Binding of Amphiphilic Peptides to a Carboxy-Terminal Tryptic Fragment of Calmodulin†

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ABSTRACT: Calmodulin (CaM) fragments 1–77 (CaM 1–77) and 78–148 (CaM 78–148) were prepared by tryptic cleavage of CaM. CaM 78–148 exhibited Ca^{2+}-dependent binding to mastoparan X, *Polistes* mastoparan, and melittin with apparent dissociation constants <0.2 μM as judged from changes in the fluorescence spectrum and anisotropy of the single tryptophan residue of each of these cationic, amphiphilic peptides. This interaction was accompanied by a large spectral blue shift of the peptide fluorescence spectrum. These findings are consistent with earlier results [Malencik, D. A., & Anderson, S. R. (1984) *Biochemistry 23*, 2420–2428] on the binding of mastoparan X to CaM fragment 72–148. The binding of the peptide to CaM 78–148 also caused a significant loss of the accessibility of the peptide tryptophan to the fluorescence quencher acrylamide. The CaM 78–148 induced effects on the fluorescence spectra and tryptophan accessibility of the peptides were most pronounced for mastoparan X, a peptide with tryptophan on the apolar face of the putative amphiphilic helix. The data were comparable with results from parallel experiments on the Ca^{2+}-dependent interaction of these peptides with intact CaM. Difference circular dichroic spectra suggested that binding to CaM 78–148 was associated with the induction of considerable degrees of helicity in the amphiphilic peptides, which by themselves have predominantly random coil structures in aqueous solution. This finding is also reminiscent of the interaction of these peptides with intact CaM. Thus, the characteristics of the high-affinity peptide binding site of CaM appear to be largely retained in CaM 78–148. CaM 1–77 exhibits a much weaker affinity (dissociation constant >20 μM) for the mastoparans.

Calmodulin is known to bind several basic, amphiphilic peptides with high affinity (Barnette et al., 1983; Comte et al., 1983; Malencik & Anderson, 1982, 1983a). The driving force for this interaction appears to be the induction of an amphiphilic helix in the peptide by the interacting surface in CaM[1] (Cox et al., 1985; McDowell et al., 1985). Other basic, amphiphilic peptides such as β-endorphin and fragments also bind to CaM, but with much lower affinity; again, however, the formation of an amphiphilic helix in the peptides appears to be important in this interaction (Puett et al., 1983; Giedroc et al., 1983a). Peptides forming nonhelical secondary structures, such as the dynorphins, also bind to CaM (Malencik & Anderson, 1983b; Anderson & Malencik, 1986). The complementary binding region of CaM for these peptides has not been identified apart from general observations that a hydrophobic surface of CaM, exposed upon binding of Ca^{2+} (LaPorte et al., 1980), is involved (McDowell et al., 1985; Sanyal & Prendergast, 1988).

The crystal structure of CaM is characterized by a long exposed helix containing residues 65–92, in addition to shorter helices flanking the Ca^{2+}-binding regions (Babu et al., 1985). In view of the observation that the positively charged amphiphilic helix structure of mastoparans and melittin is important for recognition and binding by CaM, it is reasonable to speculate that the complementary CaM surface may also have a structure, perhaps helical, with negative charges in the vicinity. The possible role of the "central helix" (i.e., residues 65–92) of CaM is an important question in this context. It should be noted, however, that a CaM fragment containing residues 1–106 showed a moderate affinity for peptide ligands whereas the CaM fragment 72–148 exhibited a much higher affinity, all in the presence of Ca^{2+} (Malencik & Anderson, 1984). This observation was intriguing because CaM 1–106 contains the entire sequence necessary to form the CaM central helix whereas the central helix is clipped in CaM 72–148. However, to what extent the original tertiary structure is retained in these fragments is an open question. It is important in this context that the spectrum resulting from adding the ^1H NMR spectra of CaM 1–77 and CaM 78–148 is nearly identical with the proton magnetic resonance spectrum of native CaM (Dalgarno et al., 1984; Klevit et al., 1984; Ikura et al., 1984, 1985; Aulabaugh et al., 1984).

Also, it has been reported that CaM 78–148 is capable of activating phosphorylase kinase (Kuznicki et al., 1981), and the erythrocyte Ca^{2+}-ATPase (Guerini et al., 1984), albeit with a lower affinity than CaM. This fragment also binds to calcineurin and cyclic nucleotide phosphodiesterase (Ni & Klee, 1985), and although it does not activate the latter, the fragment behaves as an antagonist to CaM (Newton et al., 1984). CaM 1–77 exhibits more limited binding to enzymes that CaM 78–148. For example, it binds to cyclic nucleotide phosphodiesterase and cAMP-dependent protein kinase (Ni & Klee, 1985), but is incapable of stimulating the activities of phosphodiesterase (Walsh et al., 1977; Newton et al., 1984) and erythrocyte Ca^{2+}-ATPase (Guerini et al., 1984). Thus,

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[1] Abbreviations: CaM, calmodulin; CD, circular dichroic; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid.

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the CaM fragments 1–77 and 78–148 exhibit conformations similar to those in the natural protein as judged by high-field ^1H NMR spectroscopy, and they bind with varying selectivities to different CaM-stimulated enzymes; however, only CaM 78–148 has been reported to exhibit activity in enzyme stimulation.

In the absence of crystallographic data on CaM–peptide complexes,[2] we have taken the approach of studying interactions of peptides with CaM fragments of suspected peptide-binding regions. We have investigated the interactions of CaM tryptic fragments 1–77 and 78–148 with mastoparan X, *Polistes* mastoparan, and melittin, three amphiphilic peptides, the amino acid sequences of which are shown in footnote 3. Each of these peptides has a very high affinity for intact CaM and contains a single tryptophan, which has been used as a convenient fluorescent probe to monitor interaction (Malencik & Anderson, 1983a; McDowell et al., 1985; Maulet & Cox, 1983). The tryptophan residue (Trp-3) in mastoparan X and *Polistes* mastoparan is located on the apolar face of the putative amphiphilic helix (McDowell et al., 1985), while that in the melittin helix (Trp-19) appears to be accessible to polar solvents (Degrado et al., 1981). CaM 78–148 binds all three peptides with high affinities. We have studied the structural and environmental changes induced in the peptides by this fragment. Our results suggest that CaM 78–148 offers an interacting surface similar in nature to that provided by intact CaM for these amphiphilic peptides.

