Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.
Westman, J., & Eriksson, L. E. G. (1979) *Biochim. Biophys. Acta 557*, 62–68.
Westman, J., Boulanger, Y., Ehrenberg, A., & Smith, I. C. P. (1982) *Biochim. Biophys. Acta 685*, 315–328.
Zakrzewska, K., & Pullman, B. (1981) *FEBS Lett. 135*, 268–272.

Peptide Binding by Calmodulin and Its Proteolytic Fragments and by Troponin C†

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ABSTRACT: Calmodulin and troponin C exhibit calcium-dependent binding of 1 mol/mol of dynorphin$_{1-17}$. The dissociation constants of the complexes, determined in 0.20 N KCl–1.0 mM $CaCl_2$, pH 7.3, are 0.6 μM for calmodulin, 2.4 μM for rabbit fast skeletal muscle troponin C, and 9 μM for bovine heart troponin C. Experiments with deletion peptides of dynorphin$_{1-17}$ show that peptide chain length and especially charge affect the binding of the peptides by calmodulin. Dynorphin$_{1-17}$, but not mastoparan or melittin, inhibits adenosinetriphosphatase activity in a reconstituted rabbit skeletal muscle actomyosin assay. The inhibition is partially reversed by the addition of calmodulin or troponin C in the presence of calcium. Calmodulin also exhibits calcium-dependent binding of a synthetic peptide corresponding to positions 104–115 of rabbit fast skeletal muscle troponin I. Mastoparan is a tetradecapeptide from the vespid wasp having exceptional affinity for calmodulin, with K_d ~0.3 nM [Malencik, D. A., & Anderson, S. R. (1983) *Biochem. Biophys. Res. Commun. 114*, 50]. The addition of 1 mol/mol of mastoparan to the complex of calmodulin with dynorphin$_{1-17}$ results in complete dissociation of dynorphin. Similar titrations of the skeletal muscle troponin C–dynorphin$_{1-17}$ complex produce a gradual dissociation consistent with a dissociation constant of 0.2 μM for the troponin C–mastoparan complex. Fluorescence anisotropy measurements using the intrinsic tryptophan fluorescence of mastoparan X show strongly calcium-dependent binding by proteolytic fragments of calmodulin. Dissociation constants for the complexes with mastoparan X are ~0.9 nM for calmodulin, 0.9 μM for thrombic fragment 1–106, and ~0.15 μM for half-calmodulin fragment 72–148. Neither fragment 107–148 nor parvalbumin associates with mastoparan X in this concentration range. The fluorescence emission spectra of the tryptophan-containing peptides display varying shifts toward shorter wavelengths on the binding of either calmodulin or troponin C, indicating that the environment of the peptide binding site in these proteins is *either hydrophobic or rigid, hydrophilic*. Competition experiments show that melittin may have the highest affinity for calmodulin of any protein or peptide known to interact reversibly with it. The calculated relative affinities suggest that the dissociation constant for the melittin–calmodulin complex is smaller than 3 nM, the value originally estimated [Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269].

The binding of calcium stabilizes one or more specific conformations of the calmodulin molecule recognized by calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase, adenylate cyclase, and myosin light chain kinase [cf. reviews by Means (1981) and Cheung (1980, 1982)]. Calmodulin also shows calcium-dependent binding of a number of small basic polypeptides. Weiss et al. (1980) first noted that adrenocorticotropin (ACTH)[1] and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase and demonstrated that these peptides compete with the enzyme for calmodulin. Malencik & Anderson (1982, 1983a) performed fluorescence binding measurements showing the interaction of calmodulin with ACTH, β-endorphin, substance P, glucagon, dynorphin$_{1-13}$, secretin, the gastric inhibitory peptide (GIP), and the vasoactive intestinal peptide (VIP) with dissociation constants ranging from several micromolar to 50 nM in the case of VIP. Several toxic peptides from Hymenoptera species proved surprisingly efficient in calmodulin binding. The complex of calmodulin with melittin, a 26-residue polypeptide from honey bee venom, has a reported dissociation constant of 3 nM (Comte et al., 1983) while the complex with mastoparan, a tetradecapeptide from the vespid wasp (Hirai et al., 1979), has a dissociation constant of ~0.3 nM (Malencik & Anderson, 1983b). Coincidentally, Barnette et al. (1983) discovered that mastoparan is a potent inhibitor of calmodulin-stimulated phosphodiesterase activity. Giedroc et al. (1983a,b) have used the hydrophilic cross-linking reagent bis(sulfosuccinimidyl) suberate to couple β-endorphin and some deletion peptides from its C-terminal half to calmodulin.

The peptides which calmodulin binds well generally contain homologies consisting of a strongly basic tripeptide sequence, with at least two residues which are either Arg or Lys, three positions away from a pair of hydrophobic residues (Malencik

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3',5'-phosphate; CaM, calmodulin; TnC, troponin C; Dyn, dynorphin; ACTH, adrenocorticotropic hormone; VIP, vasoactive intestinal peptide; GIP, gastric inhibitory peptide; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; ANS, 8-anilino-1-naphthalenesulfonate; NaDodSO$_4$, sodium dodecyl sulfate; K, dissociation constant; F, observed fluorescence; F_0, fluorescence of unbound ligand; F_∞, fluorescence of totally bound ligand; $\bar{A}$, average anisotropy; A_f, anisotropy of unbound ligand; A_b, anisotropy of bound ligand; [X_0], total concentration of ligand; [X], concentration of unbound ligand; [P_0], total concentration of protein (usually CaM or TnC); [P], concentration of unbound protein; f_b, fraction of fluorescent ligand bound; ϕ, fractional degree of saturation of calmodulin with peptide.

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& Anderson, 1982). We suggested that the cluster of basic amino acid side chains interacts with a complementary anionic site on calmodulin such as residues 78–84 (Asp-Thr-Asp-Ser-Glu-Glu-Glu) or 118–123 (Asp-Glu-Val-Asp-Glu) (Malencik & Anderson, 1983a). The hydrophobic residues often appear in the center of a predicted α-helix or β-strand. The peptide binding specificity of calmodulin resembles that of the cAMP-dependent protein kinase (Malencik & Anderson, 1982; Malencik et al., 1982a,b). The definite stoichiometry, the strong calcium dependence, and the competition for a common binding site indicate that these peptides are useful models for the calmodulin binding sites in calmodulin-dependent enzymes.

The present experiments describe the association of calmodulin and some of its proteolytically derived fragments and troponin C with the dynorphins, the mastoparans, and melittin. They provide insight into the location of the peptide binding site in calmodulin and into the overlapping binding specificities of calmodulin and troponin C.

Materials and Methods

All fluorescence experiments were carried out in the presence of the specified concentrations of KCl (usually 0.20 N), 5–50 mM Mops, and the indicated concentrations of EDTA or $CaCl_2$, pH 7.3 at 25 °C. Glass-distilled water was used throughout. All chemicals were reagent grade.

Glucagon and melittin, which was further purified as described by Maulet et al. (1982), were purchased from Sigma Chemical Co. The deletion peptides of dynorphin$_{1-17}$, all of which were free acids, and the mastoparans were products of Peninsula Labs. Dynorphin$_{1-17}$, free-acid form, was obtained from Vega Biochemicals. The synthetic peptide corresponding to positions 104–115 of rabbit skeletal muscle troponin I was a gift from Prof. Robert Hodges. The concentrations of the peptides in the stock solutions (usually 1–2 mg/mL) were determined by weight and by UV absorbance when aromatic residues were present.

