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Calmodulin-Linked Equilibria in Smooth Muscle Myosin Light Chain Kinase†

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ABSTRACT: Competition experiments using 9-anthroylcholine, a fluorescent dye that undergoes calmodulin-dependent binding by smooth muscle myosin light chain kinase [Malencik, D. A., Anderson, S. R., Bohnert, J. L., & Shalitin, Y. S. (1982) *Biochemistry 21*, 4031], demonstrate a strongly stabilizing interaction between the adenosine 5′-triphosphate and myosin light chain binding sites operating within the enzyme–calmodulin complex but probably not in the free enzyme. The interactions in the latter case may be even slightly destabilizing. The fluorescence enhancement in solutions containing 5.0 μM each of the enzyme and calmodulin is directly proportional to the maximum possible concentration of bound calcium on the basis of four calcium binding sites. Evidently, all four calcium binding sites of calmodulin contribute about equally to the enhanced binding of 9-anthroylcholine by the enzyme. Fluorescence titrations on solutions containing 1.0 μM enzyme plus calmodulin yield a Hill coefficient of 1.2 and $K = 0.35 \pm 0.08$ μM calcium. Three proteolytic fragments of smooth muscle myosin light chain kinase, apparent products of endogenous proteolysis, were isolated and characterized. All three possess calmodulin-dependent catalytic activity. Their interactions with 9-anthroylcholine, in both the presence and absence of calmodulin, are similar to those of the native enzyme. However, the stabilities of their complexes with calmodulin vary. The corresponding dissociation constants range from 2.8 nM for the native enzyme and 8.5 nM for the 96K fragment to ~15 nM for the 68K and 90K fragments [0.20 N KCl, 50 mM 3-(*N*-morpholino)propanesulfonic acid, and 1 mM $CaCl_2$, pH 7.3, 25 °C]. A coupled fluorometric assay, modified from a spectrophotometric assay for adenosine cyclic 3′,5′-phosphate dependent protein kinase [Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R. (1982) *Biochemistry 21*, 5794], has provided the first continuous recordings of myosin light chain kinase phosphotransferase activity. The results show that smooth muscle myosin light chain kinase is a responsive enzyme, whose activity adjusts rapidly to changes in solution conditions.

Myosin light chain kinase is a calmodulin-dependent enzyme catalyzing phosphorylation of two of the regulatory light chains of myosin [cf. reviews by Stull (1980), Small & Sobieszek (1980), Hartshorne & Siemankowski (1981), and Perry et al. (1984)]. This reversible phosphorylation is apparently necessary for cross-bridging and contraction in smooth muscle as well as in the generalized motility systems of non-muscle cells. Light chain phosphorylation stimulates chicken gizzard actomyosin adenosinetriphosphatase activity (Sobieszek & Small, 1977) and assembly of gizzard myosin into bipolar filaments (Suzuki et al., 1978). Tension development correlates with myosin phosphorylation in both functionally skinned (Hoar et al., 1979) and intact smooth muscle fibers (deLanerolle & Stull, 1980). A role for light chain phosphorylation in the regulation of actomyosin adenosinetriphosphatase has also been shown for platelets (Adelstein & Conti, 1975), macrophages (Trotter & Adelstein, 1979), lymphocytes (Fechheimer & Cebra, 1982), the brain (Barylko

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& Sobieszek, 1983), and cultured cells (Yerna et al., 1978; Holzapfel et al., 1983). Calcium control in all these cases resides in calmodulin rather than troponin, the calcium binding protein complex characteristic of striated muscle.

Skeletal muscle and cardiac muscle both contain myosin light chain kinases of largely unknown physiological function (Pires et al., 1974; Yazawa & Yagi, 1977; Nairn & Perry, 1979; Pires & Perry, 1977; Wolf & Hofmann, 1980). They differ substantially from the corresponding enzymes isolated from smooth muscle in both structure and regulation. Rabbit skeletal muscle myosin light chain kinase has a molecular weight of 103 000 (Nagomoto & Yagi, 1984) while smooth muscle myosin light chain kinases have molecular weights in the range of 135 000 (Adachi et al., 1983) to 160 000 (Walsh et al., 1982b). In vitro phosphorylation catalyzed by the adenosine cyclic 3′,5′-phosphate (cAMP)[1] dependent protein kinase weakens the association of smooth muscle myosin light chain kinase with calmodulin (Conti & Adelstein, 1981; Malencik et al., 1982; Vallet et al., 1981; Walsh et al., 1982a,b) but has no proven effect on the properties of the rabbit skeletal muscle enzyme (Edelman & Krebs, 1982). Whether the phosphorylation of myosin light chain kinase is physiologically relevant in smooth muscle is still under investigation (Silver & Stull, 1982; Miller et al., 1983).

A number of small peptides containing clusters of two or more strongly basic amino acid residues in conjunction with hydrophobic sequences compete effectively with both myosin light chain kinase and cyclic nucleotide phosphodiesterase in calmodulin binding [cf. reviews by Anderson & Malencik (1986) and Barnette & Weiss (1983)]. We hypothesized that sequences similar to those found in the peptides occur in accessible positions on the surfaces of enzymes and other proteins that recognize calmodulin and that they are directly involved in the interaction. The sequence of a small calmodulin binding fragment recently isolated from CNBr digests of rabbit skeletal muscle myosin light chain kinase is consistent with this prediction (Blumenthal et al., 1985). We also noted similarities between the recognition sequences for calmodulin and the cAMP-dependent protein kinase, suggesting that calmodulin and protein kinase interact with common sequences in some proteins (Malencik & Anderson, 1982; Malencik et al., 1982a,b). This might explain the effect of in vitro phosphorylation on the calmodulin binding properties of troponin I, histones, the myelin basic protein (Malencik et al., 1982a), or possibly of smooth muscle myosin light chain kinase. The changes that accompany phosphorylation of the enzyme, however, are much larger than those found with the other proteins and peptides (Malencik et al., 1982b; Anderson & Malencik, 1986).

We discovered that 9-anthroylcholine, a fluorescent dye previously found to interact moderately with calmodulin (LaPorte et al., 1980), forms a relatively high-affinity complex with turkey gizzard myosin light chain kinase. The association of the dye with the enzyme is related to several of its specific functions, including the binding of calmodulin and ATP. The changes in 9-anthroylcholine fluorescence are useful in stoichiometric titrations of the enzyme with calmodulin and in measurements of nucleotide binding (Malencik et al., 1982; Anderson & Malencik, 1985). 9-Anthroylcholine does not associate appreciably with rabbit skeletal muscle myosin light chain kinase, phosphorylase kinase, or other kinases such as phosphofructokinase.

Recent applications of 9-anthroylcholine have answered several questions about the smooth muscle myosin light chain kinase–calmodulin complex. This paper considers the number of calcium binding sites involved in the association of calmodulin with the enzyme, the mutual interactions between the two substrate binding sites of myosin kinase, both in the presence and absence of calmodulin, and the distinctive effects of ionic strength variation on the binding of 9-anthroylcholine. Comparisons are also made between the properties of native smooth muscle myosin light chain kinase and some of its proteolytic fragments.

Materials and Methods

Materials. Turkey gizzard myosin light chain kinase containing a single band on $NaDodSO_4$ electrophoresis was prepared essentially according to the procedure of Sobieszek & Barylko (1985). Stock solutions of the enzyme (2–3 mg/mL) containing 0.10 M KCl, 50 mM Mops, 1 mM dithiothreitol, and 1 mM EDTA (pH 7.3) were stored at −70 °C. In preparation for calcium binding measurements, enzyme samples were exhaustively dialyzed for 24 h against 200 volumes of calcium-free buffer containing 0.20 M KCl and 50 mM Mops, pH 7.3 (refer to the last part of this section for details). Six changes of the buffer solution were made. The enzyme concentrations are based on $E^{1\%}_{280} = 10$ and on the results of stoichiometric fluorescence titrations with calmodulin (Malencik et al., 1982a). The assays for enzymatic activity are described separately.

Three catalytically active fragments of myosin light chain kinase, apparent products of endogenous proteolysis, were obtained as byproducts of the enzyme fractionation. During final purification, the enzyme was eluted from a column of diethylaminoethylcellulose—equilibrated in 20 mM Tris-HCl, 1 mM EDTA, and 15 mM β-mercaptoethanol, pH 7.5—by application of a gradient ranging from 0 to 0.5 M NaCl. The leading edge of the peak contained active fragments accounting for a few percent of the enzyme activity. After concentration and dialysis against the 20 mM Tris-HCl buffer described above, the pooled fractions containing the fragments were applied to a Fractogel TSK DEAE-650S ion-exchange column. Application of the 0–0.5 M NaCl gradient produced three partially overlapping peaks of myosin light chain kinase activity. Their apparent molecular weights determined from $NaDodSO_4$ electrophoresis, listed in the order of elution, are 96 000, 68 000, and 90 000. Selected fractions were pooled, concentrated, and applied to a sizing column, Fractogel HF55SW, equilibrated in 0.5 M NaCl, 20 mM Tris, 2 mM EDTA, and 15 mM β-mercaptoethanol, pH 7.5, for final purification.

Turkey gizzard myosin was isolated according to the procedure of Sobieszek & Bremel (1975). The 15 000- and 20 000-dalton light chains were extracted from the purified myosin and resolved according to a modification of the procedure used for the rabbit skeletal muscle myosin light chains (Blumenthal & Stull, 1980; D. A. Malencik and S. R. Anderson, unpublished results). Porcine brain calmodulin, prepared according to the procedure of Schreiber et al. (1981), was subjected to a final purification step by using affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). The calmodulin concen-

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; 9AC, 9-anthroylcholine; ANS, 8-anilinonaphthalene-1-sulfonate; MLCK, myosin light chain kinase; CaM, calmodulin; LC, light chain or light chain analogue; *K*, dissociation constant; *F*, fluorescence intensity; $NaDodSO_4$, sodium dodecyl sulfate; NADH, reduced nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvic acid.

trations are based on $E^{1\%}_{280} = 2.0$ and a molecular weight of 16 680 (Watterson et al., 1980).