Materials and Methods

Preparation of CaM and Fragments. CaM was purified as described elsewhere (Jackson & Puett, 1984) and partially digested with trypsin in the presence of Ca^{2+} to yield nearly "half-molecule" fragments by using slight modifications of the conditions described by others (Walsh et al., 1977; Newton et al., 1984). The protein (40 mg) was dissolved (2.5 mg/mL) in 50 mM ammonium bicarbonate containing 2 mM $CaCl_2$ and 50 mM NaCl. Trypsin was added from a stock solution in 10 mM acetic acid to give a 212.5:1 weight ratio of calmodulin to trypsin, and the mixture was incubated at 37 °C for 50 min, after which time lyophilized soybean trypsin inhibitor was added to give a concentration of 0.1 mg/mL. The mixture was first dialyzed against 0.1 M ammonium bicarbonate containing 1 mM EGTA and then against water and was finally lyophilized. The lyophilized material was resuspended in 1 mL of water and desalted on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate containing 2 mM EDTA. The fragments were separated by reverse-phase HPLC using a Perkin-Elmer Series 4 solvent delivery system with a 25-cm Synchrompak C18 column and the following conditions with A = 10 mM potassium phosphate, pH 6.0, and 1 mM EDTA and B = 10 mM potassium phosphate, 1 mM EDTA, and 50% acetonitrile. After equilibration for 10 min with 60% A and 40% B, the sample was injected and eluted with a 1-min linear gradient to 48% B, followed by a 25-min linear gradient to 64% B; the flow rate was 1.5 mL/min. Seven major peptide peaks were resolved, and the identity of each was determined by amino acid analysis (6 N HCl, 110 °C, 24 h) using *o*-phthalaldehyde for detection. Methionine was determined, but the values were invariably low, probably due to oxidation, and thus were not included in the composition data. Peaks 2 and 6, which corresponded to CaM 78–148 and 1–77, respectively, were lyophilized, resuspended in 1 mL of water, desalted on a Sephadex G-25 column equilibrated with water, and lyophilized. The absorption and fluorescence spectra of peak 6 showed no contaminating tyrosine (from CaM 78–148).

Fluorescence Measurements. Fluorescence spectra and anisotropy measurements were made on an ISS GREG 1 photon-counting spectrofluorometer interfaced with an AT&T 6300 personal computer. Tryptophan fluorescence spectra of the peptides were obtained by exciting the samples at 295 nm to avoid interference from the tyrosines of CaM or CaM 78–148. Tryptophan fluorescence anisotropy was measured in the L format by use of polarized light at an excitation wavelength of 295 nm. The polarized components of emission were monitored at 355 nm, and a *G*-factor correction for the emission monochromator was introduced (Lakowicz, 1983).

Acrylamide quenching of fluorescence intensity was achieved by progressive additions of 10-μL aliquots of a 5 M acrylamide solution (pH 7.0, using the same buffer in which the sample was dissolved) to 2.0-mL samples in the cuvette. The measured fluorescence intensity was corrected for dilution effects and for inner-filter effect. In order to calculate the bimolecular quenching constant (k_q) from the slope (K_{SV}) of the Stern–Volmer plot, it was necessary to use fluorescence lifetime values for the peptides. The lifetime values for free and CaM-bound peptides were taken from a recent study that used multiple-frequency phase-modulation fluorometry.[4] The lifetime of the single tryptophan of each peptide was heterogeneous, in both free and intact CaM-bound forms. Average lifetimes (τ_{av}) were determined from fractional contributions of individual components. For CaM 78–148 bound peptides the same τ_{av} values as those obtained for CaM-bound peptides were used.

Unless stated otherwise all samples used in fluorescence measurements were prepared in a 20 mM MOPS buffer, pH 7.0, containing 100 mM NaCl and 2 mM $CaCl_2$. Some measurements were also made in a Ca^{2+}-free medium where the 2 mM $CaCl_2$ of the buffer solution was replaced with 10 mM EGTA. All spectra were recorded at 25 °C.

Circular Dichroism. CD spectra were measured at room temperature with an AVIV Model 60 DS CD spectropmeter (AVIV Associates, Lakewood, NJ) and a cell of 2-mm path length. Four scans were made on each sample and base line, with data accumulation every 0.2 nm. Following signal averaging at each wavelength and base-line correction, the spectrum was subjected to a four-point smoothing operation, and difference spectra were determined from the final spectra at 0.2-nm intervals. CaM 78–148, *Polistes* mastoparan, and mastoparan X were present at 51, 57, and 48 μM, respectively, in the same Ca^{2+}-containing buffer used for fluorescence. Spectra on mixtures of the peptides and CaM 78–148 were obtained with these same concentrations to give about 1:1 molar ratios. The results are reported as measured ellipticity, rather than molar or mean residue ellipticity, since there is no objective method to assign concentration with two distinct optically active species.

Results

Characterization of the CaM Half-Molecule Fragments. A typical HPLC separation of the half-molecule fragments is shown in Figure 1, and the amino acid compositions of the various HPLC peaks are given in Table I. All spectral

[2] Tanaka et al. (1985) obtained crystals of CaM and melittin of a 1:1 molar ratio, but no structural information on the complex has appeared.

[3] Sequence of peptides: H_2N-I-N-W-K-G-I-A-A-M-A-K-K-L-L-$CONH_2$ (mastoparan X); H_2N-V-D-W-K-K-I-G-Q-H-I-L-S-V-L-$CONH_2$ (*Polistes* mastoparan); H_2N-G-I-G-A-V-L-K-V-L-T-T-G-L-P-A-L-I-S-W-I-K-R-K-R-Q-Q-$CONH_2$ (melittin).

[4] G. Sanyal, S. Sedarous, P. Fisher, W. F. Degrado, and F. G. Prendergast, unpublished results.