Porcine brain calmodulin, prepared according to Schreiber et al. (1981), was subjected to a final purification step by using affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). This additional purification removes tryptophan-containing impurities. The calmodulin concentrations are based on $E^{1\%}_{280nm} = 2.0$ and a molecular weight of 16 680 (Watterson et al., 1980). Dansylcalmodulin, containing an average of 1.7 mol of the dansyl moiety, was prepared by using 5-(dimethylamino)-1-naphthalenesulfonyl chloride as described by Malencik & Anderson (1982). The C-terminal fragment of human brain calmodulin had been prepared by Schreiber et al. (1981). The thrombic fragments of calmodulin were prepared according to the procedure of Wall et al. (1981), following the details given by Andersson et al. (1983). The thrombin was obtained from Sigma. The fragments were characterized by $NaDodSO_4$ electrophoresis, UV absorption, and amino acid analysis. Rabbit skeletal muscle troponin C and bovine cardiac muscle troponin C were purified according to Potter's procedure (1982) and dogfish parvalbumin according to Pechere et al. (1971).

Fluorescence spectra were recorded with a Hitachi Perkin-Elmer MPF2A fluorometer and corrected for grating transmission and detector response. Fluorescence intensity measurements at fixed excitation and emission wavelengths were also obtained with the Hitachi Perkin-Elmer MPF2A fluorometer. The details of the titrations of dansylcalmodulin and of the tryptophan-containing peptides were described by Malencik & Anderson (1982). Following the probability of binding principle developed by Weber (1965), the peptide and protein concentrations used in experiments to determine dissociation constants were of the same magnitude as the constants whenever possible. Since direct determination of very small K_d's ($<10^{-7}$ M) is not feasible, we used competition experiments in the case of the high-affinity peptides. The titrations involve the addition of microliter quantities of titrant, using Hamilton syringes, to 1.5 mL of solution in the fluorescence cuvette. Dilution factors were minimized to 2% or less. Corrections were made for the background fluorescence of protein and solvent blanks, which were less than 5% of the sample intensity. Mathematical analysis of the fluorescence titrations is described in detail by Anderson (1974), Malencik & Anderson (1982, 1983a), and Malencik et al. (1982a).

Measurements of fluorescence anisotropy and total intensity ($I_\parallel + 2I_\perp$) were obtained by using the SLM 4000 fluorescence polarization spectrophotometer. The excitation wavelength was set at 294 nm, which gives a favorable limiting anisotropy for tryptophan and largely excludes tyrosine fluorescence. Glass cutoff filters (Corning Glass CSO-54) were placed between the sample and the photomultiplier tubes. These filters transmit most of the tryptophan emission spectrum. Both fluorometers were connected to circulating water baths to maintain a constant temperature (25.0 ± 0.1 °C) in the samples.

The inhibitory effect of dynorphin$_{1-17}$ on actomyosin adenosinetriphosphatase was determined in a reconstituted system containing 3.3 μM heavy meromyosin, 34 μM F-actin, and 4.9 μM tropomyosin in a solution of 10 mM imidazole hydrochloride, pH 7.0, 40 mM KCl, 3.0 mM ATP, 5.0 mM magnesium acetate, ±0.2 mM calcium acetate, and ±4 mM EGTA. The reconstitution and fixed-time assays of inorganic phosphate using the Fiske–SubbaRow method were performed as described by Potter (1982). Rabbit muscle myosin and heavy meromyosin were prepared according to Margossian & Lowey (1982), F-actin according to Pardee & Spudich (1982), tropomyosin according to Smillie (1982), and troponin according to Potter (1982).

Results

Binding of the Dynorphins by Calmodulin and Troponin C. The dynorphins are opioid peptides of unknown physiological function (Goldstein et al., 1979). Dynorphin$_{1-13}$ forms a complex with calmodulin, having a dissociation constant in the micromolar range (Malencik & Anderson, 1983a), and inhibits cyclic nucleotide phosphodiesterase (Sellinger-Barnette & Weiss, 1982). Unlike most of the calmodulin binding peptides, dynorphin$_{1-13}$ (see Table II for sequence) probably contains no repetitive secondary structure. However, some of the binding sites in calmodulin-dependent enzymes or other proteins which recognize calmodulin may contain similar strongly basic "coils". For example, the sequences of the dynorphins remind us of the inhibitory region of troponin I, the most widely used model calmodulin binding protein (Grand et al., 1979). Troponin C, the calcium binding subunit in the troponin complex, is ancestrally related to calmodulin (Barker et al., 1977).

The binding of dynorphin$_{1-17}$,[2] which contains tryptophan at position 14, can be detected in measurements of the intrinsic peptide fluorescence. The fluorescence spectra of other tryptophan-containing peptides—ACTH, glucagon (Malencik

[2] Sequences: dynorphin$_{1-17}$, Tyr-Gly$_2$-Phe-Leu-Arg$_2$-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln; mastoparan, Ile-Asn-Leu-Lys-Ala-Leu-Ala$_2$-Leu-Ala-Lys$_2$-Ile-Leu-NH_2; mastoparan X, Ile-Asn-Trp-Lys-Gly-Ile-Ala$_2$-Met-Ala-Lys$_2$-Leu$_2$-NH_2.

& Anderson, 1982), gastric inhibitory peptide (Malencik & Anderson, 1983a), *Polistes* mastoparan, mastoparan X (Malencik & Anderson, 1983b), and melittin (Comte et al., 1983)—are known to change when calmodulin binds. This effect is especially useful for binding measurements since calmodulin contains no tryptophan; the intrinsic tyrosine fluorescence of calmodulin or troponin C is largely excluded by excitation at 290–295 nm (Teale, 1960; Weber, 1961). Determination of the fluorescence *anisotropy* is an independent method for the detection of complex formation which is based on molecular weight changes and is responsive to binding even when the fluorescence spectrum and quantum yield do not change. The anisotropy, $\bar{A} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$,[3] is related to the lifetime of the excited state (τ) and to the rotational relaxation time (ρ) of the molecule or complex to which the fluorescent moiety (tryptophan) is attached:

$$A = \frac{A_0}{1 + 3\tau/\rho} \quad (1)$$

A_0, the anisotropy obtained in the absence of rotational diffusion, is a constant determined by intrinsic effects, including the electronic transitions involved in absorption. The rotational relaxation time is related to the size of the molecule; in the case of an anhydrous sphere, $\rho = 3\eta V/(RT)$ where V is the molecular volume [cf. reviews by Weber (1953, 1966)]. Although τ may vary to some extent, the largest changes occurring when peptides in the molecular weight range 1500–3000 associate with calmodulin (M_r 16 700) are in ρ. If changes in shape are disregarded, ρ is expected to increase 7–12-fold.

In the case of a simple dissociation involving two fluorescent components (X and PX), PX $\rightleftharpoons$ P + X, the values of $\bar{A}$ range from that of the unbound ligand X (A_f) to that of the bound ligand PX (A_b). Following the principle of additivity of anisotropies (Weber, 1952), the fraction of the fluorescent peptide bound is simply calculated when the relative fluorescence yields of X and PX are identical under the observation conditions:

$$f_b = (\bar{A} - A_f)/(A_b - A_f) \quad (2)$$

When the yields are not identical, a related equation in which F_∞/F_0 is the ratio of the fluorescence intensities of the totally bound and free ligand is used (Evett & Isenberg, 1969; S. R. Anderson and G. Weber, unpublished results):

$$f_b = \frac{\bar{A} - A_f}{(\bar{A} - A_f) + (F_\infty/F_0)(A_b - \bar{A})} \quad (3)$$

The following experiments on the binding of tryptophan-containing peptides—dynorphin$_{1-17}$,[2] dynorphin$_{6-17}$, mastoparan X,[2] and melittin—use measurements of both fluorescence intensity and anisotropy. Equation 2 or 3 is applied whenever equilibrium constants are calculated from anisotropy data. They are also used to calculate the values of $\bar{A}$ corresponding to specified values of f_b.