Peptides—mastoparan and the synthetic substrate for smooth muscle myosin light chain kinase—were purchased from Peninsula Laboratories. ATP (disodium salt, 99–100% from equine muscle), phosphoenolpyruvate (tricyclohexylammonium salt), NADH (disodium salt, grade III), dithiothreitol, Quin 2, and rabbit muscle pyruvate kinase (type VII in 50% glycerol) were obtained from Sigma Chemical Co. [γ-^{32}P]ATP, 0.3–0.7 Ci/mmol, was supplied by New England Nuclear. The chicken heart lactate dehydrogenase was prepared according to the procedure of Pesce et al. (1964). 5′-Adenylyl imidodiphosphate (AMP-PNP) was obtained from P-L Biochemicals and 9-anthroylcholine (9AC) from Molecular Probes. Reagent-grade (or best available) chemicals and distilled water passed through a Milli-Q reagent water system were used exclusively.

Catalytic Activity Measurements. In earlier stages of this work, we determined the phosphotransferase activity of myosin light chain kinase in radioassays using adenosine 5′-[γ-^{32}P]-triphosphate (Malencik et al., 1982a). In view of the discontinuous nature of the assay as well as the expense and short half-life of [γ-^{32}P]ATP, we have adopted and modified the coupled, spectrophotometric method described by Cook et al. (1982) for assays of cAMP-dependent protein kinase. Initially, we used the same solutions for both coupled and isotopic assays, i.e., 50 mM Mops, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10 μM 20 000-dalton myosin light chain, 1 mg/mL bovine serum albumin, 0.1 mM ATP, and specified concentrations of enzyme and calmodulin (pH 7.3, 25 °C). The pH of the buffer was adjusted by the addition of 2 M KOH. The coupled assay mixture contained, in addition, 8.0 μM NADH, 1.0 mM PEP, 2 units/mL pyruvate kinase, and 0.4 unit/mL lactate dehydrogenase. The original conditions of Cook et al. (1982) differed in several details, including the concentrations of NADH (200 μM), ATP (1 mM), acceptor peptide (250 μM), pyruvate kinase (7 units/mL), and lactate dehydrogenase (15 units/mL). The changes were made in order to adapt the assay for fluorescence measurements and to accommodate the lower concentrations of substrate (10 μM) used in activity measurements on myosin light chain kinase. The fluorescence intensities of NADH solutions are directly proportional to concentration, with 2.7% deviation from linearity, over the range of 1–8 μM.

Continuous measurements of the changes in NADH fluorescence were obtained with the Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer, using excitation and emission wavelengths of 348 and 455 nm, respectively. Quartz fluorescence cuvettes were used exclusively. After experimenting with several different orders of addition of the reagents, we have adopted the following protocol. We begin by measuring the fluorescence of 1.4 mL of a solution that contains NADH, ATP, and PEP in addition to the buffer components. The coupling enzymes are then added; any immediate drop in fluorescence indicates contamination with pyruvate or ADP. If the fractional change is small, more NADH can be added. Residual adenosinetriphosphatase activity in the coupling enzymes is briefly recorded so that this background rate can be subtracted from the final rate. Myosin light chain kinase, about 0.10 μg/mL, may be added next in order to check it for contaminating enolase or adenosinetriphosphatase activity. (Both the purified enzyme and fractions from later stages of purification are free of these activities.) Addition of the myosin light chain results in a small increase in the rate of NADH oxidation (about 7% of the maximum rate) that can be suppressed by the addition of EGTA, suggesting a fractional contamination of the light chain preparation with calmodulin. Finally, the reaction is initiated by the addition of 28–280 μM calmodulin. After a barely discernible lag period, the NADH fluorescence declines at a linear rate for ~40% of the time course. Total oxidation occurs when the light chain is in excess over NADH.

Specific activities obtained from the coupled fluorometric assay agree with those determined in the radioassay: 14.1 μmol of NADH min^{-1} mg^{-1} vs. 13.2 μmol of P min^{-1} mg^{-1}. In recent applications of the coupled assay, we have reduced the concentration of $MgCl_2$ to 2.0 mM (higher concentrations produce opalescence in the light chain solutions) and deleted the bovine serum albumin since the myosin light chain—present at 0.2 mg/mL—adequately protects the enzyme from adsorption losses. However, albumin is still included in stock solutions of the diluted enzyme. The rates of NADH oxidation are proportional to myosin light chain kinase concentration over a large range (0.05–0.30 μg/mL concentrations are ordinarily used) and are unaffected by increases in the concentrations of the coupling enzymes. Various preparations of turkey gizzard myosin light chain kinase have specific activities of 9–10 μmol of NADH min^{-1} mg^{-1} in the final version of the coupled assay (2 mM $MgCl_2$). Representative time courses showing the stoichiometry of the reaction, the effect of a peptide calmodulin antagonist, mastoparan (Malencik & Anderson, 1983a), and the inhibition by EGTA are given in Figure 1.

Fluorescence Binding Measurements. Equilibrium fluorescence intensity measurements were made with the Hitachi Perkin-Elmer MPF-2A fluorometer. Determinations of both fluorescence intensity and anisotropy were obtained with the SLM-4000 fluorescence polarization spectrophotometer. The details of the titrations with 9-anthroylcholine were previously described by us (Malencik et al., 1982a). Anderson (1974) has reviewed the general principles of fluorescence binding measurements as they are applied here. Constant temperature was maintained at 25.0 ± 0.1 °C with a circulating water bath.

Calcium. "Calcium-free" solutions were prepared by passage of the buffers over columns contained washed Chelex 100. The amount of residual calcium (usually about 0.9 μM) was measured by both atomic absorption and the fluorescent calcium indicator, Quin 2. The latter was also used to check for accidental contamination of buffers in the course of the experiments. The calcium content of the stock $CaCl_2$ or $Ca(OAc)_2$ was determined by atomic absorption using $CaCO_3$ as a standard.

Results

Interactions between the Myosin Light Chain and Nucleotide Binding Sites. The first continuous assays of smooth muscle myosin light chain kinase (Figure 1) reveal a responsive enzyme whose catalytic activity adjusts rapidly to changes in solution conditions. These include mixing with calmodulin (or with calcium when calmodulin is already present), the removal of calcium with excess EGTA, and the addition of the peptide calmodulin antagonist mastoparan.[2] How calmodulin affects the activation of myosin light chain kinase and other enzymes is still largely unknown. Using 9-anthroylcholine (9AC), we

[2] Mastoparan forms both a calcium-dependent complex with calmodulin (K_d < 1 nM) and a low-affinity complex with the myosin light chain (K_d ~ 20 μM) (Malencik & Anderson, 1983a; Anderson & Malencik, 1986). The latter interaction does not contribute substantially to the present measurements, nor does mastoparan affect the activities of the coupling enzymes.

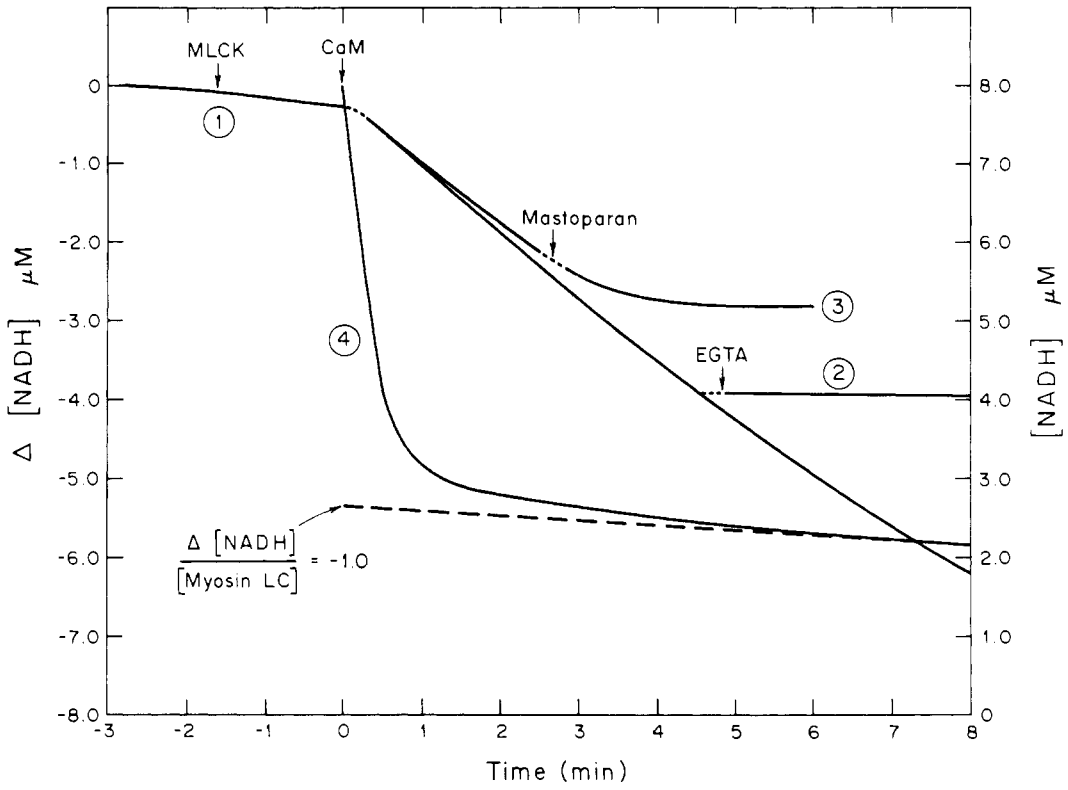

FIGURE 1: Continuous fluorometric assays of smooth muscle myosin light chain kinase phosphotransferase activity. Initial conditions (t = −3 min): 10 μM 20000-dalton smooth muscle myosin light chain, 0.10 mM ATP, 1.0 mM PEP, 8.0 μM NADH, 2 units/mL rabbit muscle pyruvate kinase, 0.4 unit/mL chicken heart lactate dehydrogenase, 0.2 mM $CaCl_2$, 2.0 mM $MgCl_2$, and 50 mM Mops, pH 7.3 (25 °C). In assay 1, MLCK was added (0.082 μg/mL) at t = −1.7 min and calmodulin (0.29 μM) at t = 0. The reaction was followed for 8 min, until [NADH] = 1.8 μM. Assay 2 is the same except that 1 mM EGTA was added at t = 4.5 min. In assay 3, the calmodulin concentration was reduced to 10 nM, and 7.0 μM mastoparan was added at t = 2.5 min. In assay 4, a limiting quantity of myosin light chain was used, 5.4 μM as indicated by the radioassay, and the MLCK concentration was increased to 2.4 μg/mL. The dashed line indicates the background adenosinetriphosphatase activity of the coupling enzymes. The NADH concentration on the right-hand axis is proportional to the fluorescence intensity. Excitation, 348 nm; emission, 455 nm.