Table I: Amino Acid Compositions of the Half-Molecule Tryptic Fragments of CaM

residue	peak no. (assigned sequence)[a]						
	1 (1–74)	2 (78–148)	3 (76–148)	4 (75–148)	5 (1–75)	6 (1–77)	7 (CaM)
Asp	14.0 (11)	11.8 (12)	11.7 (12)	11.8 (12)	11.6 (11)	12.4 (11)	23.2 (23)
Thr	5.7 (8)	3.6 (4)	4.0 (4)	4.0 (4)	7.7 (8)	8.6 (8)	11.4 (12)
Ser	1.9 (2)	2.2 (2)	1.7 (2)	1.9 (2)	2.2 (2)	2.5 (2)	3.8 (4)
Glu	13.2 (13)	13.2 (14)	13.7 (14)	14.9 (14)	13.0 (13)	15.8 (13)	27.6 (27)
Gly	6.3 (6)	5.4 (5)	5.2 (5)	5.1 (5)	6.5 (6)	6.3 (6)	11.2 (11)
Ala	6.0 (6)	4.8 (5)	4.9 (5)	4.8 (5)	5.9 (6)	6.0 (6)	10.6 (11)
Val	2.0 (2)	4.7 (5)	4.5 (5)	4.4 (5)	2.0 (2)	2.3 (2)	6.5 (7)
Ile	3.7 (4)	3.2 (4)	3.6 (4)	3.5 (4)	3.5 (4)	4.1 (4)	7.9 (8)
Leu	6.0 (6)	3.0 (3)	3.0 (3)	3.0 (3)	6.0 (6)	6.0 (6)	9.0 (9)
Tyr	0 (0)	1.7 (2)	2.0 (2)	1.8 (2)	0 (0)	0 (0)	1.6 (2)
Phe	4.6 (5)	3.2 (3)	3.1 (3)	2.9 (3)	5.1 (5)	4.4 (5)	8.2 (8)
Lys	2.8 (3)	2.0 (2)	3.0 (3)	4.0 (4)	3.5 (4)	5.1 (5)	6.8 (7)
His	0 (0)	0.6 (1)	0.6 (1)	0.6 (1)	0 (0)	0 (0)	0.7 (1)
Arg	2.0 (2)	3.9 (4)	3.9 (4)	4.1 (4)	1.8 (2)	1.7 (2)	5.4 (6)

[a] The peak numbers correspond to those shown in Figure 1, and the numbers in parentheses denote the amino acid sequence assignment. The experimental values given (moles per mole of peptide) have been normalized to Leu; the integers in parentheses correspond to the moles per mole of peptide based on the reported amino acid sequence (Watterson et al., 1980). Methionine was not included (cf. text), and proline was not detected with the system used.

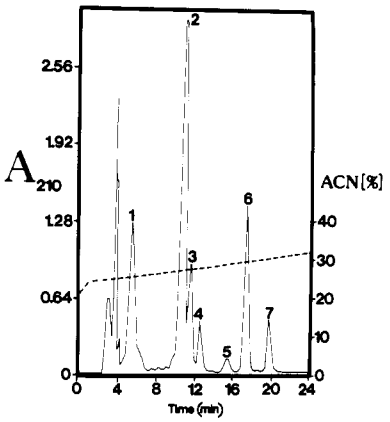

FIGURE 1: Reverse-phase HPLC separation of half-molecule tryptic fragments of CaM using a Synchrompak C18 column and the acetonitrile gradient described in the text. The amino acid compositions of each peak are given in Table I; peaks 2 and 6 correspond to CaM 78–148 and 1–77, respectively. ACN = acetonitrile.

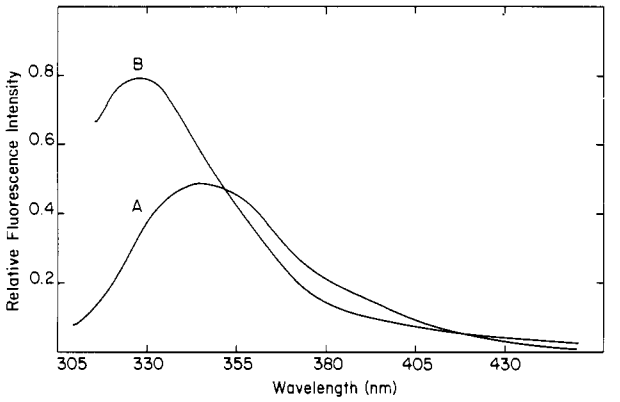

FIGURE 2: Tryptophan fluorescence spectra of (A) free and (B) CaM 78–148 bound mastoparan X; the concentrations of mastoparan X and CaM 78–148 were 10 μM each. The samples were excited at 295 nm, and the excitation and emission band-passes were 4 and 8 nm, respectively.

measurements were made on peaks 2 and 6, which corresponded respectively to CaM fragments 78–148 and 1–77. Fragments corresponding to tryptic cleavages in the central helix other than Lys-77 were also identified, e.g., Arg-74 and Lys-75, but under the conditions used (i.e., tryptic digestion in the presence of Ca^{2+}), there was no evidence of cleavage at Arg-86 and Arg-90, an observation consistent with the findings of others (Walsh et al., 1977; Newton et al., 1984; Dalgarno et al., 1984). Some variability was noted in the relative amounts of each peak in Table I, but peaks 2, 6, and 1 were generally in greatest abundance on the basis of absorbance at 210 nm.

Fluorescence Spectral Evidence of Peptide Binding to CaM 78–148. The wavelength maximum (λ_{max}) for tryptophan fluorescence of mastoparan X was blue-shifted from 345 nm in the free peptide in aqueous solution to 325 nm upon addition of an equimolar concentration of CaM 78–148 in the presence of a saturating concentration of Ca^{2+} (Figure 2). The observed fluorescence blue shift of 20 nm is comparable with the blue shift of 23 nm reported for mastoparan X interaction with intact CaM (Malencik & Anderson, 1983a; McDowell et al., 1985). Spectral blue shifts (for λ_{max}) of 17 and 13 nm were found to be associated with the binding of *Polistes* mastoparan and melittin, respectively, with CaM 78–148 (Table II). For comparison, the blue shifts induced by intact CaM in the tryptophan fluorescence λ_{max} of these peptides are 23 and 17 nm, respectively (Malencik & Anderson, 1983a; Sanyal & Prendergast, 1988).

Table II: Changes in Steady-State Fluorescence Spectral Maxima (λ_{max}) and Anisotropies (r_{ss}) Observed upon Ca^{2+}-Dependent Binding of Peptides to Calmodulin Fragment 78–148[a]

peptide	λ_{max} (nm)	r_{ss}
mastoparan X	345	0.042
Polistes mastoparan	345	0.033
melittin	345	0.042
mastoparan X–CaM 78–148	325	0.135
Polistes mastoparan–CaM 78–148	328	0.138
melittin–CaM 78–148	332	0.100

[a] The values were obtained at 25 °C for the free peptide and for the 1:1 stoichiometric complex of each peptide with CaM 78–148. The samples were excited at 295 nm.