Figure 1 shows the increase in fluorescence anisotropy occurring when 4.0 μM dynorphin$_{1-17}$ is titrated with calmodulin, rabbit skeletal muscle troponin C, or bovine cardiac troponin C. Although skeletal muscle troponin C showed little interaction with ACTH and glucagon (Malencik & Anderson, 1982; unpublished data), its affinity for dynorphin$_{1-17}$ approaches that of calmodulin. The binding is calcium dependent, with slight interaction occurring in solutions containing 10^{-4} M EDTA and no added calcium. The changes in the fluorescence intensity (data not shown) and anisotropy values were extrapolated to infinite protein concentration, giving F_∞/F_0 and A_b. The values of f_b obtained from eq 3 were then used in the calculation of [X], [P], and [PX] (Anderson, 1974). Dissociation constants were calculated from plots of $1/f_b$ vs. $1/[P]$. Table I shows the values of K, A_b, and F_∞/F_0 determined in 0.10 and 0.20 N KCl. The marked ionic strength dependence of dynorphin$_{1-17}$ binding is expected in such a highly charged peptide.[2] Parallel titrations of glucagon with calmodulin (K_d = 3.4 μM) are essentially independent of ionic strength in this range. The dissociation constant for the complex of calmodulin with dynorphin$_{1-17}$ agrees with that determined later by using dansylcalmodulin. The latter value is more reliable since a protein concentration comparable to K_d could be used. The fluorescence emission spectra of these complexes suggest differences in the microscopic environments of the peptide binding sites in calmodulin and troponin C (Figure 2). The inset to Figure 2 shows a stoichiometric fluorescence titration of dynorphin$_{1-17}$ with calmodulin.

We performed competition experiments to investigate relationships between the binding of dynorphin$_{1-17}$ and the binding of other peptides. Mastoparan[2] is a tetradecapeptide possessing a high α-helical potential, no aromatic amino acids, and exceptional affinity for calmodulin (K_d ~0.3 nM) (Malencik & Anderson, 1983b). Figure 3A shows complete release of dynorphin$_{1-17}$ on the addition of 1 mol of masto-

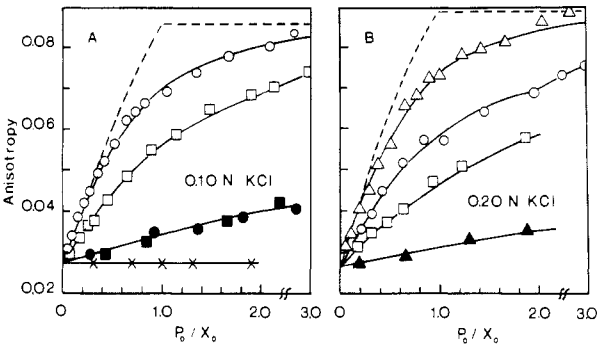

FIGURE 1: Fluorescence anisotropy titrations of 4.0 μM dynorphin$_{1-17}$ with calmodulin (▲), rabbit skeletal muscle troponin C (○), bovine cardiac troponin C (□), and dogfish parvalbumin (×). Conditions: 0.10 (A) or 0.20 (B) N KCl plus 10.0 mM Mops–1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. The solid lines are theoretical curves calculated from the constants summarized in the text. The dashed line in (A) was calculated for complete binding of skeletal troponin C up to saturation; the dashed line in (B) was similarly calculated for calmodulin. The solid symbols designate the corresponding control experiments performed with solutions containing 10^{-4} M EDTA and no added calcium. P_0/X_0 is the ratio of the concentration of calmodulin or troponin C to the concentration of dynorphin. Excitation, 294 nm with 2-nm band-pass; emission filters, Corning glass CSO-54.

Table I: Binding of Dynorphin$_{1-17}$ by Calmodulin and Troponin C[a]

protein	[KCl] (N)	K_d (μM)	A_b	F_∞/F_0
CaM	0.20	0.6 ± 0.15	0.089	1.45
skeletal TnC	0.10	0.6 ± 0.15	0.086	1.34
skeletal TnC	0.20	2.4 ± 0.2	0.086	1.34
heart TnC	0.10	4.0 ± 0.4	0.090	1.40
heart TnC	0.20	9.0 ± 1.0	0.090	1.40

[a] Conditions: 10 mM Mops-1 mM $CaCl_2$, pH 7.3, 25.0 °C. A_b is the anisotropy of the complex and F_∞/F_0 the fluorescence enhancement factor.

[3] $\bar{A} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$ where $I_\parallel$ and $I_\perp$ are the intensities of the two linearly polarized components of the light emitted at right angles to the excitation direction. $I_\parallel$ vibrates in the direction of propagation of the exciting light, and $I_\perp$ vibrates normally to the plane corresponding to the directions of excitation and observation. In a mixture of fluorescent species, the average anisotropy equals the sum of the individual anisotropies weighed by the individual fractional contributions to the total fluorescence intensity (Weber, 1952): $\bar{A} = \sum F_i A_i$.

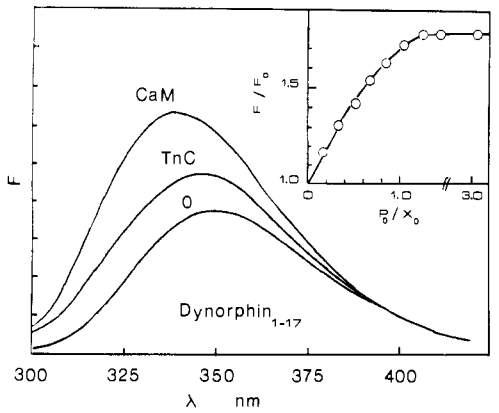

FIGURE 2: Fluorescence spectra of the complexes of dynorphin$_{1-17}$ with calmodulin and rabbit skeletal muscle troponin C. Conditions: 2.7 μM dynorphin$_{1-17}$ plus 8.0 μM calmodulin or 17.0 μM troponin C in 0.05 N KCl, 10 mM Mops, and 1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. Excitation, 290 nm. The band widths of excitation and emission were 4 and 5 nm, respectively. The inset contains a fluorescence titration of 4.8 μM dynorphin$_{1-17}$ with calmodulin followed at 340 nm.

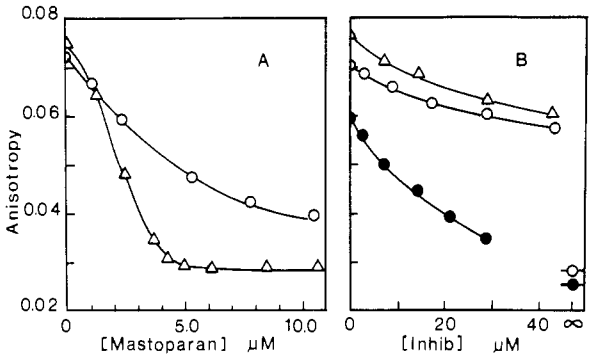

FIGURE 3: Displacement of bound dynorphin$_{1-17}$ by mastoparan and the peptide analogue corresponding to positions 104–115 of rabbit skeletal muscle troponin I. Panel A demonstrates the effects of mastoparan on the dynorphin$_{1-17}$–calmodulin complex (4.0 μM, Δ) and on the dynorphin$_{1-17}$–troponin C complex (4.0 μM dynorphin + 4.2 μM TnC, ○). Panel B shows the corresponding effects of the inhibitory peptide analogue. Conditions: 0.10 N KCl, 10 mM Mops, and 1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. The dissociation obtained with 4.0 μM dynorphin$_{6-17}$ plus 6.4 μM troponin C in 0.05 N KCl (●) is also given. The measurement conditions are described under Figure 1.

paran/mol of calmodulin. (This titration is nearly the reverse of that resulting when dynorphin$_{1-17}$ is titrated with calmodulin.) Even though the two bound peptides may have different conformations, they compete in binding. The addition of mastoparan to the glucagon–calmodulin complex also gives stoichiometric displacement, as did VIP and secretin (Malencik & Anderson, 1983a). Titration of the skeletal muscle troponin C–dynorphin$_{1-17}$ complex with mastoparan results in gradual dissociation (Figure 3A). By calculating changes in the concentration of bound dynorphin$_{1-17}$, we calculate a K_d of 0.2 μM for the troponin C–mastoparan complex.[4] Evidently the exceptional affinity of mastoparan for calmodulin is a unique feature not shared with troponin C.