demonstrated a stabilizing interaction between the calmodulin and ATP binding sites of turkey gizzard myosin light chain kinase corresponding to ΔG = −0.6 to −0.7 kcal/mol (Malencik et al., 1982a). Enhanced association of the myosin light chain with the enzyme–calmodulin complex has been detected with bovine cardiac myosin light chain kinase (Zimmer et al., 1984). Changes in the binding of the two substrates ATP and the myosin light chain may explain the activation of the enzyme by calmodulin, as suggested by Nairn & Perry (1979) and Crouch et al. (1981). The addition of ATP to solutions containing the turkey gizzard myosin light chain kinase–9-anthroylcholine complex results in decreases in fluorescence similar to those shown in Figure 2 for 5′-adenylyl imidodiphosphate (AMP-PNP). The results are described by the noncompetitive model of McClure & Edelman (1967), for which apparent dissociation constants (K_{app}) are calculated from plots of the changes in fluorescence ($I_0 - I$) vs. ($I_0 - I$)/[ATP]. K_{app} is related to the dissociation constants corresponding to the three independent equilibria and to P_0 and X_0, the total concentrations of enzyme and 9AC, respectively. ("MLCK" designates either the enzyme–calmodulin complex or the free enzyme, whichever is under consideration.)

$$K_{app} = \frac{(P_0 + X_0)/K_2 + 1}{(P_0 + X_0)/K_3 + 1} K_1 \qquad (1)$$

$$\text{MLCK–ATP} \underset{K_1}{\rightleftharpoons} \text{MLCK} + \text{ATP}$$

$$\text{MLCK–9AC} \underset{K_2}{\rightleftharpoons} \text{MLCK} + \text{9AC}$$

$$\text{9AC–MLCK–ATP} \underset{K_3}{\rightleftharpoons} \text{MLCK–ATP} + \text{9AC}$$

Whenever $K_3 \gg K_2$, the results approach those expected for a competitive model. That is

$$K_{app} = [(P_0 + X_0)/K_2 + 1]K_1 \qquad (2)$$

We have previously used the second equation to calculate K_1 for both the ATP–MLCK and ATP–MLCK–CaM complexes (Malencik et al., 1982a). In order to establish the applicability of the equation, we have compiled values of K_1 from ATP titrations conducted in the presence of widely varying 9AC concentrations. Titrations of the MLCK–CaM complex, using fixed 9AC concentrations of 1–25 μM, give K_1 = 44 ± 2.8 μM (0.20 M KCl, 50 mM Mops, 1 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.3, 25 °C). Measurements with the enzyme alone, covering 9AC concentrations from 5 to 40 μM, give K_1 = 140 ± 15 μM. (There was no consistent trend in the variability found for the latter value.) The range of 9AC concentrations examined was chosen in order to overlap the values for K_2: 5.2 μM for the enzyme–calmodulin complex and 18.0 μM for the enzyme alone. The relative constancy of K_1 would not have been obtained if terms in the denominator of eq 1 contributed significantly to K_{app}. Hence, the use of eq 2 is justified. The strong, mutually antagonistic interaction between ATP and 9-anthroylcholine may result from competition for a common binding site. Extrapolation of the intensity changes to infinite ATP concentration leads to 87–93% reversal of the 9AC fluorescence enhancement.

Experiments to determine the influence of the second substrate—the myosin light chain—required several changes in procedure. To prevent reaction from occurring, we substituted the ATP analogue 5′-adenylyl imidodiphosphate

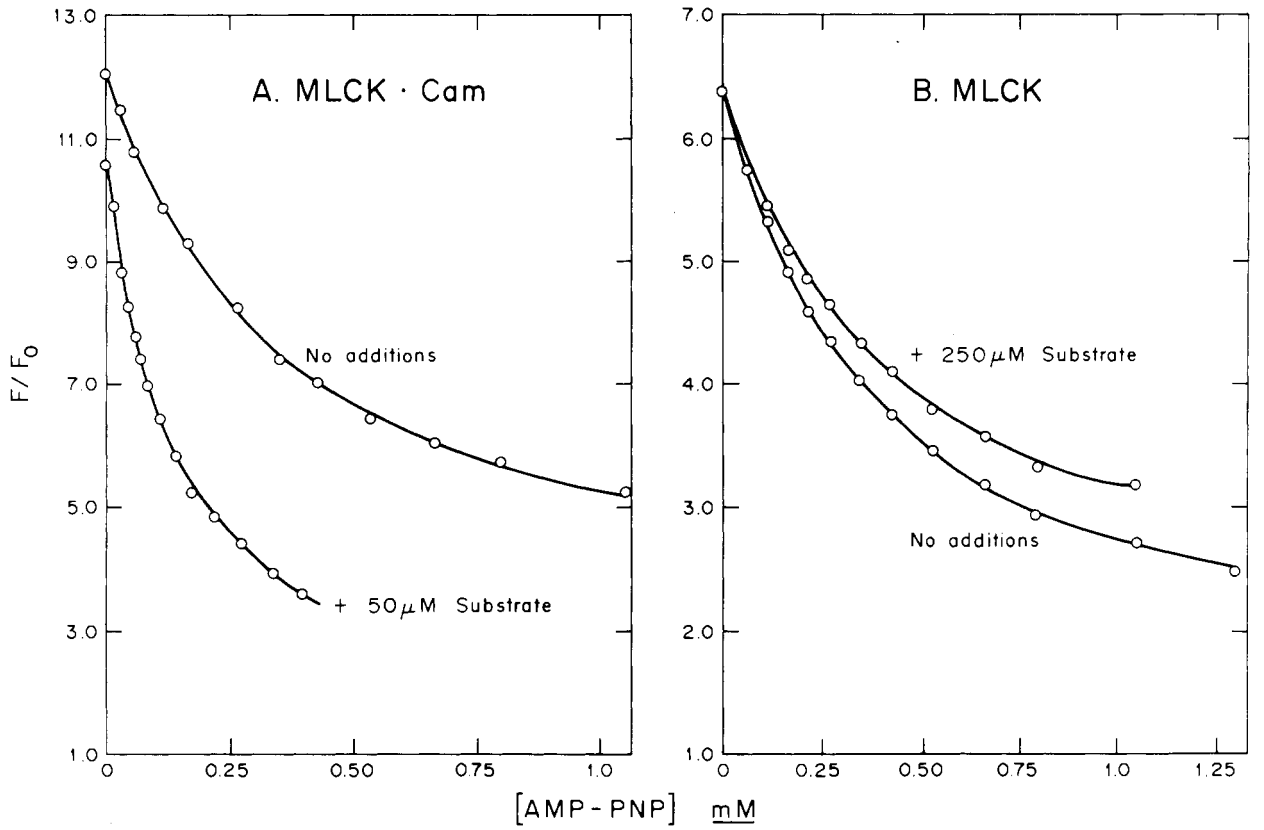

FIGURE 2: Effect of AMP-PNP concentration on 9-anthroylcholine binding by smooth muscle myosin light chain kinase. Panel A shows the results obtained with the enzyme–calmodulin complex (0.20 μM) and 1.0 μM 9AC, in the presence and absence of the myosin light chain analogue (KKRPQRATSNVFS-NH_2). Panel B contains parallel experiments with the free enzyme (0.6 μM) and 5.0 μM 9AC. The ratio F/F_0 is the fluorescence enhancement factor, with F_0 representing the intensity of 9AC in the absence of the proteins. Excitation, 360 nm; emission, 460 nm. Conditions: 50 mM Mops, 1.0 mM $CaCl_2$, and 2.0 mM $MgCl_2$, pH 7.3 (25 °C).

(AMP-PNP). Secondly, we adopted the synthetic myosin light chain kinase substrate Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2 (Kemp et al., 1983) when we found that the myosin light chain binds 9-anthroylcholine. Although this association is weak, considerable enhancement of 9AC fluorescence occurs at the excess concentrations of light chain required. Finally, we adjusted the ionic strength of the solution in order to bring substrate binding within a measurable range. Accordingly, we had to determine the effect of ionic strength variation on the binding of 9AC by both the enzyme and the enzyme–calmodulin complex. Since the results proved interesting in themselves, they are presented separately in the third section of this paper. The values for K_2 (refer to eq 2) at different KCl concentrations are included there. As we like to limit the correction factor $[(P_0 + X_0)/K_2 + 1]$ to values less than 2, we performed our initial experiments with 1.0 μM 9AC plus 0.20 μM enzyme–calmodulin complex (K_2 = 1.6 μM in 50 mM Mops) and with 5.0 μM 9AC plus 0.60 μM enzyme (K_2 = 20 μM).

Fortunately, the light chain analogue does not significantly alter the binding of 9-anthroylcholine by myosin light chain kinase. It does show a weak calcium-dependent interaction with calmodulin, accounting for the 13% decrease in the initial fluorescence intensity shown in Figure 2A. Titrations of dansylcalmodulin (Malencik & Anderson, 1982) with the analogue yield a dissociation constant of 33 μM under these conditions. The cluster of basic amino residues in the substrate is similar to those found in more effective calmodulin binding peptides [review by Anderson & Malencik (1986)]. However, the distance between the basic and hydrophobic centers is larger than that in the ideal model envisioned by us. The myosin light chain also associates weakly with calmodulin (D. A. Malencik, A. Sobieszek, and S. R. Anderson, unpublished results). In later analyses, we assume that the fluorescence changes are *directly* related only to AMP-PNP binding.