Fluorescence spectra of stoichiometric (1:1 molar ratio) complexes of all three peptides with CaM 78–148 were taken over a concentration range of 0.2–10 μM in the presence of 2 mM Ca^{2+}. For each peptide, the λ_{max} was independent of concentration and no spectral shift was observed with decreasing concentration. The fluorescence intensity was linearly dependent on concentration, as expected. Thus, there was no evidence for any dissociation of these complexes even at the lowest concentration that we examined. The dissociation constant of these complexes are, therefore, below 0.2 μM. Due to the relatively poor quantum yield of the single tryptophan in these peptides (e.g., a quantum yield of 0.13 was measured

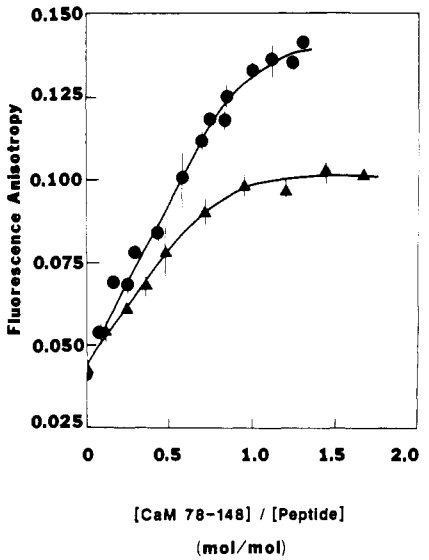

FIGURE 3: Stoichiometric titrations of the peptides melittin (▲) and mastoparan X (●) with CaM 78–148, as monitored by measuring fluorescence anisotropy as a function of addition of CaM 78–148 to the free peptides, the initial peptide concentration being 10 μM in each case. The samples were excited at 295 nm (band-pass of 4 nm), and emission was monitored at 355 nm (band-pass of 8 nm). A *G*-factor correction for the emission monochromator was used in calculating anisotropies. The mean and SEM of at least three measurements are shown for each data point.

for free mastoparan X), the fluorescence measurements could not be made with confidence below a peptide concentration of 0.2 μM. Malencik and Anderson (1984) estimated a value of ≤0.15 μM for the dissociation constant of the mastoparan X complex with CaM 72–148.

Fluorescence Anisotropy Measurements as Evidence for Peptide Binding to CaM 78–148. The Ca^{2+}-dependent interactions of mastoparan X, *Polistes* mastoparan, and melittin with CaM 78–148 were also monitored by measuring the increases in fluorescence anisotropy of the peptides that occurred upon titration with CaM 78–148. These results are shown in Figure 3, and the data suggest a 1:1 stoichiometry for binding of each peptide up to the maximum concentration of peptide examined (10 μM). The anisotropies of the free peptides were between 0.030 and 0.045 (for 295-nm excitation). With the addition of increasing concentrations of CaM 78–148, the anisotropy progressively increased and then, at a molar ratio of approximately 1:1 for CaM 78–148–peptide, leveled off at 0.135 ± 0.005, 0.138 ± 0.002, and 0.100 ± 0.001 for mastoparan X, *Polistes* mastoparan, and melittin, respectively. With the addition of increasing concentrations of CaM 78–148, the anisotropy progressively increased and then, at a molar ratio of approximately 1:1 for CaM 78–148–peptide, leveled off at 0.135 ± 0.005, 0.138 ± 0.002, and 0.100 ± 0.001 for mastoparan X, *Polistes* mastoparan, and melittin, respectively.

Ca^{2+} Requirement for Binding. The high-affinity binding of the peptides to CaM 78–148 was dependent upon the presence of Ca^{2+}. In the absence of Ca^{2+} (i.e., solutions prepared with 10 mM EGTA and no external Ca^{2+}), a weak partial (nonstoichiometric) binding was apparent from small linear increases in fluorescence anisotropy and lack of saturation when 11 μM mastroparan X was titrated with CaM 78–148 in the concentration range of 0.9–13 μM at pH 7.0 (data not shown). Even for a mixture containing 24 μM each of mastoparan X and CaM 78–148, the tryptophan fluorescence anisotropy (for 295-nm excitation) was only 0.055 at 25 °C, compared to a value of 0.030 for the free peptide. For a 1:1 stoichiometric complex an anisotropy value of ~0.135 is expected under the same conditions on the basis of measurements in the presence of Ca^{2+} (cf. Figure 3).

Table III: Changes in Bimolecular Rate Constants (k_q) for Collisional Quenching of Tryptophan Fluorescence of Peptides by Acrylamide upon Ca^{2+}-Dependent Interaction with CaM 78–148: Comparison with Changes Induced by Intact CaM

peptide	K_{SV}[a] (M^{-1})	$k_q \times 10^{-9}$ [b] (M^{-1} s^{-1})	k_q^{bound}/k_q^{free}
mastoparan X	16.9	7.25	
mastoparan X–CaM	0.601	0.222	0.031
mastoparan X–CaM 78–148	0.791	0.292	0.040
Polistes mastoparan	19.6	6.66	
Polistes mastoparan–CaM	0.984	0.360	0.054
Polistes mastoparan–CaM 78–148	3.02	1.11	0.167
melittin	18.0	5.45	
melittin–CaM	2.89	0.781	0.143
melittin–CaM 78–148	3.62	0.978	0.179

[a] K_{SV} is the slope of linear Stern–Volmer plot (Figure 4). Acrylamide concentrations up to 0.2 and 0.5 M, respectively, were used for free and bound peptides. [b] k_q was obtained by dividing K_{SV} by the average lifetime; τ_{av} values were obtained in a separate study[4] by multiple-frequency phase-modulation determination of heterogeneous tryptophan lifetimes.

Interaction of Peptides with CaM 1–77. CaM 1–77 showed a much weaker affinity for mastoparan X and *Polistes* mastoparan than did CaM 78–148. No fluorescence spectral shift or change in anisotropy was observed when 2 μM peptide was mixed with 2 μM of this fragment in the presence of 2 mM Ca^{2+} at pH 7.0. Addition of 10 μM CaM 1–77 to 10 μM mastoparan X, under the same conditions, caused a spectral blue shift of the peptide from 346 to 339 nm and a change in fluorescence anisotropy from 0.045 to 0.062 at 25 °C. At 10 μM concentrations of *Polistes* mastoparan and CaM 1–77, under conditions identical with those described above, the λ_{max} and anisotropy values were respectively 342 nm and 0.051 relative to the values of 345 nm and 0.040 for the free peptide.