The commercial availability of deletion peptides of dynorphin$_{1-17}$ permitted systematic study of the effects of peptide

[4] When there are two competing equilibria with fluorescent ligand X and nonfluorescent ligand Y:

$$PX \rightleftharpoons P + X \qquad PY \rightleftharpoons P + Y$$

$$K_x/K_y = ([X]/[PX])/([Y]/[PY])$$

where $[X]/[PX] = (1 - f_b)/f_b$, $[PY] = [P_0] - f_b[X_0] - f_bK_x/(1 - f_b)$, and $[Y] = [Y_0] - [PY]$, $[Y_0]$ being the total concentration of ligand Y.

Table II: Binding of Deletion Peptides of Dynorphin$_{1-17}$ by Dansylcalmodulin[a]

sequence position	K_d (μM) at a KCl concn (N) of[b]			
1 5 10 15	0.20	0.10	0.05	0.025
Y G G F L R R I R P K L K W D N Q	0.7			
R R I R P K L K W D N Q	>40	4.3	1.8	
Y G G F L R R I R P K L K	2.5	0.82		
Y G G F L R R I R P K L	7.1	2.4	0.7	
Y G G F L R R I R P K	14	3.6	1.6	
Y G G F L R R I R P		18	11	
Y G G F L R R I R		45	20	
Y G G F L R R I				>200

[a] Conditions: 5.0 nM Mops–1.0 mM $CaCl_2$, pH 7.8 (25.0 °C). The concentration of dansylcalmodulin was 0.2–2.0 μM, with the lower concentrations used for the peptide giving the smaller dissociation constants. [b] Reproducibility was ±10–15%.

chain length and charge. Since most of these peptides contain no tryptophan, we used the covalent conjugate of calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride. The binding of specific peptides and proteins by dansylcalmodulin causes the quantum yield to increase and the fluorescence emission maximum to shift from 522 to 500–510 nm (Malencik & Anderson, 1982). The fractional degree of saturation (ϕ) of dansylcalmodulin with the peptide is related to the fluorescence enhancement:

$$\phi = (F/F_0 - 1)/(F_\infty/F_0 - 1)$$

Table II summarizes the dissociation constants calculated from the changes in fluorescence intensity when dansylcalmodulin was titrated with the deletion peptides in solutions containing 0.025–0.20 N KCl. The removal of a basic amino acid residue consistently has a larger effect on calmodulin binding than the removal of a neutral residue. Peptide charge becomes increasingly critical with decreasing chain length. Even though calmodulin does not bind Leu-enkephalin, removal of the N-terminal opioid pentapeptide from dynorphin$_{1-17}$ has a large effect on binding. The sharp decrease in binding found with dynorphin$_{1-8}$ agrees with that determined by Sellinger-Barnette & Weiss (1984) in inhibition experiments.

Talbot & Hodges (1981a,b) synthesized several peptide analogues of the inhibitory region of troponin I. One of these, corresponding to positions 104–115 of rabbit fast skeletal muscle troponin I, was donated to us: Ac-Gly-Lys-FPhe-Lys-Arg-(Pro)$_2$-Leu-(Arg)$_2$-Val-Arg-NH_2. This fluorinated derivative had been used in proton magnetic resonance experiments, suggesting its interaction with the N-terminal section of region III in troponin C (Cachia et al., 1983). Fluorescence intensity titrations of dansylcalmodulin with the inhibitory peptide gave dissociation constants of 4.8 μM in solutions containing 0.20 N KCl, 5.0 mM Mops, and 1 mM $CaCl_2$, pH 7.8 (25 °C) and of 0.94 μM in comparable solutions containing 0.10 N KCl. No interaction in the micromolar concentration range occurs in solutions containing 10^{-4} M EDTA, no added, calcium, and 0.20 N KCl. Addition of the fluorinated inhibitory peptide to the complexes of rabbit skeletal muscle troponin C and calmodulin produces gradual dissociation (Figure 3B). The results are consistent with a dissociation constant of 10–13 μM for the complex of troponin C with the inhibitory peptide.[4] The weak binding of the inhibitory peptide, in comparison to that of dynorphin$_{1-17}$, probably reflects its shorter chain length. Displacement occurs more readily when dynorphin$_{6-17}$ is substituted for dynorphin$_{1-17}$. The dynorphins and the inhibitory peptide probably

Table III: Effects of Calmodulin Binding Peptides on the Catalytic Activity of Reconstituted Rabbit Muscle Adenosinetriphosphatase[a]

protein or peptide additions	ΔA[b] in 0.2 mM $CaCl_2$	ΔA[b] in 4 mM EGTA
none	0.45	0.44
5 μM troponin	0.46	0.13
40 μM TnC	0.39	0.42
20 μM CaM	0.50	0.34
5 μM TnI	0.11	0.10
5 μM Dyn_{1-17}	0.25	0.25
15 μM Dyn_{1-17}	0.19	0.22
10 μM mastoparan	0.48	0.43
60 μM melittin	0.66	0.60
5 μM TnI + 40 μM TnC	0.43	0.38
5 μM TnI + 20 μM CaM	0.36	0.28
15 μM Dyn_{1-17} + 40 μM TnC	0.51	0.38
15 μM Dyn_{1-17} + 20 μM CaM	0.64	0.37

[a] Conditions: 3.3 μM heavy meromyosin, 34 μM F-actin, 4.9 μM tropomyosin in 10 mM imidazole hydrochloride, pH 7.0, 40 mM KCl, 3.0 mM ATP, and 5.0 mM magnesium acetate. [b] Absorbancy change measured at 720 nm in a Fiske-SubbaRow assay performed after 5-min incubation. The reproducibility of these values was generally ±5%.

compete for common sites on both calmodulin and troponin C.

In view of the similarities between the dynorphins and the inhibitory region of troponin I, we determined the effect of added dynorphin$_{1-17}$ in a reconstituted rabbit skeletal muscle actomyosin assay system [cf. Potter (1982)]. The peptide analogues of Talbot & Hodges (1981a–c) had exerted an inhibitory effect in this assay similar to that of troponin I. Table III shows that the inclusion of dynorphin$_{1-17}$ in the assay also results in an inhibition of actomyosin adenosinetriphosphatase which is partially reversed by the addition of either troponin C or calmodulin in the presence of calcium. Mastoparan, in contrast, has no effect on the rate of ATP hydrolysis. The stimulatory effects of melittin and of the dynorphin–calmodulin complex were reproducible, as was the moderate inhibotory action of calmodulin obtained in the presence of EGTA. The effects of troponin C on the inhibition by troponin I were previously described by Eisenberg & Kielly (1974) and Amphlett et al. (1976). The calcium sensitivity that we find with calmodulin is less than that reported by Amphlett et al. (1976). However, calmodulin is known to interact with troponin I to some extent in the absence of calcium (LaPorte et al., 1981; Keller et al., 1982).