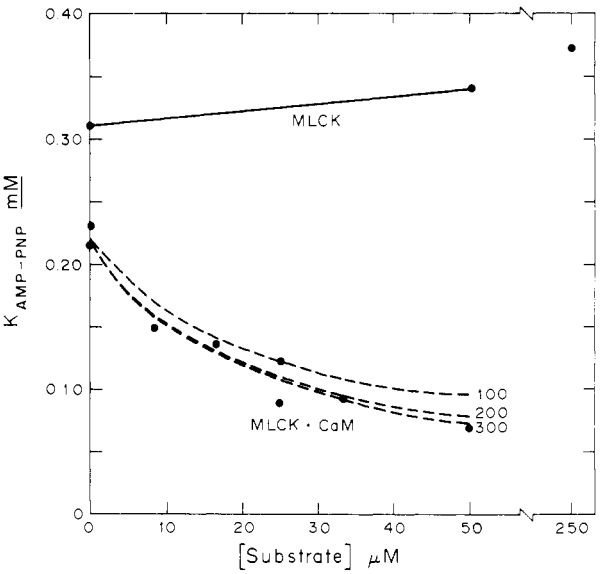

FIGURE 3: Dependence of AMP-PNP binding on the concentration of the light chain analogue. $K_{AMP\text{-}PNP}$ is the dissociation constant of the MLCK–AMP-PNP or the (CaM–MLCK)–AMP-PNP complex calculated from eq 2. The dashed lines represent theoretical curves calculated for K_1 = 220 μM, K_{LC}'' = 20 μM, and K_{LC}' = 100, 200, or 300 μM in the case of the enzyme–calmodulin complex. See Figure 2 for conditions.

Figure 2 shows that the light chain analogue has a strong stabilizing effect on AMP-PNP binding by the myosin light chain kinase–calmodulin complex, contrasting with possible

destabilization in the case of the free enzyme. We have conducted additional titrations in order to establish the concentration dependence of $K_{AMP\text{-}PNP}$, the value for K_1 obtained when eq 2 is applied to an experiment conducted in the presence of a fixed concentration ([LC]) of light chain analogue. Substrate concentrations from 0 to 50 μM demonstrate an excellent range of binding in the case of the enzyme–calmodulin complex (Figure 3). This result was facilitated by the use of a buffer (50 mM Mops) solution containing no KCl. (Note that the effect of calmodulin on AMP-PNP binding in the absence of substrate is smaller than that found with ATP. We have obtained this result under several different conditions.)

The 12 linked equilibria involved in the associations of AMP-PNP, light chain analogue, calcium–calmodulin complex, and myosin light chain kinase can be studied in sets of four at a time. In this case, we are concerned with

$$\text{CaM–MLCK–AMP-PNP} \underset{K_1}{\rightleftharpoons} \text{CaM–MLCK} + \text{AMP-PNP}$$

$$\text{CaM–MLCK–LC} \underset{K_{LC}'}{\rightleftharpoons} \text{CaM–MLCK} + \text{LC}$$

$$\text{CaM–MLCK–LC–AMP-PNP} \underset{K_4''}{\rightleftharpoons} \text{CaM–MLCK–LC} + \text{AMP-PNP}$$

$$\text{CaM–MLCK–LC–AMP-PNP} \underset{K_{LC}''}{\rightleftharpoons} \text{CaM–MLCK–AMP-PNP} + \text{LC}$$

for which $K_1K_{LC}'' = K_4''K_{LC}'$. $K_{AMP\text{-}PNP}$, defined in the last paragraph, is related to these equilibria according to the equation

$$K_{AMP\text{-}PNP} = \frac{1 + [LC]/K_{LC}'}{1 + [LC]/K_{LC}''}K_1 \qquad (3)$$

The dependence of $K_{AMP\text{-}PNP}$ on the concentration of the light chain analogue (Figure 3) corresponds to $K_1 = 220$ μM, $K_4'' \leq 22$ μM, $K_{LC}' \geq 200$ μM, and $K_{LC}'' = 20$ μM. The stabilizing effect of the substrate is so large that precise determination of K_{LC}' or K_4'' is difficult. The slight dissociation of the enzyme–calmodulin complex caused by the interaction of the substrate with calmodulin does not substantially alter this analysis. Any error thus introduced leads to underestimation of the stabilizing effects.

The binding of AMP-PNP by the free enzyme is only moderately affected by the light chain analogue over the concentration range examined, making calculation of equilibrium constants other than K_1 difficult. However, the trend toward destabilization indicates that, in a set of equilibria analogous to those listed above, $K_{LC}'' > K_{LC}'$ and $K_4'' > K_1$.

The relationship between our observations and those from other laboratories is considered in the discussion. In concluding, we repaired several of the above titrations in solutions containing 0.20 N KCl. The results in Table I show that similar effects occur—but at much higher concentrations of the substrate.

Calcium Binding. The binding of 9-anthroylcholine by turkey gizzard myosin light chain kinase provides a unique opportunity to follow the development of a calmodulin-dependent property as a function of calcium ligation. We have previously applied the increased binding of 9AC, corresponding to a decrease in K_2 from 18 to 5.2 μM under the conditions used here, in stoichiometric titrations of the enzyme with calmodulin (Malencik et al., 1982a; Anderson & Malencik, 1986). The direct connection between calmodulin binding by the enzyme and the observed changes in 9AC fluorescence has been established by the following observations. (1) Fluorescence intensity and anisotropy measurements performed over a wide range of enzyme concentrations, 50 nM to 5.0 μM, reveal precise stoichiometric end points consistent with the known molecular weights and extinction coefficients of both the enzyme and calmodulin. (2) Parvalbumin, troponin C, and proteolytic fragments of calmodulin comprising residues 1–106, 73–148, and 107–148 have no effect on the binding of 9AC by the enzyme (unpublished results). (3) The enhancement is reversed by the addition of either excess EDTA or high-affinity calmodulin binding proteins and peptides.[3] These agents have no effect on either the free 9AC or the enzyme–9AC complex. (4) The extent of dissociation of the enzyme–calmodulin complex in the presence of calmodulin binding proteins or peptides, as determined from the changes in 9AC fluorescence, agrees with that predicted in the cases where the dissociation constants of the competing complexes are known. (5) In 4 years of experience, the enhanced binding of 9AC by the enzyme–calmodulin complex has always correlated with its catalytic activity. (The enhancement is useful for the rapid detection of the enzyme during purification procedures.) Malencik et al. (1982a) and Anderson & Malencik (1986) dealt with points 1, 3, and 4 and Malencik & Anderson (1983a,b) with points of 3 and 4.

Table I: Summary of Nucleotide Binding by Smooth Muscle Myosin Light Chain Kinase and Its Complex with Calmodulin

nucleotide	[KCl] (M)	[LC analogue] (μM)	K_1[a] (μM) MLCK	K_1[a] (μM) MLCK–CaM
ATP	0	0	51 ± 4	16.9 ± 1
ATP	0.20	0	140 ± 15	44 ± 3
AMP-PNP	0	0	310 ± 30	220 ± 15
AMP-PNP	0	50	330 ± 20	68 ± 4
AMP-PNP	0.20	0	580 ± 30	350 ± 20
AMP-PNP	0.20	500	750 ± 30	220 ± 15

[a] K_1 is the dissociation constant of the nucleotide–enzyme or nucleotide–(enzyme–calmodulin) complex. Conditions: 50 mM Mops, 1.0 mM $CaCl_2$, and 2.0 mM $MgCl_2$, pH 7.3 (25 °C).

The conditions for the titrations with calcium were chosen to facilitate the direct demonstration of the stoichiometry of calcium binding necessary for optimal enhancement of 9AC fluorescence. An excess concentration (20 μM) of 9AC was used to ensure that the concentration of unbound probe remains relatively constant throughout the titration. Using the relationship $dY/Y = (d[9AC]/[9AC])(1 - Y)$ (Anderson & Weber, 1966), where Y represents the fractional saturation of any specified component with 9AC, we calculate that the *maximum* error in Y resulting from changes in 9AC is 5% for the 9AC–MLCK–CaM–/Ca_{sat}^{2+} complex and 6.9% for the 9AC–MLCK complex. The matching concentrations of the enzyme and calmodulin (5.0 μM each), which are at least 2800-fold larger than the dissociation constant for the enzyme–calmodulin complex, guarantee essentially total association (>98%) of the proteins when sufficient calcium is present with significant residual concentrations of neither remaining.

The relationship between the fractional saturation of a protein with its ligand (ϕ), the total ligand concentration (X_0), and the total protein concentration (P_0) is obtained by rearrangement of the empirical Hill equation (Anderson & Antonini, 1968).

$$\phi = \left(\frac{X_0}{NP_0} - \frac{K}{NP_0}\right)\sqrt[n]{\phi/(1-\phi)} \qquad (4)$$

[3] Calmidazolium also reversed the enhancement.