Accessibility of Tryptophan to Acrylamide in CaM 78–148 Bound Peptides. The rate constants for collisional quenching of tryptophan fluorescence of peptides by acrylamide are reduced upon binding of the peptides to CaM (McDowell et al., 1985). It has also been found that peptides with tryptophan on the apolar face of the amphiphilic helix, e.g., mastoparan X, experience a greater loss of accessibility to acryamide when bound to CaM than those with tryptophan on the polar face, e.g., melittin (McDowell et al., 1985; O'Neil et al., 1987). We have compared under identical conditions, the acrylamide accessibility of the tryptophan residues of mastoparan X, *Polistes* mastoparan, and melittin bound to CaM 78–148 with those of the peptides bound to intact CaM. The Stern–Volmer plots for free peptides and their 1:1 stoichiometric complexes with CaM and CaM 78–148 are shown in Figure 4. For the peptide–protein complexes, the plots were linear, within experimental error, up to 0.5 M acrylamide concentrations, indicating that quenching was dynamic. For the free peptides, which exhibited a significantly higher degree of tryptophan accessibilty, the plots were linear up to 0.2 M acrylamide. Acrylamide was found to be totally nonfluorescenct (i.e., emission spectrum was indistinguishable from dark counts) up to a concentration of 0.5 M. The slopes of these plots (i.e., the Stern–Volmer constant or K_{SV}) are listed in Table III. The bimolecular rate constants for collisional quenching, also given in Table III, were obtained by dividing K_{SV} by the lifetime, τ_{av}, as described under Materials and Methods. It is clear that both intact CaM and CaM 78–148 induce a large decrease (range, 6–32-fold) in the accessibility of the peptide

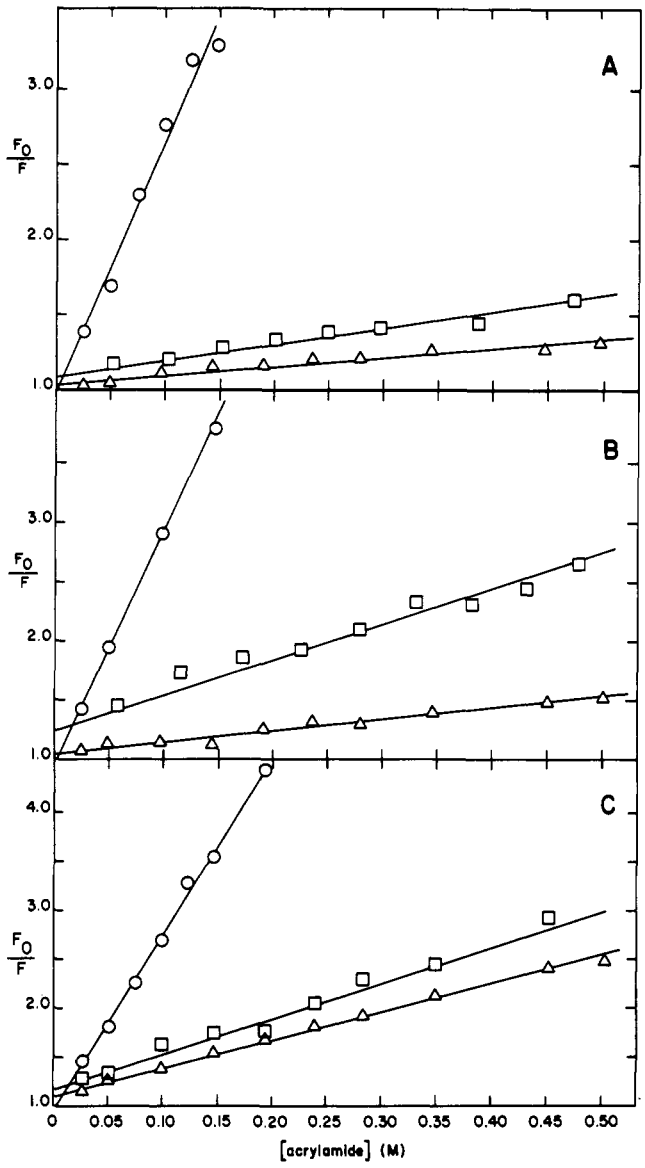

FIGURE 4: Stern–Volmer plots for acrylamide quenching of peptides in free (○), CaM-bound (△), and CaM 78–148 bound (□) states: (A) mastoparan X, (B) *Polistes* mastoparan, and (C) melittin. The protein and peptide concentrations were 10 μM in each case. Fluorescence spectra were taken upon excitation at 295 nm, and the extents of quenching were determined at the wavelength of maximum fluorescence.

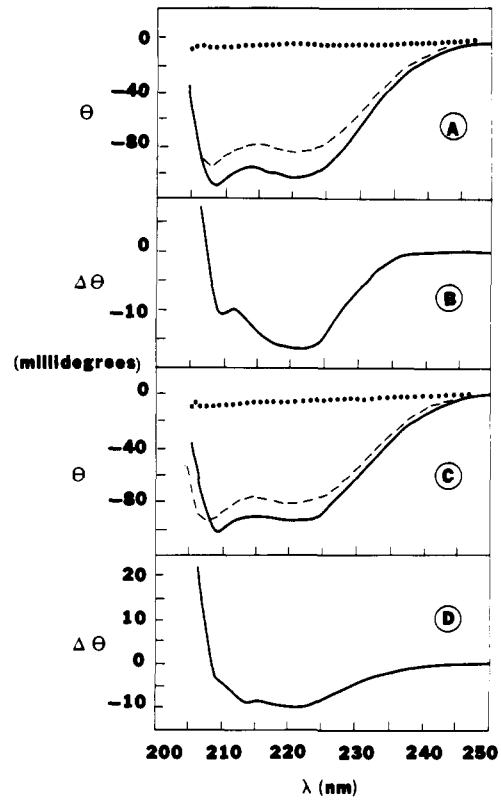

FIGURE 5: Ultraviolet CD spectra of CaM 78–148 (51 μM), mastoparan X (48 μM), *Polistes* mastoparan (57 μM), and difference CD spectra (i.e., the spectrum of the CaM 78–148–peptide complex minus the added spectra of CaM 78–148 and the peptide): (A) CD spectra of CaM 78–148 (- - -), mastoparan X (···), and a mixture of the two (—); (B) difference CD spectrum of the spectra in panel A; (C) CD spectra of CaM 78–148 (- - -), *Polistes* mastoparan (···), and a mixture of the two (—); (D) difference CD spectrum of the spectra in panel C.

tryptophan to acrylamide. For both mastoparan X and melittin, the ratio of the k_q values between the CaM 78–148 bound peptide and free peptide is only 1.3-fold larger than the ratio between CaM-bound peptide and free peptide. These two ratios are larger for mastoparan X than for melittin, meaning that the tryptophan of mastoparan X experiences a greater loss of accessibility as a result of interaction with either CaM or CaM 78–148 than does melittin. The effect of CaM 78–148 on the k_q for tryptophan of *Polistes* mastoparan (6-fold) is not as pronounced, however, as that produced by intact CaM (19-fold). We have found, in this study, a larger reduction of k_q to be induced in general by intact CaM for these three peptides than that originally reported (McDowell et al., 1985). Our present data are completely consistent, however, with the original conclusions regarding acrylamide accessibility of intact CaM–peptide complexes.