Binding of Mastoparan X by Calmodulin and Proteolytically Derived Fragments of Calmodulin. The exceptional stabilities of the calmodulin–mastoparan complexes suggested that the mastoparans could be useful for the detection of peptide binding by fragments of calmodulin, possibly permitting identification of the peptide binding site. We selected three proteolytically derived fragments of calmodulin for study: an N-terminal fragment containing residues 1–106, obtained by thrombin-catalyzed cleavage of calmodulin in the absence of calcium (Wall et al., 1981); the corresponding C-terminal fragment, comprising residues 107–148; and a C-terminal half-calmodulin fragment, corresponding to residues 72–148, obtained as result of endogenous proteolysis by Schreiber et al. (1981).

We carried out fluorescence anisotropy titrations of mastoparan X,[2] which contains tryptophan, with the three fragments as well as with calmodulin, rabbit muscle troponin C, and dogfish parvalbumin. Only fragment 107–148 and parvalbumin failed to interact with mastoparan X in this concentration range. Figure 4 shows that the addition of either calmodulin or fragment 72–148 gives nearly stoichiometric (complete) binding at the peptide concentration used (3.0 μM). The titration with fragment 72–148 indicates that $K_d \leq 0.15$ μM while that with fragment 1–106, the least strongly bound, gives a value for K_d of 0.9 μM. All the equilibria are strongly calcium dependent, with negligible interaction occurring in solutions containing EDTA. The values of A_b, the anisotropy of the bound peptide, approach the value obtained with calmodulin. This reflects the fact that the anisotropy approaches the limiting anisotropy, A_0, as the π/τ ratio increases (eq 1). Similar titrations of dynorphin$_{1-17}$ with fragment 72–148 revealed only a weak interaction which was not calcium dependent.

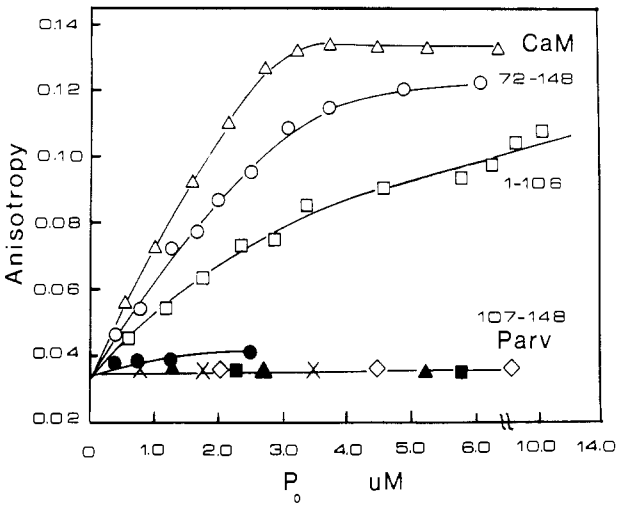

FIGURE 4: Fluorescence anisotropy titrations of 3.0 μM mastoparan X with calmodulin (△), half-calmodulin fragment 72–148 (○), thrombic fragments 1–106 (□) and 107–148 (◇), and dogfish parvalbumin (×). Conditions: 0.20 N KCl, 5.0 mM Mops, and 1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. The theoretical curve for fragment 72–148 was calculated for K_d = 0.15 μM, A_b = 0.125, and F_∞/F_0 = 1.0; the curve for fragment 1–106 corresponds to K_d = 0.9 μM, A_b = 0.11, and F_∞/F_0 = 1.0. The solid symbols represent the control experiments performed with 10^{-4} M EDTA and no added calcium. The measurement conditions are given under Figure 1.

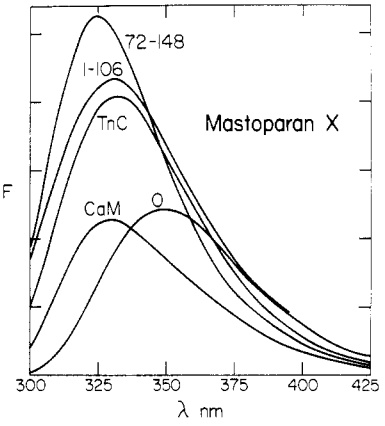

FIGURE 5: Fluorescence spectra of the complexes of mastoparan X with calmodulin, thrombic fragment 1–106, half-calmodulin fragment 72–148, and rabbit skeletal muscle troponin C. Conditions: 3.3 μM mastoparan X plus 3.3 μM calmodulin, 14 μM fragment 1–106, 9.0 μM fragment 72–148, or 4.0 μM troponin C in 0.20 N KCl–5.0 mM Mops–1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. The measurement conditions are outlined under Figure 2.

Figure 5 shows the distinctive fluorescence emission spectra of the complexes of mastoparan X with calmodulin, fragments 1–106 and 72–148, and rabbit skeletal muscle troponin C.

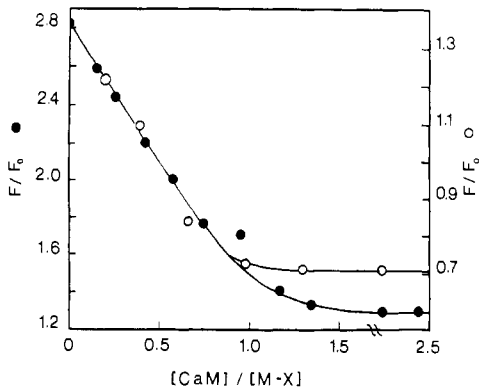

FIGURE 6: Effect of calmodulin addition on the complexes of mastoparan X with half-calmodulin fragment 72–148 and rabbit skeletal muscle troponin C. Conditions: 2.0 μM mastoparan X plus 3.0 μM fragment 72–148 with fluorescence emission measured at 330 nm (●) and 6.2 μM mastoparan X plus 6.4 μM troponin C with emission measured at 350 nm (○). The fluorescence changes reflect the differences between the corresponding spectra in Figure 5. The solutions contained 0.20 N KCl, 5.0 mM Mops, and 1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. Excitation wavelength, 290 nm with 2-nm band-pass. F/F_0 is the ratio of the observed fluorescence intensity to the intensity of dynorphin$_{1-17}$ in the absence of protein.

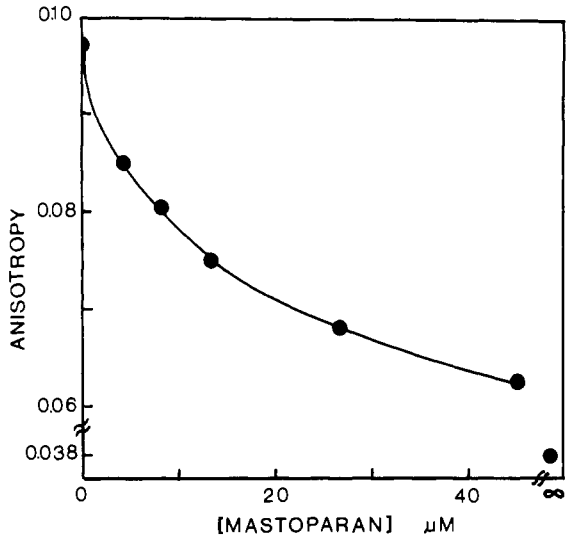

FIGURE 7: Competition experiments with melittin. This figure shows the dissociation of the melittin–calmodulin complex (3.3 μM) by varying concentrations of mastoparan up to 45 μM as detected in fluorescence anisotropy. The smooth curve was calculated for $K_{\text{mastoparan-CaM}}/K_{\text{melittin-CaM}} = 12.5$, $A_b = 0.097$, $A_f = 0.038$, and $F_\infty/F_0 = 1.20$. Conditions: 0.20 N KCl, 45 mM Mops, and 1.0 mM $CaCl_2$, pH 7.3, 25.0 °C. Excitation, 294 nm with 2-nm band-pass; emission, Corning glass CSO-54.

Large shifts (17–24 nm) of the emission maximum toward shorter wavelengths accompany complex formation in each case. The quantum yields of the complexes of mastoparan X with the two fragments actually surpass the yield of the complex with calmodulin. Tyrosine makes a negligible contribution to these spectra. Residual tyrosine fluorescence, minimized by excitation at 290 nm, was eliminated by subtraction. Mastoparan has no effect (±1%) on the fluorescence of calmodulin.

