}) and 1.2 ns versus 9.9 ns (plus Ca^{2+}). This result shows that the intermolecularly cross-linked segments of calmodulin possess a high degree of local mobility at all concentrations of Ca^{2+} examined. In addition, however, the widths of the distribution curves indicate that the relative contributions of slower rotations become increasingly evident at the higher calcium concentrations (Figure 4, panels A−F). The extended widths obtained in the presence of calcium may include the global rotations of some of the individual polymer molecules, which would have minimum global rotational correlation times of 6.5*N* ns. (The value of *N* represents the degree of polymerization of a spherical monomer having a value for ϕ of 6.5 ns.) However, the longer values of ϕ also approach the limits for experimental determination when the excited-state lifetime is 4 ns. Table 2 illustrates the uncertainty in width σ obtained at the higher calcium concentrations, where the low modulation frequencies at which the longer rotational correlation times need to be analyzed correspond to unfavorably low phase angles and little demodulation.

We also performed frequency domain anisotropy determinations on the internally cross-linked calmodulin monomer (Figure 5) in order to compare rotational correlation times to the values just determined for the enzymatically cross-linked polymers. Analysis of the decays according to the multicorrelation time model for a discrete sum of exponentials (*27*) reveals a set of two rotational correlation times determined in the presence and absence of Ca^{2+} (Table 3). At zero Ca^{2+}, ϕ_1 = 7.9 ns (fractional or component anisotropy $r_1 = 0.25$) and $\phi_2 = 0.33$ ns (fractional anisotropy $r_2 = 0.11$). When the Ca^{2+} concentration is increased to 20 mM, which approaches saturation with this derivative (*7*), ϕ_1 = 11.4 ns (fractional anisotropy $r_1 = 0.278$) and ϕ_2 = 0.2 ns (fractional anisotropy $r_2 = 0.082$). These quantities are related in the time domain equation (*22*, *27*).

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) \qquad (1)$$

Note that the sum of the two component or fractional anisotropies equals the limiting anisotropy (r_o), which in this case has a value of 0.36.

Table 3 compares our results with those obtained in time-domain measurements performed on the cross-linked calmodulin monomer (*18*). Both experiments demonstrate a 44−45% increase in the value of ϕ_1 that takes place upon Ca^{2+} binding. The present values of ϕ_1 are 15% greater than the previous values. However, the largest difference between the results obtained in our two reports involves the values of ϕ_2 and the zero time or limiting anisotropy (r_o). The frequency domain data point to a fast depolarization corresponding to ϕ_2 = 0.2 ns − 0.3 ns that is detected at the higher modulation frequencies. Depending on the width of the excitation pulse and the mathematical methods applied, this fast rotation may have remained unresolved in the earlier study. Varying resolution of a fast depolarization is consistent with the different values reported for the zero time anisotropy of the cross-linked monomer—0.36 versus 0.265 (Table 3).[3]

The fact that the limiting anisotropy of the cross-linked polymers (0.33) is slightly smaller than that of the monomer (0.36) may result from energy transfer among the dityrosyl moieties of the polymers. No transfer is possible in the monomer since there is only a single dityrosine moiety. We calculated a value for R_0, the characteristic energy transfer distance (*33*), which is based on the overlap of the corrected excitation and emission spectra of dityrosyl calmodulin (*7*), the extinction coefficients of dityrosine (*34*), a quantum yield of 0.85 (*17*), and the assumption of random orientation. The result is $R_0 \approx 6.6$ Å.[4] If a single pair of dityrosyl chromophores is separated by distances comparable to the range (12−22 Å) of intramolecular distances between Tyr^{99} and Tyr^{138} that were reported for native calmodulin (*35*), the corresponding transfer efficiencies $[1 + (R/R_0)^6]^{-1}$ would be 0.027 to 0.0007. However, transfer efficiencies within polymers larger than the trimer may be higher than the calculated values since the presence of more than one potential acceptor per excited donor increases the average probability of transfer. The effect of energy transfer on fluorescence polarization measurements carried out with systems that contain more than one possible acceptor for each excited donor was demonstrated in the binding of 1-anilino-8-naphthalenesulfonate to the five binding sites of bovine serum albumin (*36*). Moens and dos Remedios (*37*) recently

[3] We also recovered a limiting anisotropy of 0.36 in experiments with a cross-linked dimer of [Tyr^8] substance P (unpublished).