Circular Dichroism. The far-UV CD spectra of melittin and the mastoparans in aqueous solution at pH 7.0 are typical of a predominantly random coil structure and a negligible helical contribution (Maulet & Cox, 1983; McDowell et al., 1985), while CaM exhibits a CD spectrum clearly indicative of a significant degree of helical structure (Klee et al., 1980). However, the CD spectra of samples obtained upon interaction of CaM with the mastoparans show substantially increased helical structures over that which could be accounted for by additivity of the CaM spectrum and the peptide spectrum. This observation has been interpreted as evidence that binding to CaM is accompanied by a transition from a random to a helical structure for these amphiphilic peptides (McDowell et al., 1985). We have measured the far-UV CD spectra of complexes of mastoparan X and *Polistes* mastoparan with CaM 78–148, and difference CD spectra have been obtained after subtraction of the peptide and CaM 78–148 spectra from that of each of the complexes. The actual experimental spectra and the difference CD spectra are shown in Figure 5. The enhancement of negative ellipticity at the 221-nm ($n-\pi^*$ transition) and 208–209-nm ($\pi-\pi^*$ transition) extrema upon the addition of the peptides to the CaM 78–148 solutions offers strong evidence of induced helicity. The difference CD spectra are consistent with this interpretation; moreover, the weak apparent negative difference band at 215 ± 1 nm may reflect some induced β structure, this being more pronounced with *Polistes* mastoparan than with mastoparan X. Caution must be exercised, however, against overinterpreting difference CD spectra in view of the inherent errors in the measurements.

Discussion

Our major findings are that CaM fragment 78–148 binds amphiphilic peptides in a Ca^{2+}-dependent manner and that

this interaction appears to induce conformational changes in the peptides similar to those induced by intact CaM. Malencik and Anderson (1984) found CaM fragment 72–148 to bind mastoparan X with a high affinity in the presence of Ca^{2+}. It is interesting, upon comparing the two sets of results, that the deletion of residues 72–77 in the tryptic fragment does not have a dramatic effect on peptide binding.

The magnitudes of fluorescence spectral blue shifts exhibited by the single tryptophan residue of each peptide upon the interactions with CaM 78–148 are similar to those induced by intact CaM and CaM 72–148 (Malencik & Anderson, 1983a, 1984; McDowell et al., 1985). These large blue shifts are typical of a change in the environment of tryptophan from polar to apolar or rigid polar. The smaller blue shift exhibited by melittin, compared to mastoparan X and *Polistes* mastoparan, indicates a direct involvement of the tryptophan-containing apolar face of the mastoparans in the interface with a presumably hydrophobic surface in CaM 78–148. In CaM 78–148 bound melittin, the position of tryptophan-19 in the putative amphiphilic helix is such as to possibly allow it more contact with water.

The CaM-induced spectral shift on the position of tryptophan in a series of CaM-binding amphiphilic peptides has recently been shown to follow a periodic repeat of ~3.4 (O'Neil et al., 1987). This observation is an elegant demonstration that these peptides form amphiphilic helices when bound to CaM and reinforces earlier suggestions that the apolar face of the peptide helix forms an interface with an apolar CaM surface (Cox et al., 1985; McDowell et al., 1985; Sanyal & Prendergast, 1988).

The similarity of the present findings on the tryptophan environment of CaM 78–148 bound peptides with those of CaM–peptide complexes suggests that the CaM 78–148 fragment offers an environment to the amphiphilic peptides that is similar to that provided by intact CaM. Further support for this inference comes from the difference CD spectra, which indicate that largely helical structures are induced by CaM 78–148, in consonance with earlier CD observations on complexes of melittin and the mastoparans with intact CaM (Maulet & Cox, 1983; McDowell et al., 1985). It should be pointed out, however, that the generation of a difference spectrum upon subtracting the CaM or the CaM 78–148 spectrum from that of the corresponding protein–peptide complex does not necessarily mean that the residual signal can be unambiguously assigned to the peptide. It is an implicit assumption in this subtraction that the CaM or the CaM 78–148 spectrum remains unaffected by complex formation with the peptide. The justification for this assumption lies in the observation that (i) all of the three peptides studied have large helical hydrophobic moments and hence a high potential for forming amphiphilic helices (Eisenberg et al., 1982; McDowell et al., 1985) and (ii) these peptides exhibit CD spectra typical of random coil structure in aqueous solution, but a transition to helical structures becomes apparent from spectral changes that accompany the addition of trifluoroethanol or methanol to the peptide solution (Higashijima et al., 1983; McDowell et al., 1985).

We have used fluorescence anisotropy measurements to monitor CaM fragment–peptide interaction and to establish the stoichiometry of Ca^{2+}-dependent binding of the peptides to CaM 78–148. Malencik and Anderson (1984) titrated mastoparan X with the CaM fragment 72–148 and reported fluorescence anisotropy values (also using 295-nm excitation and 25 °C) that are similar to those that we have obtained for titration of mastoparan X with CaM 78–148. The smaller anisotropy of the melittin–CaM 78–148 complex, compared to the complexes of the two mastoparans with CaM 78–148, is probably due to either or both of the following reasons: (i) higher motional freedom of tryptophan located in the more polar peptide face of melittin; (ii) longer lifetime of protein-bound melittin. The generally smaller steady-state anisotropies of CaM-bound peptides containing tryptophan on the polar face, compared to those with tryptophan on the apolar face, have been interpreted as indicative of higher motional freedom for the tryptophan residue in the former class of peptides, and a periodic dependence of anisotropy on tryptophan position has been suggested (O'Neil et al., 1987). Such interpretations, however, can be made only from time-resolved anisotropy measurements and after a careful consideration of different factors that may contribute to the decay of anisotropy. For example, an increase in excited-state lifetime will result in a decreased steady-state anisotropy. The tryptophan fluorescence lifetime of CaM-bound melittin is, in fact, longer than that of CaM-bound mastoparan X or *Polistes* mastoparan (McDowell et al., 1985; Sanyal et al.[4]).