We used the above spectral differences in competition experiments to compare the affinities of the proteins. Titrations of the troponin C–mastoparan X and fragment 72–148–mastoparan X complexes with calmodulin, followed at 350 and 330 nm, respectively, result in linear changes in the fluorescence intensity and virtually complete dissociation on addition of 1 mol of calmodulin/mol of mastoparan X (Figure 6). These stoichiometric titrations demonstrate that the dissociation constant of the mastoparan X–calmodulin complex (~0.9 nM) is at least 2 orders of magnitude smaller than the dissociation constants for the other complexes.

Reexamination of the Binding of Melittin by Calmodulin. Fluorescence anisotropy titrations of melittin with calmodulin confirm the 1:1 stoichiometry and strong calcium dependence previously reported by Comte et al. (1983) and Maulet & Cox (1983). However, competition experiments by us indicate that the dissociation constant for the calmodulin–melittin complex is probably smaller than the value previously reported. Fluorescence anisotropy titration of the melittin–calmodulin complex with mastoparan reveals a degree of dissociation consistent with a dissociation constant which is 12 times smaller than the one for the mastoparan–calmodulin complex (Figure 7). Estimations of the dissociation constant for the melittin–calmodulin complex based on this and other competition experiments[5] consistently give values *less* than 0.1 nM.

The dissociation constants for many of the peptide–calmodulin and protein–calmodulin complexes are beyond the range of *direct* determination. The limit for reliable values determined fluorometrically is usually 10^{-7} M; in a few cases, this can be extended to 10^{-8} M. Radiotracer methods permit the use of lower concentrations. However, loss of both proteins and peptides by adsorption to glass etc. becomes a major source of error. Added carriers, such as bovine serum albumin, may interact with one of the ligands. Competition experiments permit *comparison* of dissociation constants even when the individual values are too small for direct determination. We have previously used competitive displacement methods with smooth muscle myosin light chain kinase, the myelin basic protein, troponin I, histone H2A, the mastoparans, glucagon, VIP, GIP, secretin, ACTH, β-endorphin, and substance P. These proteins and peptides form a hierarchy in which mastoparan has the highest affinity for calmodulin. That melittin has a still higher affinity is evident in Figure 7.

The directly determined dissociation constants for the calmodulin–secretin complex, 0.14 μM (Malencik & Anderson, 1983a), and the calmodulin–troponin I complex,[6] 20 nM (Keller et al., 1982) to 60 nM (LaPorte et al., 1981), are used to estimate the K_d's for the other ligands. Moderate error is expected in this determination, reflecting errors in the reference values in addition to errors occurring in the competition experiments. However, both the hierarchy of binding and the actual calculation of K_d indicate that the dissociation constant for the melittin–calmodulin complex is smaller than the value (3 nM) estimated by Comte et al. (1982). The latter authors measured the catalytic activity of cyclic nucleotide phosphodiesterase in solutions containing 60 nM melittin, varying concentrations of calmodulin, and 5 μM bovine serum albumin. The accuracy of the dissociation constant obtained from the experiment (3 nM) depends *critically* on the precise determination of the concentration of free melittin when ~95% of the total melittin is bound. Possible binding of even a small

[5] Other experiments indicate that the affinity of melittin for calmodulin considerably exceeds that of smooth muscle myosin light chain kinase. Fluorescence spectra of equimolar mixtures of melittin, the enzyme, and dansylcalmodulin are close to the spectrum characteristic of the melittin–dansylcalmodulin complex. Gel filtration on Bio-Gel P-60, performed at concentrations (17 μM calmodulin) where little dissociation of the complexes occurs, gives a distribution in which ~85% of the dansylcalmodulin is bound to melittin and ~15% to the enzyme. Fluorescence titration of the enzyme–calmodulin complex with melittin, performed in the presence of 9-anthroylcholine (Malencik et al., 1982a; Malencik & Anderson, 1983a), shows a nearly linear stoichiometric decrease in the fluorescence of the probe.

[6] We used an average value of 40 nM in our calculations (Malencik & Anderson, 1983a).

portion of melittin by serum albumin and the appreciable base-line activity of the enzyme in the absence of calmodulin make an accurate determination difficult. The *multiplication* of error in the subsequent calculation of K_d is very large whenever 95% of the ligand is bound (Weber, 1965). The dissociation constant which Comte et al. (1982) calculated from chromatographic separation is subject to overestimation as a result of both dissociation and general adsorption occurring at the low concentration used (2.4 nM).

Discussion

The dynorphins[2] have similar affinities (within a factor of 4) for calmodulin and rabbit skeletal muscle troponin C. That electrostatic interactions are vital to the stabilization of the complexes with dynorphins is evident in the marked ionic strength dependence of binding and in the effects of peptide charge, demonstrated with the deletion peptides of dynorphin$_{1-17}$. Nonetheless, these associations are highly specific as indicated by the definite stoichiometries and strong calcium dependence. Dogfish parvalbumin, a protein descended from calmodulin which is considered to have lost the specific protein binding site (Blum et al., 1977), does not bind the dynorphins. The dynorphins remind us of the inhibitory region of troponin I: both contain highly basic sequences with interspersed hydrophobic residues. The synthetic peptide analogue corresponding to the inhibitory region of rabbit skeletal muscle troponin I, believed to interact with the N-terminal section of region III in troponin C (Cachia et al., 1983), also binds calmodulin. Dynorphin$_{1-17}$ exerts an inhibitory effect in the rabbit skeletal muscle actomyosin adenosinetriphosphatase assay similar to that previously found for troponin I and the inhibitory peptide analogues (Talbot & Hodges, 1981a,b). Neither mastoparan nor melittin inhibits actomyosin adenosinetriphosphatase. Overall, the dynorphins seem to be the most similar to troponin I of the naturally occurring calmodulin binding peptides. Cardiac troponin C binds dynorphin$_{1-17}$ less well than rabbit skeletal troponin C. Mastoparan and dynorphin$_{1-17}$ compete in calmodulin binding, with virtually complete displacement of dynorphin$_{1-17}$ taking place on the addition of 1 mol of mastoparan/mol of calmodulin. These two peptides also compete in the binding of rabbit skeletal muscle troponin C. However, the dissociation constant for the mastoparan–troponin C complex is 700-fold larger than that for the calmodulin complex.

Competition experiments with melittin[7] show that it has the highest affinity for calmodulin of any of the peptides and that the dissociation constant for the melittin–calmodulin complex is probably smaller than the value originally estimated by Comte et al. (1982). The C-terminal sequence of melittin, -Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2, conforms to the ideal model or recognition sequence envisioned by us.

Two proteolytic fragments of calmodulin show strongly calcium-dependent binding of mastoparan X. Fragment 1–106, containing postulated calcium binding sites I, II, and III, gives a complex with mastoparan X having $K_d = 0.9$ μM. This fragment is known to interact with troponin I and cyclic nucleotide phosphodiesterase; the concentration of the fragment required for enzyme activation is 200-fold larger than that of calmodulin (Walsh et al., 1977; Wall et al., 1981). The C-terminal fragment 72–148 forms a stable complex with mastoparan X having a $K_d \leq 0.15$ μM and at least 100-fold larger than the K_d for native calmodulin (0.9 nM). This fragment contains calcium binding sites III and IV, probably the two high-affinity calcium binding sites of calmodulin (Andersson et al., 1983). A related fragment, 78–148, binds troponin I and activates the phosphodiesterase poorly, with micromolar concentrations required (Walsh et al., 1977). In contrast, fragment 78–148 activates phosphorylase kinase quite efficiently (Kuznicki et al., 1981). This may reflect the fact that *diverse* proteins activate phosphorylase kinase by stimulating autophosphorylation (Malencik & Fischer, 1982). Neither fragment 107–148 nor parvalbumin interacts with the calmodulin binding peptides in the micromolar concentration range.