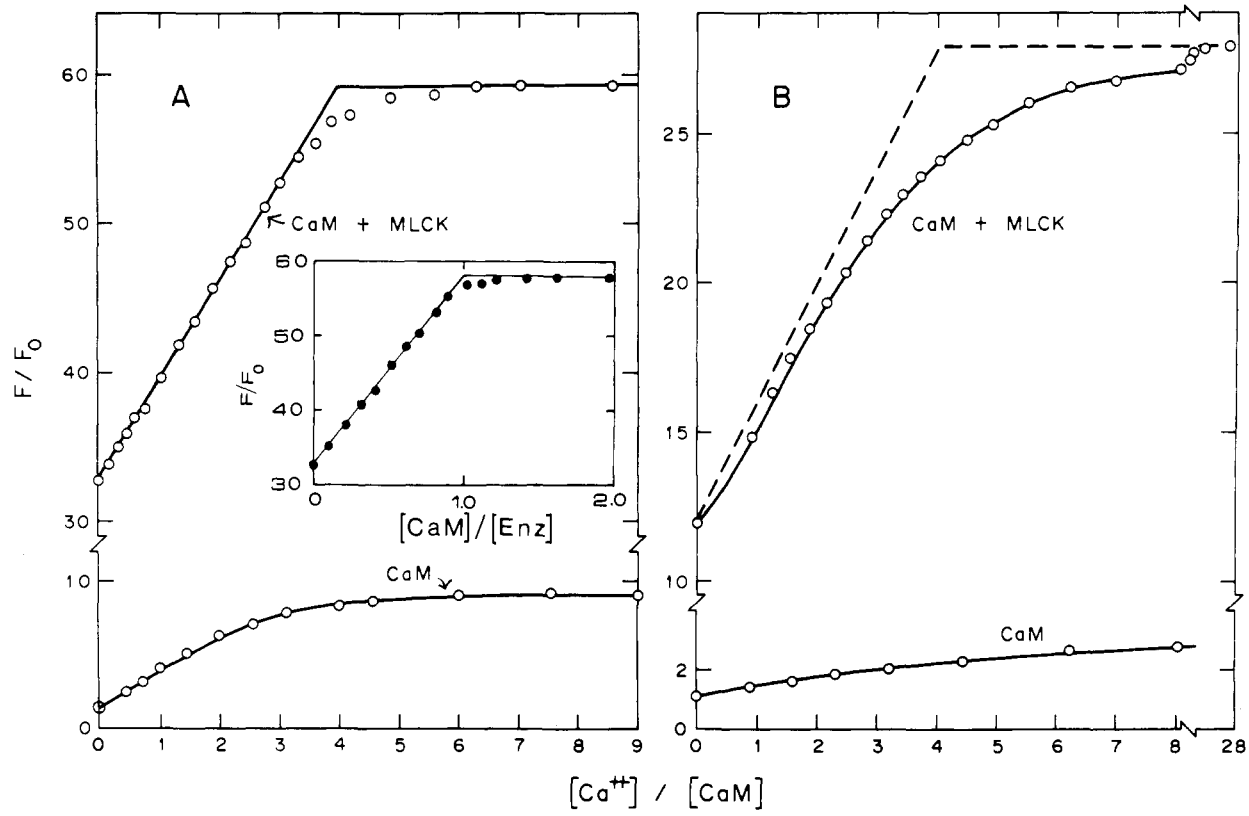

FIGURE 4: Calcium dependence of the calmodulin-linked association of 9-anthroylcholine with smooth muscle myosin light chain kinase. Panel A demonstrates the stoichiometry of calcium binding determined with a solution containing 5.0 μM each of the enzyme and calmodulin plus 20 μM 9AC. The calcium-dependent binding of 9AC by 5.0 μM calmodulin alone is also shown (see text). The inset illustrates the stoichiometry of calmodulin binding in a solution containing 5.0 μM enzyme, 20.0 μM calcium, and 20 μM 9AC. Panel B illustrates the calcium dependence obtained with 1.0 μM each of the enzyme and calmodulin plus 5.0 μM 9AC. The results are consistent with a free calcium concentration at half-saturation of 0.35 μM and a Hill coefficient of 1.2 (see text). F/F_0 is the fluorescence enhancement factor, with F_0 representing the fluorescence intensity of 9AC in the absence of the proteins. Excitation, 360 nm; emission, 460 nm. Conditions: 0.20 N KCl and 50 mM Mops, pH 7.3 (25 °C).

N is the total number of ligand binding sites; n, the Hill coefficient; and $\bar{K}$, the concentration of free ligand occurring when $\phi = 0.5$. In our case, X_0 is the total calcium concentration and P_0, the molar concentration of calmodulin calculated from a molecular weight of 16 680. When varying amounts of ligand are added to a solution containing a fixed concentration of protein in excess of $\bar{K}$ ($NP_0 \gg \bar{K}$), plots of ϕ vs. X_0/P_0 contains two linear segments intersecting at $X_0/P_0 = N$. The addition of the first 0–N mol of ligand gives essentially complete binding, with $\phi = X_0/NP_0$. At higher proportions of ligand, $\phi = 1$.

Values of ϕ are routinely determined either directly (such as by equilibrium dialysis) or indirectly through evaluation of changes in some intrinsic property—such as absorbance (ΔA) or fluorescence (ΔF)—of either the protein, as in our case, or the ligand as a function of X_0/P_0. The latter methods facilitate the rapid acquisition of data spanning the full range of ϕ with minimal sample deterioration and adsorption losses. When the condition $NP_0 \gg \bar{K}$ is met, plots of ΔA or ΔF vs. X_0/P_0 directly reflect those predicted for ϕ vs. X_0/P_0 [cf. Weber (1965), Daniel & Weber (1966), Anderson & Antonini (1968), and Anderson (1974)]. A lack of proportionality between the values of ΔA or ΔF and X_0 (up to $X_0 = NP_0$) usually means that the protein concentration is too low. Nonlinearity may also occur when the protein has multiple binding sites with widely varying affinities for the ligand.

The addition of varying concentrations of calcium to a "calcium-free" solution containing 5.0 μM enzyme plus 5.0 μM calmodulin results in a largely linear increase in 9AC fluorescence up to a total[4] of 4 mol of Ca^{2+}/mol of calmodulin (Figure 4A). Little further change occurs at higher concentrations of calcium, through 1 mM. This ideal stoichiometric titration is consistent with essentially total calcium binding up to saturation, with all four calcium binding sites of calmodulin contributing to the association with smooth muscle myosin light chain kinase and the accompanying enhancement of 9AC fluorescence. Calcium has no effect on the binding of 9AC by the enzyme alone. In order to show that the *fluorescence enhancement is directly proportional to the maximum possible concentration of bound calcium*, we performed a second titration in which varying amounts of calmodulin were added to a solution containing 5.0 μM enzyme + 20.0 μM calcium (inset to Figure 4A). The results of the two experiments—where the limiting component is different in each case—are superimposable on the basis that $N = 4$. The completeness of calcium binding compares to that expected for rabbit skeletal muscle myosin light chain kinase and calmodulin. The values of $\bar{K} = 0.40$ μM and $n = 1.6$ reported by Olwin et al. (1984) correspond to at least 95% binding in a solution containing 20 μM calcium and 5.0 μM enzyme plus calmodulin. Equilibrium dialysis experiments confirmed the binding of 4.0 ± 0.4 mol of Ca^{2+}/mon of calmodulin–enzyme under conditions corresponding to the stoichiometric end point (not shown).

[4] The total calcium concentration includes the background level 0.9 μM. This represents 4.5% of the calcium present at the end point. The fluorescence intensities at "zero" calcium were obtained on samples to which 2–3 μM EDTA was added. Fluorescence anisotropy measurements on a solution containing 5.0 μM enzyme plus 5.0 μM dansylcalmodulin show no interaction in the absence of calcium.

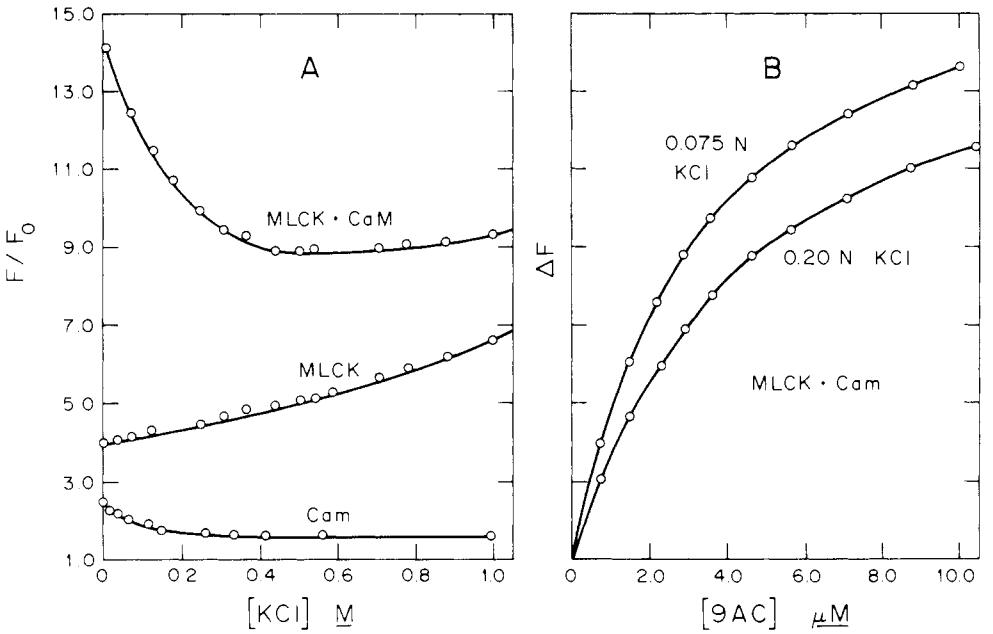

FIGURE 5: Effects of varying KCl concentrations on the fluorescence of 9-anthroylcholine adsorbates. Panel A shows the fluorescence enhancements (F/F_0) obtained with 0.25 μM calmodulin, with 0.25 μM myosin light chain kinase, and with the 0.25 μM enzyme–calmodulin complex plotted as a function of [KCl]. The 9AC concentration was 3.0 μM. Panel B shows two fluorescence titrations of the enzyme–calmodulin complex (0.5 μM) with 9AC. ΔF is the difference between the fluorescence intensities of 9AC measured in the presence and absence of the proteins. Excitation, 360 nm; emission, 460 nm. Conditions: 50 mM Mops and 1.0 mM $CaCl_2$, pH 7.3 (25 °C).

Consideration of the complete system—enzyme, calmodulin, and enzyme–calmodulin—needs to take into account the calcium-dependent binding of 9-anthroylcholine by calmodulin, reported by LaPorte et al. (1980) to involve four to six 9AC binding sites with an average dissociation constant of 440 μM. Figure 4A shows the smaller enhancement found when calcium is added to a solution containing 5.0 μM calmodulin and 20 μM 9AC alone. To evaluate the contribution of the third component, the calmodulin–9AC complex, the titrations were also followed in measurements of the fluorescence anisotropy. Probably because of its lower molecular weight, the calmodulin–9AC complex has a smaller anisotropy (0.106 ± 0.001) than either the enzyme–9AC (0.1464 ± 0.0005) or the calmodulin–enzyme–9AC (0.1535 ± 0.0005) complex. In the two preceding titrations, the changes in anisotropy followed a two-state model with an increase from 0.1464 to 0.1535 at the end point. The addition of either excess calmodulin or calcium had no further effect on the anisotropy. Substantial amounts (>10%) of the free calmodulin–9AC complex apparently do not occur in the limiting mixtures of calmodulin, enzyme, and calcium. When calcium and calmodulin are both in excess, the appearance of the calmodulin–9AC complex is signaled by a decrease in anisotropy, which eventually approaches 0.106. Malencik et al. (1982a) utilized this phenomenon to verify the end points of stoichiometric titrations performed in solutions containing 1.0 mM calcium and to characterize the weaker binding of the phosphorylated enzyme by calmodulin.