[4] The spectra of free dityrosine give smaller values of R_0 since there is less overlap.

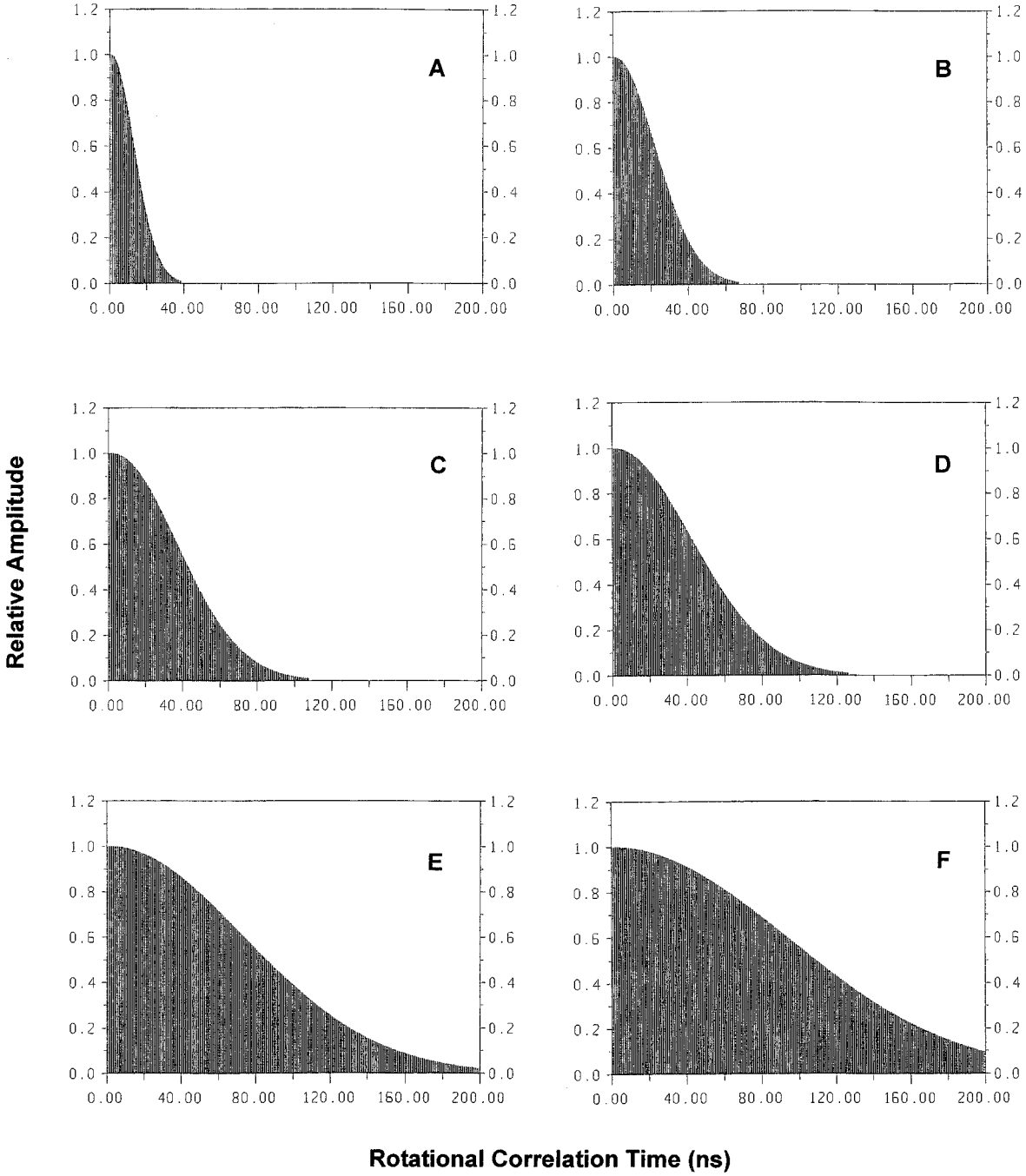

FIGURE 4: Normal distribution analyses showing the relative amplitudes and range of rotational correlation times consistent with frequency domain anisotropy measurements on a population of calmodulin polymers. Changes in distribution as a function of Ca^{2+} concentration are shown in panels A, 0.1 mM EDTA; B, 10 μM $CaCl_2$; C, 30 μM $CaCl_2$; D, 100 μM $CaCl_2$; E, 1 mM $CaCl_2$; and F, 5 mM $CaCl_2$.

showed that the average efficiency of transfer increases with the mole fraction of the acceptor in F-actin that has been reconstituted from varying proportions of donor- and acceptor-labeled G-actin.