Perhaps the region of sequence overlap of the two fragments (residues 72–106) contains a major portion of the peptide binding site of calmodulin. In the case of troponin C, fragments containing amino acid residues 89–100 (homologous to calmodulin residues 79–90) plus at least one pair of calcium binding sites associate with troponin I (Grabarek et al., 1981). X-ray crystallography of calmodulin shows that the sequence region between calcium binding sites II and III (residues 68–92) is exposed to solvent and hence accessible to ligands (Weissman & Kretsinger, 1983). We previously suggested several points of interaction of the model peptides with calmodulin. These include anionic sites at positons 78–84 and 118–123 and several loci within the α-helical regions of calmodulin which might also exist in β-strand conformations, allowing hydrogen bonding to the peptides (Malencik & Anderson, 1983a). All but the first site occur outside the region of sequence overlap.

The anionic sequence (Asp-Thr-Asp-Ser-Glu-Glu-Glu) at positions 78–84 is a possible subsite for ion pairing or salt bridging with the basic sequences found in all the efficient calmodulin binding peptides. The calmodulin antagonist trifluoperazine inhibits the binding of β-endorphin by calmodulin, as shown in the cross-linking experiments of Giedroc et al. (1983a). Head et al. (1982) used CNBr cleavage to obtain a fragment of calmodulin, containing residues 77–124, which binds the phenthiazines. Gariepy & Hodges (1983) have ^{1}H NMR spectra indicating close proximity of the phenothiazine aromatic region to Ala, Leu, and Ile residues within sequence 95–100 of troponin C (homologous to calmodulin sequence 85–92). These authors suggested that the positive charge on the piperazine nitrogen atoms of trifluoperazine interacts with residues such as Glu-92, -93, -94, and -97, preventing the binding of tropinin I by troponin C and possibly the binding of peptides and proteins by calmodulin. Later experiments with the synthetic peptide corresponding to the inhibitory region of rabbit skeletal troponin I also suggest interactions with the N-terminal region of site III of troponin C (Cachia et al., 1983). Association of the basic domain characteristic of the calmodulin binding peptides with the Glu-Glu-Glu sequence could place the peptide hydrophobic domain in close proximity to nonpolar amino acid side chains within calmodulin sequence 88–92.

Glutamic acid has a below average rate of occurrence in calmodulin binding peptides while glutamine, arginine, and lysine occur with above average frequencies (Malencik & Anderson, 1983a). We originally proposed that the low frequency of Glu is related to the secondary structures of the bound peptides since Glu is strongly stabilizing in α-helices but destabilizing in β-strands and β-turns. However, the exceptional stabilities of the complexes with melittin and mastoparan tend to rule out this suggestion. The bound melittin molecule is largely α-helical (Maulet & Cox, 1983) while

[7] We have since found that apamin, another toxic peptide from the honey bee, binds dansylcalmodulin moderately ($K_d \geq 30$ μM).

mastoparan has a high potential for α-helix formation. The low incidence of Glu in the peptides may relate instead to the presence of Glu or Asp in the peptide binding site of calmodulin. Glu residues in the peptides could have a larger destabilizing effect than Asp residues for steric reasons.

The fluorescence emission maxima of the tryptophan-containing peptides shift toward shorter wavelengths on the binding of calmodulin or troponin C. The magnitude of this change varies among the peptides, suggesting that the individual tryptophan residues occupy different subsites within the peptide binding site. This effect is especially large for *Polistes* mastoparan, with a shift from 348 to 325 nm (Malencik & Anderson, 1983b). These changes are similar to those resulting when tryptophan or indole is transferred from an aqueous solution to a nonpolar solvent. Such general solvent effects led to the promotion of reagents such as 8-anilino-1-naphthalenesulfonate (ANS) as fluorescent probes of hydrophobic binding sites in proteins [cf. review by Slavik (1982)]. However, Brand et al. (1971) and Seliskar & Brand (1971) pointed out important differences between solvents and protein molecules. The red shift characteristic of polar solvents requires a degree of mobility which allows the solvent dipoles to reorient during the excited-state lifetime of the fluorescent molecule. This freedom may be absent in polar amino acid side chains or immobilized water molecules. X-ray crystallography of the fluorescent complex of chymotrypsin with ANS bore out this possibility: the ANS binding site proved to be rigid, hydrophilic (Weber et al., 1979). The proposal of a hydrophobic protein binding domain in calmodulin is based in part on the calcium-dependent binding of fluorescent probes such as ANS and 9-anthroylcholine (LaPorte et al., 1980; Tanaka & Hidaka, 1980). The fluorescence spectra of these complexes, as well as our spectra of the complexes of calmodulin with tryptophan-containing peptides, are consistent with *either a hydrophobic or a rigid hydrophilic* binding site. The sequences of the model peptides suggest that the peptide binding site of calmodulin has both hydrophobic and anionic domains close together in the three-dimensional structure. The effects of pH, ionic strength, and temperature on the activation of skeletal muscle myosin light chain kinase indicated that both hydrophobic and other types of interaction contribute to the binding and activation (Blumenthal & Stull, 1982).

Acknowledgments

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Registry No. Ac-Gly-Lys-FPhe-Lys-Arg-$(Pro)_2$-Leu-$(Arg)_2$-Val-Arg-NH_2, 86129-34-2; dynorphin$_{1-17}$, 80448-90-4; dynorphin$_{6-17}$, 87079-95-6; dynorphin$_{1-13}$, 72957-38-1; dynorphin$_{1-12}$, 79985-35-6; dynorphin$_{1-11}$, 79985-34-5; dynorphin$_{1-10}$, 79994-24-4; dynorphin$_{1-9}$, 77259-54-2; dynorphin$_{1-8}$, 75790-53-3; mastoparan, 72093-21-1; mastoparan X, 72093-22-2; ATPase, 9000-83-3; calcium, 7440-70-2; melittin, 37231-28-0.

References

Amphlett, G. W., Vanaman, T. C., & Perry, S. V. (1976) *FEBS Lett. 72*, 163.

Anderson, S. R. (1974) in *Experimental Techniques in Biochemistry* (Brewer, J. M., Pesce, A. B., & Ashworth, R. B., Eds.) p 248, Prentice-Hall, Englewood Cliffs, NJ.

Andersson, A., Forsen, S., Thulin, E., & Vogel, H. J. (1983) *Biochemistry 22*, 2309.

Barker, W. C., Ketcham, L. K., & Dayhoff, M. O. (1977) in *Calcium Binding Proteins and Calcium Functions* (Wasserman, R. H., et al., Eds.) p 73, Elsevier/North-Holland, New York.

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

Blum, H. E., Lehky, P., Kohler, L., Stein, E. A., & Fischer, E. H. (1977) *J. Biol. Chem. 252*, 2834.

Blumenthal, D. K., & Stull, J. T. (1982) *Biochemistry 21*, 2386.

Brand, L., Seliskar, C. J., & Turner, D. C. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Lee, C. P., & Blasie, J. K., Eds.) Vol. I, p 17, Academic Press, New York.

Cachia, P. J., Sykes, B. D., & Hodges, R. S. (1983) *Biochemistry 22*, 4145.

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

Cheung, W. Y. (1980) *Science (Washington, D.C.) 207*, 19.

Cheung, W. Y. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 2253.

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

Eisenberg, E., & Kielly, W. W. (1974) *J. Biol. Chem. 249*, 4742.

Evett, J., & Isenberg, I. (1969) *Ann. N.Y. Acad. Sci. 158*, 210.