Both the intensity and anisotropy measurements follow a two-state model, with *two fluorometrically distinguishable* components—enzyme–9AC and calmodulin–enzyme–9AC—present under limiting conditions. The binding of 9-anthroylcholine by calmodulin probably contributes little to the fluorescence of the calmodulin–enzyme–9AC complex. Titrations of the enzyme–calmodulin complex were consistent with the binding of 1 mol of 9AC/mol of the complex. The effect of saturating levels of ATP or AMP-PNP is similar for both the enzyme and the enzyme–calmodulin complex, suggesting that the binding site is the same in both cases (Malencik et al., 1982a). Finally, calmodulin binding proteins displace most of the bound 9AC from calmodulin. Of 10 proteins investigated so far, all have this effect. They include troponin I, cyclic nucleotide phosphodiesterase (LaPorte et al., 1980), and rabbit skeletal muscle *myosin light chain kinase* (D. A. Malencik and S. R. Anderson, unpublished results).

Calcium titrations conducted at 1.0 μM smooth muscle myosin light chain kinase plus 1.0 μM calmodulin show appreciable dissociation over the final 50% of the saturation range (Figure 4B). As a result of the dilution made, the maximum possible enhancement due to calmodulin alone is only 7% of that obtained when the enzyme is also present. Assuming that ΔF is proportional to ϕ, we can fit the results with $N = 4$, $\bar{K} = 0.35 \pm 0.08$ μM, and $n = 1.2$. We had planned to follow up this experiment with measurements using a calcium–EGTA buffer to stabilize low concentrations of calcium [cf. review by Bartfai (1979)]. However, the calcium–EGTA complex has an effect—apparent quenching—on the enzyme–9AC complex obtained with neither calcium nor EGTA alone. Attempts to correct the intensities determined in the presence of calcium–EGTA result in anomalous Hill plots.

Effects of Ionic Strength Variation on 9-Anthroylcholine Binding. The gradual addition of 0–1.0 M KCl to the solutions has markedly different effects on the adsorbates of 9-anthroylcholine with calmodulin, smooth muscle myosin light chain kinase, and the myosin light chain kinase–calmodulin complex (Figure 5A). These experiments may provide clues on how the association of the enzyme with calmodulin leads to stabilization of the enzyme–9AC complex. In order to separate the effects of variation in equilibrium constant and fluorescence quantum yield, we performed complete titrations spanning 9AC concentrations of 0–60 μM and of 0–30 μM for the enzyme and the enzyme–calmodulin complexes, respectively (e.g., Figure 5B). Plots of the reciprocal of the fluorescence change (ΔF^{-1}) vs. the reciprocal of the concentration of unbound 9AC [calculated according to the method of Malencik et al. (1982a)] are linear, giving values for both K_2 (designated here as K_{9AC}) and the fluorescence intensity of the complex at saturation. The results show that the pri-

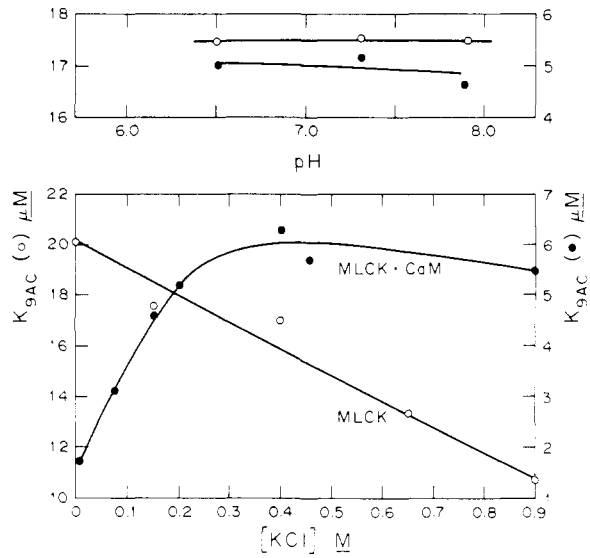

FIGURE 6: Ionic strength and pH dependence of 9-anthroylcholine binding. Values for K_{9AC}, determined in titrations similar to those given in Figure 5B, are plotted as a function of [KCl] (pH 7.3) and as a function of pH ([KCl] = 0.15 M). Results for smooth muscle myosin light chain kinase alone (○) and the kinase–calmodulin complex (●) are included. Refer to Figure 5 for details.

mary effect of ionic strength perturbation is on the binding of 9AC. Figure 6 contains a compilation of the values of K_{9AC} determined for both the enzyme and the enzyme–calmodulin complexes at various KCl concentrations. (The values measured in 0.20 N KCl—18 and 5.2 μM—are slightly smaller than those that we originally reported—20 and 6.4 μM.)

The binding of 9-anthroylcholine by smooth muscle myosin light chain kinase alone is enhanced by increasingly higher KCl concentrations, suggesting that the hydrophobic effect contributes to complex formation. Although fluorescent dyes such as 9AC have been embraced as probes of nonpolar interaction sites, X-ray diffraction studies have demonstrated that they also bind to rigid, hydrophilic regions of proteins (Weber et al., 1979; Madsen et al., 1983). That an electrostatic element is involved in the stabilization of the enzyme–9AC complex by calmodulin is indicated by the pronounced increase in K_{9AC} occurring at KCl concentrations between 0 and 0.3 M. However, there appear to be no participating amino acid side chains that ionize in the physiological pH range. Variations in pH between 6.5 and 7.9 have no effect on the values of K_{9AC} measured in the presence of 0.15 M KCl. The behavior of the enzyme–calmodulin complex at still higher KCl concentrations, between 0.3 and 1.0 M, suggests that the effects of stabilization and destabilization are about balanced (Figure 6).

Comparison of the integrated corrected fluorescence spectrum of a solution containing 1.0 μM 9-anthroylcholine, 8.5 μM smooth muscle myosin light chain kinase–calmodulin complex, 0.33 mM $CaCl_2$, and 50 mM Mops (pH 7.3, 25 °C) with that of a matching quinine sulfate standard[5] shows that the two samples have identical fluorescence quantum yields. Taking into account the quantum yield of quinine sulfate, 70% (Secrist et al., 1972), and the fraction of 9AC bound, 83% calculated for K_{9AC} = 1.6 μM, we obtain a quantum yield of 84% for the 9AC–enzyme–calmodulin complex. The 9AC–enzyme adsorbate must have a comparable fluorescence efficiency. Its extrapolated intensity at saturating dye concentrations is just 11% less than that determined for the complex (Malencik et al., 1982a).

[5] The two solutions have equal calculated absorbancies at the excitation wavelength 360 nm.

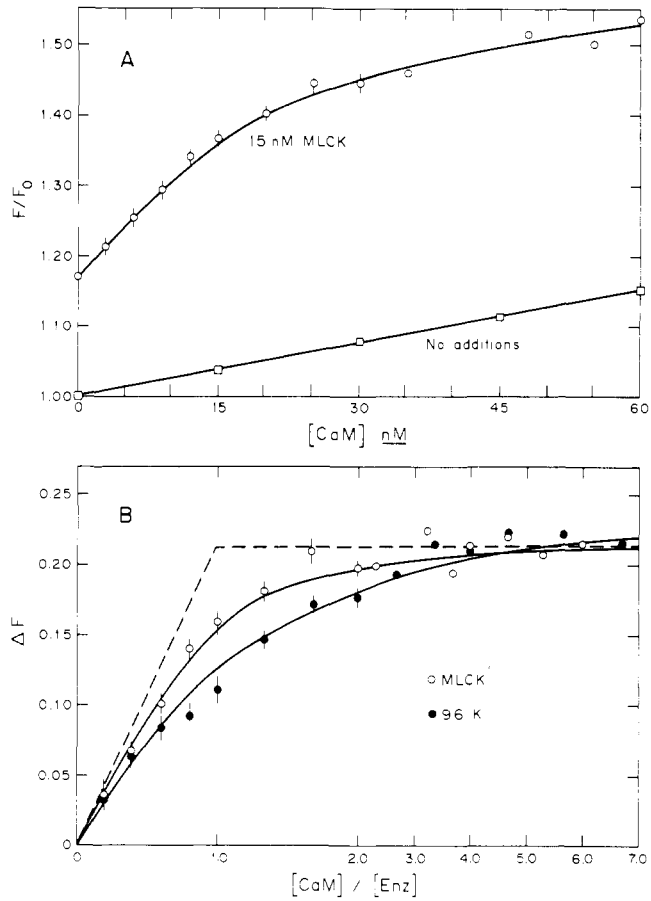

FIGURE 7: Calmodulin binding in a 15.0 nM solution of smooth muscle myosin light chain kinase. Panel A shows fluorescence titrations of 5.0 μM 9-anthroylcholine with calmodulin, both in the presence of 15 nM enzyme and with no additions. Panel B shows the difference between the above titrations, adjusted to $\Delta F = 0$ at the origin (see text). The smooth curves were calculated for dissociation constants of 1.8 nM (corresponding to ΔF_{max} = 0.212) for the enzyme (○) and 5.5 nM (corresponding to ΔF_{max} = 0.23) for a 96K fragment of the enzyme (●). F/F_0 is the fluorescence enhancement factor, where F_0 represents the fluorescence of 9AC in the absence of proteins. Excitation, 360 nm; emission, 460 nm. Conditions: 0.20 N KCl, 50 mM Mops, and 1.0 mM $CaCl_2$, pH 7.3 (25 °C).