DISCUSSION

Our frequency domain fluorescence anisotropy experiments, which were carried out on a selected fraction of enzymatically polymerized calmodulin exhibiting molecular masses greater than 96 000, demonstrate fast local rotations involving the dityrosyl cross-links. Normal distribution analyses of the data show peak rotational correlation times—0.6 (0 Ca^{2+}) and 1.2 ns (+Ca^{2+})—that are much smaller than the corresponding major values of ϕ characteristic of the calmodulin monomer. The high degree of internal mobility detected in the calmodulin polymers is probably related to the short rotational correlation times that were demonstrated in 1000 MHz frequency domain anisotropy measurements on the intrinsic tyrosine fluorescence of native calmodulin. In experiments conducted at 37 °C, Gryczynski et al. (*38*) obtained values of ϕ_2 and ϕ_1 (corrected to 20 °C) of 0.11 ns (39% of recovered anisotropy) and 2.1 ns (61% of recovered anisotropy) for calmodulin in the absence of Ca^{2+} and of 0.38 ns (28%) and 5.5 ns (72%) in the presence of calcium.[5] The intermolecularly cross-linked segments of the polymers evidently retain a degree of the

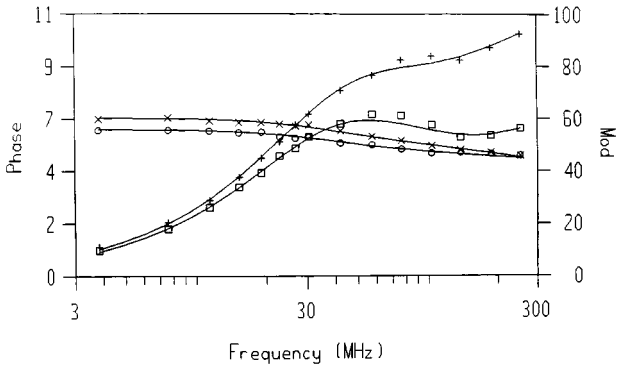

FIGURE 5: Frequency domain anisotropy data for intramolecularly cross-linked calmodulin monomer. The plot shows the differential polarized phase angle as a function of modulation frequency determined in the presence of either 0.10 mM EDTA (+) or 20 mM $CaCl_2$ (□) and the difference in modulation ratio determined in 0.10 mM EDTA (X) and 20 mM $CaCl_2$ (○). The smooth curves were calculated for the constants shown in Table 2. Conditions: 3.0 μM calmodulin in 50 mM Mops, pH 7.4, 20 °C. Excitation: 310 nm. Emission: 420 nm.

Table 3: Anisotropy Decays of Cross-linked Calmodulin Monomer. Comparison of Results Obtained by Frequency Domain and Time-Correlated Monophoton Counting Fluorometry[a]

	Ca^{2+}-free CaM		CaM + 20 mM Ca^{2+}	
	freq. domain	time-corr.[b]	freq. domain	time-corr.[b]
ϕ_1 (ns)	7.9 (+0.55,−0.47)[d]	7.17	11.4 (+0.69,−0.61)[d]	9.91
r_1	0.250	0.225	0.278	0.265
ϕ_2 (ns)	0.33 (±0.04)[d]	2.0	0.21 (+0.04,−0.03)[d]	
r_2	0.11	0.039	0.082	
r_o	0.36[c]	0.264	0.360[c]	0.265
χ^2	0.90	1.06	1.32	0.955

[a] Conditions: 3.0 μM calmodulin, 50 mM Mops, pH 7.4, 20 °C. [b] Data compiled from Small and Anderson (*18*). [c] The value of r_o was fixed at 0.36 in the analysis. r_1 and r_2 represent the component anisotropies, with $r_o = r_1 + r_2$. [d] In parentheses, errors are reported as correlated 67% confidence limits of the reduced χ^2.

mobility characteristic of the tyrosine-containing sequences in native calmodulin.