For mastoparan X, where the single tryptophan residue occurs on the apolar face of the putative helix, binding to CaM 78–148 is accompanied by a 25-fold loss of accessibility to acrylamimde. This is comparable with a 33-fold reduction in accessibility of the peptide upon binding to CaM. For melittin, where the single tryptophan residue is on the polar face of the helix, the corresponding reductions in accessibility induced by CaM 78–148 and CaM are respectively 5.6- and 7.0-fold. This further supports our conclusion that CaM 78–148 retains a significant part of the peptide-interacting surface of CaM, which complements the apolar fact of the amphiphilic helices formed by these peptides at the interface. In contrast with mastoparan X, *Polistes* mastoparan exhibited a much smaller decrease of accessibility to acrylamide when bound to CaM 78–148 (6-fold) compared to the 20-fold decrease induced by interaction with intact CaM. Thus, the accessibility of the tryptophan in CaM 78–148 bound *Polistes* mastoparan is only slightly less than that of the tryptophan of CaM 78–148 bound melittin. This finding is in contrast with our general observations of similarity between changes induced in the peptide structure by CaM 78–148 and by intact CaM. It is conceivable that the orientation assumed by the tryptophan residue of *Polistes* mastoparan on the CaM surface is different from that at the interface with CaM 78–148. In this connection, the subtle differences between the far-UV CD spectra of the complexes of CaM 78–148 with mastoparan X and *Polistes* mastoparan (discussed above) are also worth noting.

Our finding of relatively high-affinity of the amphiphilic peptides to CaM 78–148, contrasted to lower affinity binding of the two mastoparan peptides to CaM 1–77, may relate to the recent observation of Linse et al. (1986). They found that 2 mol of mastoparan/mol of Cd_4CaM broadened the four ^{113}Cd NMR signals over that found at 1:1 molar ratio without affecting the chemical shifts. This observation was interpreted as binding of a second molecule of mastoparan with lower affinity than the first or a faster exchange of the second bound molecule relative to the first; the absence of changes in the ^{113}Cd NMR chemical shifts argues against major conformational changes in calmodulin, at least near the metal binding sites, accompanying the bining of the second peptide molecule to the protein. In the presence of Ca^{2+}, stoichiometries greater than 1:1 are known to occur with a lower affinity binding β-endorphin-derived peptide (Giedroc & Puett, 1985); moreover, a stoichiometry of 2:1 for the binding of a synthetic

amphiphilic peptide to CaM has been recently reported, and sequential interactions with a high-affinity site and a low-affinity site have been proposed (Erickson-Viitanen & Degrado, 1987). Phenothiazines, as well as certain peptides, are known to bind to calmodulin in a Ca^{2+}-dependent manner and inhibit the ability of the protein to activate target enzymes (Levin & Weiss, 1976). Various calmodulin fragments and a skeletal muscle troponin C synthetic peptide have also been shown to bind phenothiazines. Newton et al. (1984) reported that several tryptic fragments of calmodulin, 1–77, 1–90, 1–106, and 78–148, bound to a norchlorpromazine affinity column, while CaM 107–148 did not. Using ^{113}Cd NMR spectroscopy to monitor binding of trifluoperazine to several tryptic and thrombic fragments of calmodulin, Thulin et al. (1984) also concluded that fragments 1–77, 1–106, and 78–148 bound the phenothiazine, but not fragment 107–148. The fragment 107–148 also failed to bind mastoparan X at concentrations up to the micromolar range (Malencik & Anderson, 1984).

A cyanogen bromide fragment of calmodulin corresponding to residues 77–124 was also reported to bind fluphenazine (Head et al., 1982), and several phenothiazines altered the CD spectrum, indicative of binding, of a synthetic pepide (residues 90–123) of rabbit skeletal muscle troponin C (Reid et al., 1983). These results strongly suggest the presence of multiple binding sites on calmodulin, a conclusion that is supported by recent binding studies of chlorpromazine and trifluoperazine to calmodulin and various adducts (Marshak et al., 1985; Giedroc et al., 1985a; Jackson & Puett, 1986), where stoichiometries of 6–8 mol of phenothiazine/mol of protein were reported in the presence of Ca^{2+}. Moreover, there appears to be some commonality in the binding sites of phenothiazines and at least one calmodulin-binding peptide, β-endorphin, to calmodulin (Giedroc et al., 1983b, 1985a,b).

Further work with synthetic analogues of parts or all of the central helix and with analogues of other helical segments in the carboxy-terminal half should help in determining precisely the high-affinity peptide-binding regions of CaM. Interestingly, on the basis of ^{113}Cd NMR, Linse et al. (1986) have suggested that mastoparan induces conformational changes in the two globular lobes of calmodulin, and Yazawa et al. (1987) have recently proposed that mastoparan facilitates "communication" of the two lobes. It is anticipated that elucidation of the nature of peptide binding to calmodulin may reveal interesting information on the association of calmodulin with target enzymes.

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REFERENCES

Anderson, S. R., & Malencik, D. A. (1986) in *Calcium and Cell Function*, Vol. VI, pp 1–42, Academic, New York.

Aulabaugh, A., Niemczura, W. P., & Gibbons, W. A. (1984) *Biochem. Biophys. Res. Commun. 118*, 225–232.

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

Barnette, M. S., Daly, P., & Weiss, B. (1983) *Biochem. Pharmacol. 32*, 2929–2933.

Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269–272.

Cox, J. A., Comte, M., Fitton, J. E., & Degrado, W. F. (1985) *J. Biol. Chem. 260*, 2527–2534.

Dalgarno, D. C., Klevit, R. E., Levine, B. A., Williams, R. J. P., Dobrowolski, Z., & Drabikowski, W. (1984) *Eur. J. Biochem. 138*, 281–289.

Degrado, W. F., Kezdy, F. J., & Kaiser, E. T. (1981) *J. Am. Chem. Soc. 103*, 679–681.

Eisenberg, D., Weiss, R. M., & Terwilliger, T. C. (1982) *Nature (London) 299*, 371–374.

Erickson-Viitanen, S., & Degrado, W. F. (1987) *Methods Enzymol. 139*, 455–478.

Giedroc, D. P., & Puett, D. (1985) *Mol. Pharmacol. 28*, 588–593.

Giedroc, D. P., Ling, N., & Puett, D. (1983a) *Biochemistry 22*, 5584–5591.

Giedroc, D. P., Puett, D., Ling, N., & Staros, J. V. (1983b) *J. Biol. Chem. 258*, 16–19.

Giedroc, D. P., Keravis, T. M., Staros, J. V., Ling, N., Wells, J. N., & Puett, D. (1985a) *Biochemistry 24*, 1203–1211.

Giedroc, D. P., Sinha, S. K. Brew, K., & Puett, D. (1985b) *J. Biol. Chem. 260*, 13406–13413.

Guerine, D., Krebs, J., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 15172–15177.

Head, J. F., Masure, H. R., & Kaminer, B. (1982) *FEBS Lett. 137*, 71–74.

Higashijima, T., Wakamatsu, K., Takemitsu, M., Fujino, M. Nakajima, T., & Mivazawa, T. (1983) *FEBS Lett. 152*, 227–230.