The high quantum yields of the 9-anthroylcholine adsorbates facilitate binding measurements at low concentrations of smooth muscle myosin light chain kinase. The changes in 9AC fluorescence taking place when 0.5 μM enzyme is titrated with calmodulin occur in two distinctly linear stages, indicating that the dissociation constant of the enzyme–calmodulin complex is at least 2 orders of magnitude smaller than the enzyme concentration used (Malencik et al., 1982a). Since a definite value for this constant would be generally useful in the interpretation of our results, we lowered the enzyme concentration until dissociation could be detected. A solution containing 15 nM enzyme, 5.0 μM 9AC, 0.20 N KCl, 50 mM Mops, and 1 mM $CaCl_2$ (not limiting), pH 7.3, offers a favorable compromise of fluorescence enhancement and measurable dissociation. Titration of this solution with calmodulin results in a nonlinear rate of increase in the 9AC fluorescence as expected for incomplete binding of calmodulin (Figure 7A). Considering that the calmodulin–9AC complex is somewhat unstable in dilute solutions, we actually used a replacement technique in which each point on the titration was obtained with a fresh sample.

The fraction of myosin light chain kinase bound (f_b) by

Table II: Properties of Turkey Gizzard Myosin Light Chain Kinase and Its Proteolytic Fragments

sample	sp act.[a] (μmol of NADH min^{-1} mg^{-1})	$K_{m,CaM}$ (nM)[a]	K_{CaM} (nM)[b]	K_{9AC} (μM)[b] −CaM	K_{9AC} (μM)[b] +CaM	K_{ATP} (μM)[c] −CaM	K_{ATP} (μM)[c] +CaM
native	9–10	1.2 ± 0.2	2.8 ± 0.7	18.2 ± 0.8	5.2 ± 0.3	140 ± 15	44 ± 3
96K	6	2.7 ± 0.3	8.5 ± 1.0	16.6 ± 0.7	5.4 ± 0.3	116 ± 10	67 ± 3
90K	2	20 ± 3	~15	16.5 ± 0.8	6.6 ± 0.3		
68K	2.5	30 ± 5	~15	16.8 ± 0.8	6.6 ± 0.3		

[a] Determined in an assay system containing 10 μM 20K smooth muscle myosin light chain, 0.29 μM calmodulin, 0.1 mM ATP, 0.2 mM $CaCl_2$, 2.0 mM $MgCl_2$, and 50 mM Mops, pH 7.3 (25 °C), plus the coupling system described under Figure 1. [b] Determined in 0.20 M KCl, 1.0 mM $CaCl_2$, and 50 mM Mops, pH 7.3 (25 °C). Correction was made for 9AC binding. The enzyme concentration was 0.2–0.5 μM. [c] Same as in footnote *b* plus 2 mM $MgCl_2$.

calmodulin is related to the fluorescence enhancement obtained with the equilibrium mixture (F_{obsd}/F_0), the fluorescence enhancement with the enzyme alone (F_{MLCK}/F_0), the enhancement with calmodulin alone (F_{CaM}/F_0), and the enhancement characteristic of the enzyme–calmodulin complex (F_c/F_0).

$$f_b = \frac{(F_{obsd}/F_0 - 1) - (F_{MLCK}/F_0 - 1) - (F_{CaM}/F_0 - 1)}{(F_c/F_0 - 1) - (F_{MLCK}/F_0 - 1) - (F_{CaM}/F_0 - 1)} \quad (5)$$

The numerator of this equation, abbreviated as ΔF, is obtained by subtracting the enhancement found with calmodulin alone from the corresponding enhancement determined for the equilibrium mixture followed by a final subtraction of $F_{MLCK}/F_0 - 1$.

Figure 7B shows the values of ΔF plotted as a function of the moles of calmodulin added per mole of enzyme. The smooth curve was calculated for a dissociation constant of 1.8 nM and a value for ΔF of 0.212 at saturating calmodulin concentrations. The close approach of the results to the stoichiometric titration expected for complete binding of calmodulin (dashed line) as well as the proportionality of the fluorescence enhancements to those measured at higher concentrations (not shown) indicates that all of the enzyme is accounted for.

The dissociation constant of the enzyme–calmodulin complex in the absence of 9-anthroylcholine is readily calculated from the value obtained in its presence, K_{app}.

$$K_{MLCK\text{–}CaM} = \frac{1 + [9AC]/K_2'}{1 + [9AC]/K_2} K_{app} \quad (6)$$

K_2 is the dissociation constant for the enzyme–9AC complex (18 μM) and K_2', the dissociation constant for the (calmodulin–enzyme)–9AC complex (5.2 μM). We thus determine a value for $K_{MLCK\text{–}CaM}$, 2.8 ± 0.7 nM, which is in good agreement with that previously found in competition experiments, ~2 nM (Malencik et al., 1982a; Malencik & Anderson, 1983b). Evidently, the enzyme–calmodulin complex remains tightly associated under the conditions of the previous experiments, ruling out one possible explanation for the effects on ionic strength variation on the binding of 9AC.

Figure 7B also shows a titration (K_{app} = 5.5 nM) of a 96 000-dalton proteolytic fragment of myosin light chain kinase, which is described further in the next section.

Proteolytic Fragments of Myosin Light Chain Kinase. Several proteolytic fragments of turkey gizzard myosin light chain kinase—obtained as products of endogenous proteolysis occurring either in the muscle or during fractionation procedures—exhibit calmodulin-dependent activity in our coupled assay system. Figure 8 shows the dependence of the relative reaction rate on calmodulin concentration for the native enzyme and for fragments having apparent molecular weights of 68 000, 90 000, and 96 000. The 96K fragment is close to the native enzyme in its K_m for calmodulin and specific

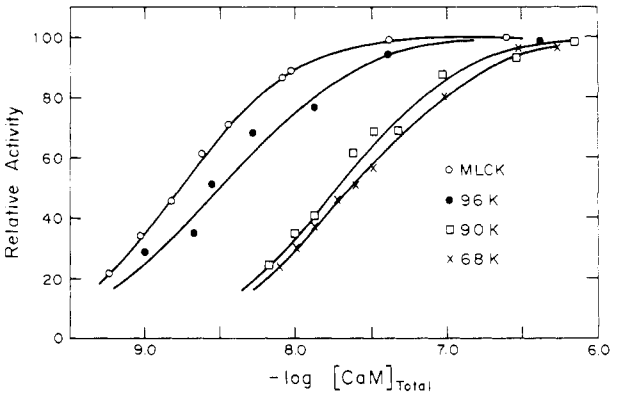

FIGURE 8: Dependence of myosin light chain kinase phosphotransferase activity on the total calmodulin concentration. Assays were performed following the protocol given in Figure 1. Results are included for the native enzyme, 0.15 μg/mL (○); the 96K fragment, 0.15 μg/mL (●); the 90K fragment (□); and the 68K fragment (×). The latter were assayed at 0.4 μg/mL.

activity, while the 68K and 90K fragments have apparently lower affinities for calmodulin as well as lower specific activities (Table II). Note that the K_m of the native enzyme for calmodulin obtained in this assay agrees with the values previously determined with the radioassay (Conti & Adelstein, 1981; Malencik et al., 1982a).

The native enzyme and the fragments have about equal affinities for 9-anthroylcholine. The stabilizing effect of calmodulin on 9AC binding is also similar in the four cases. (The slight variations seen in Table II probably reflect incomplete binding of calmodulin at the concentrations of the fragments used.) The titrations with ATP reveal moderate differences between the native enzyme and the 96K fragment. The stabilizing interaction between the ATP and calmodulin binding sites is somewhat less in the 96K fragment: −0.35 kcal/mol vs. −0.70 kcal/mol.

Discussion

9-Anthroylcholine is a uniquely advantageous fluorescent probe for turkey gizzard myosin light chain kinase. It undergoes calmodulin-dependent binding by the enzyme, with large increases in fluorescence intensity corresponding to a quantum yield for the adsorbate of 84%. The free energy of interaction between the 9AC and calmodulin binding sites ranges from −0.37 to −1.5 kcal/mol, depending on the ionic strength of the medium. These changes are at once large enough to facilitate sensitive binding measurements and small enough to avoid pronounced perturbation of the equilibria. By limiting the concentrations of 9AC used, we are able to apply modest correction factors (usually less than 2-fold) in the calculation of true equilibrium constants. In fact, the perturbation obtained under our conditions is less than that found with any known covalent fluorescent conjugate of calmodulin. 9AC may be specific for smooth muscle myosin light chain

kinases since it does not associate appreciably with the rabbit skeletal muscle enzyme.

The interaction sites for 9-anthroylcholine and ATP may coincide since the binding of the two ligands by the enzyme is very nearly mutually exclusive or competitive. There are similarities as well as differences in the behavior of ATP and 9AC. The association with smooth muscle myosin light chain kinase is calmodulin-dependent for both. The binding of ATP or AMP-PNP by the enzyme requires magnesium and, in the case of the enzyme–calmodulin complex, is strongly enhanced in the presence of the second substrate. The adsorption of 9AC, on the other hand, is independent either of magnesium or of the substrate. The effects of ionic strength variation, studied intensively for 9AC binding, also differ (cf. Table I and Figure 6). The opposite charges of the two ligands—with 9AC being cationic and the MgATP complex, anionic—may account for deviations in their local interactions.

Enhancement of substrate binding in the myosin light chain kinase–calmodulin complex was suggested by Nairn & Perry (1979) and Crouch et al. (1981). Competition experiments using 9-anthroylcholine indeed revealed a stabilizing interaction between the ATP and calmodulin binding sites of smooth muscle myosin light chain kinase corresponding to ΔG = −0.6 to −0.7 kcal/mol (Malencik et al., 1982a). The experiments presented here consider the additional effects of the second substrate, a tridecapeptide analogue of the 20 000-dalton myosin light chain. Titrations of the 9AC adsorbates with AMP-PNP demonstrate a strong positive interaction ($\Delta G \leq -1.4$ kcal/mol) between the nucleotide and light chain analogue binding sites operating within the enzyme–calmodulin complex but probably not within the free enzyme. The dissociation constants for the binary (calmodulin–enzyme)–LC and (calmodulin–enzyme)–AMP-PNP complexes are at least 10-fold larger than those of the ternary (calmodulin–enzyme)–LC–AMP-PNP complex. Measurements on the free enzyme suggest not only that stabilization is absent but also that the interactions are even slightly antagonistic. In the case of the myosin light chain kinase–calmodulin complex, the interaction energy compares to that found with other two-substrate enzymes. Kolb & Weber (1975), for example, determined that the free energy of stabilization for oxalate and NADH binding by chicken heart lactate dehydrogenase is −1.3 kcal/mol.