Although fast rotational correlation times prevail at all concentrations of Ca^{2+} examined, variations in the widths of the normal distribution curves show that the relative contributions of slower rotations increase markedly upon Ca^{2+} binding (Figure 5). The pronounced responsiveness of the distributions to changes in Ca^{2+} concentration is reminiscent of the results obtained in sedimentation velocity experiments. While the presence of Ca^{2+} has slight effect on the value of $s_{20,w}$ characteristic of the native calmodulin monomer[6] (*9*), it results in significant overall increases in the values of $s_{20,w}$ obtained with the polymers (*20*). The fluorescence anisotropy and sedimentation velocity experiments together point to a population of extended, flexible polymers that become more compact and less flexible upon Ca^{2+} binding.

Comparative experiments with the intramolecularly cross-linked dityrosyl derivative of calmodulin give principal rotational correlation times (ϕ_1) of 7.9 ($-Ca^{2+}$) and 11.4 ns ($+Ca^{2+}$). These values are similar to those that were obtained previously in time-domain experiments, i.e., 7.2 and 9.9 ns, respectively (*18*). However, the present study also demonstrates the existence of fast rotations of apparent ϕ_2 = 0.2 to 0.3 ns that account for 31% ($-Ca^{2+}$) to 23% ($+Ca^{2+}$) of the anisotropy. The intramolecular dityrosyl cross-link exhibits local mobility that is apparently similar to that found with Tyr^{138} and Tyr^{99} (*38*), and with Trp^{99} in an engineered calmodulin. Steiner et al. (*35*) found that the $Tyr^{99} \rightarrow Trp^{99}$ mutant gives a value for ϕ_2 of 0.76 ns ($-Ca^{2+}$) accounting for 16% of the recovered anisotropy in the time domain and of 0.10 to 0.12 ns ($\pm Ca^{2+}$) representing 22.6% of the recovered anisotropy in the 2000 MHz frequency domain. These authors concluded that the shorter rotational correlation times reported for calmodulin are unresolved averages that vary with the method of measurement. Since the tyrosyl residues of calmodulin are normally separated by ~15 Å (*8*), the intramolecularly cross-linked segments must be perturbed from their normal positions. This displacement may contribute to the local flexibility detected.

Calcium binding sites III and IV of native calmodulin are in contact with each other through a short segment of β-sheet containing residues 98−100 and 135−138 (*8*). The intramolecular dityrosyl cross-link thus connects Ca^{2+} binding sites III and IV, while the intermolecular cross-links join the Ca^{2+} binding sites of different calmodulin molecules—III to III, III to IV, and IV to IV. The extent of Ca^{2+} binding by these modified sites is unknown. However, the cross-linked monomer demonstrated markedly diminished interactions with both phenylagarose and smooth muscle myosin light chain kinase, with no enzyme activation detected in catalytic assays (*7*). The cross-linked polymers, in contrast, bind tightly to phenylagarose and give ~50% to ~100% of the maximum activation of myosin light chain kinase in assay systems that contain 1:1 proportions of enzyme and calmodulin (calculated in terms of monomer equivalents) (*20*). This result suggests that either all or a significant fraction of the intermolecularly cross-linked sites indeed bind Ca^{2+}.

The mobility of the intermolecular cross-links that we have demonstrated in the frequency domain fluorescence anisotropy measurements may contribute to the effectiveness of the polymers in the binding and activation of myosin light chain kinase. As the result of their ability to reorient, the individual units of a flexibly cross-linked polymer could be more accessible to the enzyme than the units of a rigidly cross-linked polymer. The experiments described in this report provide a view of the enzymatically cross-linked polymers of calmodulin as mobile, accommodating molecules. Observations on dityrosine as both cross-link and probe should provide information on the structures of naturally occurring dityrosine-containing proteins and on the various polymers and conjugates that may be obtained through in vitro cross-linking. We believe that the enzyme-catalyzed coupling of tyrosyl side chains is a useful approach to protein cross-linking that has many advantages over the previously employed technique of diazonium conjugation (*40*).

[5] Experiments performed at 5 °C also gave short values for ϕ_1 and ϕ_2.

[6] Gel filtration experiments gave Stoke's radii of 24.91 and 23.95 Å, respectively, for Ca^{2+}-free and Ca^{2+}-saturated calmodulin (*39*).

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