Ikura, M., Hiraoki, T., Hikichi, K., Minowa, O., Yamaguchi, H., Yazawa, M., & Yagi, K. (1984) *Biochemistry 23*, 3124–3128.

Ikura, M., Minowa, O., & Hikichi, K. (1985) *Biochemistry 24*, 4264–4269.

Jackson, A. E., & Puett, D. (1984) *J. Biol. Chem. 259*, 14985–14992.

Jackson, A. E., & Puett, D. (1986) *Biochem. Pharmacol. 35*, 4395–4400.

Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489–515.

Klevit, R. E., Dalgarno, D. C., Levine, B. A., & Williams, R. J. P. (1984) *Eur. J. Biochem. 139*, 109–114.

Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, N., & Cohen, P. (1981) *FEBS Lett. 130*, 141–145.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.

Laporte, D. C., Wierman, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814–3819.

Levin, R. M., & Weiss, B. (1976) *Mol. Pharmacol. 12*, 581–589.

Linse, S., Drakenberg, T., & Forsen, S. (1986) *FEBS Lett. 199*, 28–32.

Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480–3486.

Malencik, D. A., & Anderson, S. R. (1983a) *Biochem. Biophys. Res. Commun. 114*, 50–56.

Malencik, D. A., & Anderson, S. R. (1983b) *Biochemistry 22*, 1995–2001.

Malencik, D. A., & Anderson, S. R. (1984) *Biochemistry 23*, 2420–2428.

Marshall, D. R., Lucas, T. J., & Watterson, D. M. (1985) *Biochemistry 24*, 144–150.

Maulet, Y., & Cox, J. A. (1983) *Biochemistry 22*, 5680–5686.

McDowell, L. M., Sanyal, G., & Prendergast, F. G. (1985) *Biochemistry 24*, 2979-2984.
Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., & Klee, C. B. (1984) *J. Biol. Chem. 259*, 4419-4426.
Ni, W.-C., & Klee, C. B. (1985), *J. Biol. Chem. 260*, 6974-6981.
O'Neil, K. T., Wolfe, H. R., Jr., Erickson-Viitanen, S., & Degrado, W. F. (1987) *Science (Washington, D.C.) 236*, 1454-1456.
Puett, D., Giedroc, D. P., Tollefson, S., & Ling, N. (1983) *Peptides (Fayetteville, N.Y.) 4*, 191-194.
Reid, R. E., Gariepy, J., & Hodges, R. S. (1983) *FEBS Lett. 154*, 60-64.
Sanyal, G., & Prendergast, F. G. (1988) in *Fluorescent Biomolecules*, Plenum, New York (in press).
Tanaka, Y., Takahashi, S., Mitsui, Y., Itoh, S., Iitaka, Y., Kasai, H., & Okuyama, T. (1985) *J. Mol. Biol. 186*, 675-677.
Thulin, E., Andersson, A., Drakenberg, T., Forsén, & Vogel, H. J. (1984) *Biochemistry 23*, 1862-1870.
Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977) *J. Biol. Chem. 252*, 7440-7443.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962-975.
Yazawa, M., Ikura, M., Hikichi, K., Ying, L., & Yagi, K. (1987) *J. Biol. Chem. 262*, 10951-10954.

Filament Assembly and Regulation of the Actin-Activated ATPase Activity of Thymus Myosin†

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ABSTRACT: The effects of light chain phosphorylation on the actin-activated ATPase activity and filament assembly of calf thymus cytoplasmic myosin were examined under a variety of conditions. When unphosphorylated and phosphorylated thymus myosins were monomeric, their MgATPase activities were not activated or only very slightly activated by actin, but when they were filamentous, their MgATPase activities were stimulated by actin. The phosphorylated myosin remained filamentous at lower Mg^{2+} concentrations and higher KCl concentrations than did the unphosphorylated myosin, and the myosin concentration required for filament assembly was lower for phosphorylated myosin than for unphosphorylated myosin. By varying the myosin concentration, it was possible to have under the same assay conditions mostly monomeric myosin or mostly filamentous myosin; under these conditions, the actin-activated ATPase activities of the filamentous myosins were much greater than those of the monomeric myosins. The addition of phosphorylated myosin to unphosphorylated myosin promoted the assembly of unphosphorylated myosin into filaments. These results suggest that phosphorylation may regulate the actomyosin-based motile activities in vertebrate nonmuscle cells by regulating myosin filament assembly.

Vertebrate smooth muscle and nonmuscle myosins contain two 200 000-Da heavy chains and two pairs of light chains, M_r 20 000 and 17 000. Phosphorylation of the 20 000-Da light chains (LC20)[1] is thought to play a central role in the regulation of smooth muscle contraction and nonmuscle contractility (Kendrick-Jones & Scholey, 1981; Sellers & Adelstein, 1987). In vitro, this phosphorylation affects both the actin-activated ATPase activities of these myosins (Sobieszek & Small, 1977; Sherry et al., 1978; Trotter & Adelstein, 1979; Sellers et al., 1981; Fechheimer & Cebra, 1982; Scholey et al., 1982; Wagner et al., 1985; Wagner & Vu, 1986) and their assembly into filaments (Suzuki et al., 1978; Scholey et al., 1980; Citi & Kendrick-Jones, 1986; Wagner & Vu, 1987). We have previously shown that the actin-activated ATPase activities of filamentous, unphosphorylated, and phosphorylated calf thymus myosins have about the same maximum rates, V_{max} (Wagner & George, 1986), but phosphorylation does cause a 15-20-fold decrease in K_{app}, the actin concentration required to achieve $^1/_2V_{max}$. Thus, at low actin concentrations phosphorylation causes a large increase in ATPase activity, but at high actin concentrations unphosphorylated and phosphorylated thymus myosins have comparable ATPase activities. Similar results were obtained with skeletal muscle and thymus actins. In the presence of either skeletal muscle or thymus tropomyosin, phosphorylation causes no change in the V_{max} of the actin-activated ATPase activity of filamentous thymus myosin and only a 3-5-fold decrease in K_{app}. This decrease in K_{app} appears to be too small for this phosphorylation to be an effective regulatory system.

The observation that the actin-activated ATPase activities of unphosphorylated and phosphorylated thymus myosins have the same V_{max} was made under conditions in which both the unphosphorylated and the phosphorylated myosins were greater than 90% filamentous (Wagner & George, 1986). These conditions were chosen to avoid the complication of comparing the actin-activated ATPase activities of monomeric and filamentous myosins. When the assays were performed

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[1] Abbreviations: ATPase, adenosinetriphosphatase; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; LC20, 20 000-dalton myosin light chain.