We believe that the principal features of this model system apply to the phosphorylation of smooth muscle myosin. AMP-PNP differs from ATP in the substitution of a nitrogen atom for an oxygen atom. The stabilizing effect of calmodulin is somewhat less for AMP-PNP than it is for ATP. The free energy of interaction between AMP-PNP and calmodulin ranges from −0.20 kcal/mol in 50 mM Mops, 2 mM $MgCl_2$, and 1 mM $CaCl_2$ to −0.44 kcal/mol in the same buffer containing 0.20 N KCl. Most of the experiments were performed at low ionic strength in order to conserve our supply of the light chain analogue. However, measurements on solutions containing 0.20 N KCl confirm the essential differences between smooth muscle myosin light chain kinase and its complex with calmodulin. The light chain analogue represents just $^1/_{300}$th of the native myosin molecule. Although it provides no information on long-range interactions between myosin and the enzyme, it is likely to be a good model for local events at the phosphorylation site of myosin.

The interactions involved in the binding of calmodulin and the substrates by the enzyme should be mutual. Olwin et al. (1984) determined the combined action of AMP-PNP and the 18 500-dalton myosin light chain on the association of rabbit skeletal muscle myosin light chain kinase with a fluorescent calmodulin conjugate, derivatized with 5-[[[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid. Their analysis, which included correction factors of 7.4–10-fold for the effects of covalent modification on calmodulin, showed that the dissociation constant for the enzyme–calmodulin complex decreases from 15 to 3 nM in the presence of 40 μM light chain plus 0.5 mM AMP-PNP. This corresponds to a combined energy of stabilization of −0.95 kcal/mol. The results of separate experiments with the two substrates were not reported.

Myosin light chain kinase often shows low levels of activity in the absence of added calmodulin, possibly reflecting contamination of either the myosin or the myosin light chain preparations used in the assays (this paper; Walsh et al., 1982b). In the case of cardiac myosin light chain kinase, Zimmer et al. (1984) demonstrated that the base-line activity is actually due to calmodulin-independent activity of the native enzyme. Assays using 1 mM [γ-^{32}P]ATP indicated that the enzyme alone has an apparent K_m for mixed myosin light chains of 8 mg/mL while the enzyme–calmodulin complex has a corresponding K_m of 0.35 mg/mL.

The binding of most of the small ligands studied here—9-anthroylcholine, ATP, AMP-PNP, and the light chain analogue—is dependent on the ionic strength of the medium. The distinctive results obtained with the 9-anthroylcholine–(enzyme–calmodulin) complex suggest that both hydrophobic and electrostatic interactions are involved in the association. Blumenthal & Stull (1982) concluded that hydrophobic and other effects contribute to the activation of skeletal muscle myosin light chain kinase by calmodulin.

Titrations of calcium-free solutions of smooth muscle myosin light chain kinase plus calmodulin (5.0 μM each) show that the fluorescence of 9-anthroylcholine increases linearly up to the addition of 4 mol of calcium/mol of calmodulin, with no further change occurring through calcium concentrations of 20 μM to 1 mM. A parallel experiment, in which varying concentrations of calmodulin are added to a solution containing 5.0 μM enzyme plus 20.0 μM calcium, confirms direct proportionality between fluorescence enhancement and the maximum possible concentration of bound calcium based on four calcium binding sites. Evidently, all four calcium binding sites of calmodulin contribute about equally to the enhanced binding of 9AC by the enzyme. Consideration of the properties of both the calmodulin–enzyme–9AC complex and the calmodulin–9AC complex (from which most of the 9AC is released upon protein binding) reinforces the conclusion that the stoichiometric titration reflects primarily the calmodulin-dependent binding of 9AC by myosin light chain kinase. The apparent equivalence of the four calcium binding sites is supported by stopped-flow kinetic studies showing that the rate of calcium dissociation from the enzyme–calmodulin complex is essentially independent of the initial degree of calcium saturation (M. I. Schimerlik, D. A. Malencik, and S. R. Anderson, unpublished results).

Cox (1984) described an equilibrium gel filtration experiment in which both Ca_3^{2+}–calmodulin and Ca_4^{2+}–calmodulin associate extrinsically with phosphorylase kinase. Being unable to detect species with less calcium, he concluded that the enzyme recognizes only the more fully liganded complexes of calmodulin. Activity measurements, performed at nanomolar concentrations of the enzymes and calmodulin, showed that the activation of skeletal muscle myosin light chain kinase (Blumenthal & Stull, 1980) and the activation of cyclic nucleotide phosphodiesterase (Huang et al., 1981) are both fourth

order with respect to calcium concentration. Although these authors originally concluded that only the Ca_4^{2+}–calmodulin complex activates the enzymes, the strong cooperativity apparent at such low protein concentrations could also be due to enhanced calcium binding resulting from the association of the proteins. Our experiments, conducted at protein concentrations in the micromolar range, yield a Hill coefficient of 1.2 for the binding of calcium by the enzyme–calmodulin complex. This value suggests that calcium is distributed among the various possible enzyme–calmodulin complexes (MLCK–CaM–Ca^{2+}, MLCK–CaM–Ca_2^{2+}, MLCK–CaM–Ca_3^{2+}, and MLCK–CaM–Ca_4^{2+}). This result would be in agreement with the model of Huang et al. (1981) in which successive calcium binding promotes a stepwise enhancement of the protein–protein interaction.

We have isolated three proteolytic fragments of smooth muscle myosin light chain kinase having apparent molecular weights of 68 000, 90 000, and 96 000 that retain calmodulin-dependent catalytic activity. Their interactions with 9-anthroylcholine, in both the presence and absence of calmodulin, are close to those of the native enzyme. However, fluorescence titrations and catalytic activity measurements show that the stabilities of their complexes with calmodulin vary considerably. The differences in chromatographic behavior and calmodulin binding suggest that the 90K and 96K fragments resulted from proteolysis at opposite ends of the myosin light chain kinase molecule. The resolution of the fragments in both ion-exchange and gel-filtration chromatographies indicates their existence as distinct entities in nondenaturing solutions. The catalytic properties of the fragments generated by endogenous proteolysis are in marked contrast to those of the 80K calmodulin-independent fragment, prepared by Walsh et al. (1982a) through limited chymotryptic digestion of the enzyme. Foyt et al. (1985) recently prepared calmodulin-dependent active fragments of chicken gizzard myosin light chain kinase of molecular weights 70 000 (from a V8 protease digestion) and 95 000 (from chymotryptic digestion). However, no quantitative information on their catalytic activities, calmodulin binding properties, or other ligand binding characteristics is available. Adoption and modification of the coupled spectrophotometric assay used by Cook et al. (1982) for cAMP-dependent protein kinase expedited both our characterization of the active fragments and the routine purification of the enzyme.

Registry No. ATP, 56-65-5; AMP-PNP, 25612-73-1; MLCK, 51845-53-5; 9AC, 74095-55-9.

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Purification and Characterization of a "Half-Molecule" α_2-Macroglobulin from the Southern Grass Frog: Absence of Binding to the Mammalian α_2-Macroglobulin Receptor†

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ABSTRACT: An α-macroglobulin (α_2M), which is a dimer consisting of two non-disulfide-bonded subunits, was identified and purified from frog plasma by Ni^{2+} chelate affinity chromatography. This frog "half-molecule" α-macroglobulin migrated as an α_2-globulin in cellulose–acetate electrophoresis rather than as the previously described frog α_1M, which exists as a tetramer formed by the noncovalent association of disulfide-bonded pairs. A molecular weight of ~380 000 was obtained by gel-filtration high-pressure liquid chromatography, and in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) the protein migrated as a single band of M_r ~180 000 before and after reduction. No evidence was obtained for association of this protein to a higher molecular weight species. After the preparation was heated, additional bands were obtained in SDS–PAGE with M_r ~60 000 and 12 000. The additional bands were not obtained after heating methylamine-treated preparations. The circular dichroic spectrum of frog α_2M exhibits negative ellipticity over the region 205–250 nm with a minimum at 216 nm. After reaction with proteinase, a decrease in the absolute mean residue rotation was obtained. Amino acid analysis demonstrated that frog α_2M and α_1M are similar in composition to avian and mammalian α-macroglobulins; however, there are sufficient differences in the composition of these two amphibian α-macroglobulins to support the conclusion that they are distinct proteins. Frog α_2M bound approximately 0.5 mol of trypsin/mol of inhibitor. This binding was abolished by pretreatment with methylamine. Frog α_2M, in contrast to α_1M, exhibited no specific binding to the mammalian α-macroglobulin receptor in either direct or indirect binding assays. It is concluded that two distinct α-macroglobulins are present in the frog and that naturally occurring half-molecule α-macroglobulins are not recognized by the mammalian α-macroglobulin receptor.

Human α_2-macroglobulin (α_2M)[1] is a high molecular weight plasma proteinase inhibitor (M_r ~720 000) formed by the noncovalent association of disulfide-bonded pairs of identical subunits (Harpel, 1973; Hall & Roberts, 1978; Swenson & Howard, 1979a; Barrett, 1981). By a unique mechanism, termed "trapping", it is capable of inhibiting endopeptidases of each of the four mechanistic classes (Barrett and Starkey, 1973). α_2M contains a region highly susceptible to proteolytic cleavage termed the "bait" region (Barrett & Starkey, 1973). On reaction of a proteinase at this site, a

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[1] Abbreviations: α_1M, α-macroglobulin; α_2M, α_2-macroglobulin; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SBTI, soybean trypsin inhibitor; DEAE, diethylaminoethyl.

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