Gariepy, J., & Hodges, R. S. (1983) *Biochemistry 22*, 1586.

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

Goldstein, A., Tachibana, S., Lowney, L. L., Hunkapiller, M., & Hood, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 72*, 64.

Grabarek, Z., Drabikowski, W., Leavis, P. C., Rosenfeld, S. S., & Gergely, J. (1981) *J. Biol. Chem. 256*, 13121.

Grand, R. J. A., & Perry, S. V. (1980) *Biochem. J. 189*, 227.

Grand, R. J. A., Perry, S. V., & Weeks, R. A. (1979) *Biochem. J. 177*, 521.

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

Hirai, Y., Yasuhara, T., Yoshida, H., Nakajima, T., Fujino, M., & Kitada, C. (1979) *Chem. Pharm. Bull. 37*, 1942.

Keller, C. B., Olwin, B. B., LaPorte, D. C., & Storm, D. R. (1982) *Biochemistry 21*, 156.

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

LaPorte, D. C., Keller, C. H., Olwin, B. B., & Storm, D. R. (1981) *Biochemistry 20*, 3965.

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

Malencik, D. A., & Fischer, E. H. (1982) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. III, p 161, Academic Press, New York.

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

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

Malencik, D. A., Anderson, S. R., Bohnert, J. L., & Shalitin, Y. (1982a) *Biochemistry 21*, 4031.

Malencik, D. A., Huang, T.-S., & Anderson, S. R. (1982b) *Biochem. Biophys. Res. Commun. 108*, 266.

Margossian, S. S., & Lowey, S. (1982) *Methods Enzymol. 85*, 55.

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

Maulet, Y., Brodbeck, U., & Fulpius, B. W. (1982) *Anal. Biochem. 127*, 61.

Means, A. R. (1981) *Recent Prog. Horm. Res. 37*, 333.

Pardee, J. D., & Spudich, J. A. (1982) *Methods Enzymol. 85*, 164.

Pechere, J.-F., Demaille, J., & Capony, J.-P. (1971) *Biochim. Biophys. Acta 236*, 391.
Potter, J. D. (1982) *Methods Enzymol. 85*, 241.
Schreiber, W. E., Sasagawa, T., Titani, K., Wade, R. D., Malencik, D. A., & Fischer, E. H. (1981) *Biochemistry 20*, 5239.
Seliskar, C. J., & Brand, L. (1971) *Science Washington D.C.) 171*, 799.
Sellinger-Barnette, M., & Weiss, B. (1982) *Mol. Pharmacol. 21*, 86.
Sellinger-Barnette, M., & Weiss, B. (1984) *Adv. Cyclic Nucleotide Res. 16*, 261.
Slavik, J. (1982) *Biochim. Biophys. Acta 694*, 1.
Smillie, L. B. (1982) *Methods Enzymol. 85*, 234.
Talbot, J. A., & Hodges, R. S. (1981a) *J. Biol. Chem. 256*, 12374.
Talbot, J. A., & Hodges, R. S. (1981b) *J. Biol. Chem. 256*, 2798.
Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078.
Teale, F. W. J. (1960) *Biochem. J. 76*, 381.
Wall, C. M., Grand, R. J. A., & Perry, S. V. (1981) *Biochem. J. 193*, 307.
Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977) *J. Biol. Chem. 252*, 7440.
Watterson, D. M., Sharief, F., & Fanaman, T. C. (1980) *J. Biol. Chem. 225*, 962.
Weber, G. (1952) *Biochem. J. 21*, 155.
Weber, G. (1953) *Adv. Protein Chem. 8*, 415.
Weber, G. (1961) *Nature (London) 190*, 27.
Weber, G. (1965) in *Molecular Biophysics* (Pullman, B., & Weissbluth, M., Eds.) Academic Press, New York.
Weber, G. (1966) in *Fluorescence and Phosphorescence Analysis* (Hercules, D., Ed.) p 499, Interscience, New York.
Weber, L. D., Tulinsky, A., Johnson, J. D., & El-Bayoumi, M. A. (1979) *Biochemistry 18*, 1297.
Weiss, B., Prozialeck, W., Cimino, M., Barnette, M. S., & Wallace, T. L. (1980) *Ann. N.Y. Acad. Sci. 356*, 319.
Weissman, L., & Kretsinger, R. H. (1983) 186th National Meeting of the American Chemical Society, Washington, DC, Aug 1983, Abstr. 263.

Transient Kinetics of the Binding of ATP to Actomyosin Subfragment 1: Evidence That the Dissociation of Actomyosin Subfragment 1 by ATP Leads to a New Conformation of Subfragment 1†

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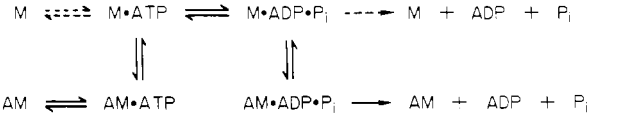
ABSTRACT: The initial steps by which ATP dissociates and binds to actomyosin subfragment 1 (acto-SF-1) were studied. Two techniques were used: stopped-flow (for acto-SF-1 dissociation kinetics) and rapid-flow quench with ATP chase quenching (for ATP binding kinetics). The experiments were carried out in 40% ethylene glycol–5 mM KCl, pH 8, at 15 °C. Under these conditions, the binding of SF-1 to actin remains very tight. As with SF-1, the ATP chase technique could be used, first, to titrate active sites and, second, to study the kinetics of ATP binding to acto-SF-1. The kinetic constants obtained were compared with those of SF-1 alone and with the acto-SF-1 dissociation kinetics under identical conditions. The kinetics of the acto-SF-1 dissociation did not vary with the actin to SF-1 ratio, but the ATP binding kinetics did, and a maximum value was reached at a mole ratio of 2.5. At high ATP (100 μM), k_{diss} = 300 s^{-1}, which compares with 49 s^{-1} and 13 s^{-1} for the ATP binding kinetics for acto-SF-1 (actin to SF-1 = 1:1) and SF-1, respectively. As with SF-1, the ATP binding to acto-SF-1 follows a hyperbolic law with the ATP concentration. This suggests a rapid equilibrium (K) followed by an essentially irreversible step (k). The values for K and k for the two proteins were significantly different: $K = 1.15 \times 10^5$ M^{-1} and k = 15.6 s^{-1} for SF-1, and $K = 3.6 \times 10^4$ M^{-1} and k = 62.7 s^{-1} for acto-SF-1. Taken together, these results show that the steps by which ATP dissociates and binds tightly to acto-SF-1 are kinetically distinct. Further, they imply that the conformation of SF-1 freshly released from acto-SF-1 by ATP is different from that of SF-1 alone. In the presence of ATP, this new structure then isomerizes to an intermediate (M*·ATP) which is also on the SF-1 ATPase pathway. Two possible mechanisms by which ATP dissociates and binds to acto-SF-1 are discussed. One of these involves one site for ATP on acto-SF-1. The other mechanism involves two sites: an acto-SF-1 dissociation site (where the ATP is not hydrolyzed) and an ATPase site (where the ATP is tightly bound and then hydrolyzed).

It is now well recognized that the various processes producing motility are intimately related to the actomyosin system. Thus, muscle contraction involves the sliding of interdigiting myosin and actin filaments. It is thought that this sliding process is driven by a cyclic interaction of myosin cross bridges (heads) with actin and coupled to ATP[1] hydrolysis. The ATPase sites are on the cross bridges, and movement is controlled by the various intermediates on the actomyosin ATPase pathway.

A model for actomyosin ATPase was first proposed by Lymn & Taylor (1971). In this, it is assumed that actin dissociates before hydrolysis occurs:



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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; P_i, inorganic orthophosphate; SF-1, myosin subfragment 1; Tris, tris(hydroxymethyl)aminomethane.

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