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Interaction of Calmodulin with Skeletal Muscle Myosin Light Chain Kinase†

Thomas H. Crouch, Michael J. Holroyde, John H. Collins, R. John Solaro, and James D. Potter*

ABSTRACT: Studies on myosin light chain kinase isolated from rabbit skeletal muscle show that the enzyme has a molecular weight of 80 000-84 000 with a sedimentation coefficient of 3.2 S and an apparent Stokes radius of 53 Å. Gel filtration chromatography with a ^{3}H-labeled calmodulin using a Hummel-Dryer technique shows that the enzyme will bind 1 mol of calmodulin per mol of enzyme, with an affinity of $(1.9 \pm 0.5) \times 10^7$ M^{-1} in the absence of substrate. The calmodulin dependence of enzyme activation at limiting Mg^{2+} and light chain concentrations confirms this observation. The calcium dependence of activation of the enzyme-calmodulin complex is characterized by a Hill coefficient of 2.5, with half-activation occurring at 6.6×10^{-7} M Ca^{2+}. The amino acid composition shows a high percentage (9.1%) of proline, which may account for the large apparent Stokes radius and no clear resemblance to other skeletal muscle proteins. A comparison of the amino acid composition with that from turkey gizzard shows some resemblance.

Phosphorylation of the 18 500-dalton light chain component of myosin (LC_2)[1] from rabbit fast skeletal muscle is catalyzed by a Ca^{2+}-dependent myosin light chain kinase (MLCK).[1] In intact skeletal muscle, this occurs during contraction (Bárány et al., 1979) and may play a role in the posttetanic potentiation of peak twitch tension in the muscle (Manning & Stull, 1979).

† From the Section of Contractile Proteins, Department of Pharmacology and Cell Biophysics (T.H.C., J.H.C., R.J.S., and J.D.P.), and the Department of Physiology (M.J.H. and R.J.S.), University of Cincinnati College of Medicine, Cincinnati, Ohio 45267. *Received April 20, 1981.* This work was supported by National Institutes of Health Grants (HL 22619-3A,B,E) HL 22231 and AM-20875, Postdoctoral Research Training Grant HL 07382, the Muscular Dystrophy Association, and the American Heart Association (78-1167). T.H.C. is a fellow of the Muscular Dystrophy Association. J.H.C. and R.J.S. are holders of Research Career Development Awards.

[1] Abbreviations used: LC_1, light chain 1; LC_2, light chain 2; LC_3, light chain 3; MLCK, myosin light chain kinase; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 4-morpholinopropanesulfonic acid; Me_2SO, dimethyl sulfoxide; Caps, 3-(cyclohexylamino)propanesulfonate; DTE, dithioerythritol; Hap (HAP in figures), hydroxylapatite; Tris, tris(hydroxymethyl)aminomethane.

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Ca^{2+} activation of MLCK is mediated by calmodulin, the ubiquitous Ca^{2+} binding protein that appears to be a mediator of Ca^{2+} function in eukaryotes (Cheung, 1980; Klee et al., 1980). Clearly, an understanding of the nature of the calmodulin–MLCK interaction is required if we are to extend our knowledge of the role of LC_2 phosphorylation in skeletal and other types of muscle. The enzyme has been reported to bind 1 mol of calmodulin per mol of MLCK (Yagi et al., 1978; Nairn & Perry, 1979a; Stull et al., 1980; Blumenthal & Stull, 1980); however, these observations have been based on indirect rather than direct binding measurements and have not been confirmed by conventional physical methods. Moreover, there is not yet agreement concerning the Ca^{2+} and calmodulin dependence of MLCK activation. Several investigators have reported different values for the apparent K_m of approximately 1 nM (Nairn & Perry, 1979a; Blumenthal & Stull, 1980) while Yagi et al. (1978) and Walsh et al. (1980) report values on the order of 10–100 nM. The concentration of Ca^{2+} required for half-maximal activation also varies among reports from these investigators. Yazawa & Yagi (1978) as well as Stull et al. (1980) place the pCa for half-maximal activation in the neighborhood of 5 while Nairn & Perry (1979a) place this value at approximately 6.8. Blumenthal & Stull (1980) have, however, shown this to be Mg^{2+} dependent.

Since both the kinase and calmodulin can be purified to homogeneity in useful quantities by procedures reported here, the system provides an opportunity to study the sedimentation properties of the enzyme, giving an estimation of the apparent Stokes radius of the enzyme as well as the thermodynamics of the association of calmodulin with one of the many enzymes with which it associates. Through the use of modified standard physical biochemical techniques, the stoichiometry as well as the equilibrium association constant of calmodulin with skeletal muscle MLCK is reported in the presence and absence of skeletal muscle LC_2. The Ca^{2+} dependence of activation is also examined where free Ca^{2+} concentrations are held constant by using EGTA as a Ca^{2+} buffer.

Materials and Methods

Purification of MLCK. Skeletal muscle MLCK was prepared by a modification of the procedure of Yazawa & Yagi (1978). Minced rabbit fast skeletal muscle (2.0 kg) was extracted in 2 volumes of 50 mM KH_2PO_4/KOH buffer, pH 6.8, containing 5 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. The suspension was centrifuged at 10960*g* for 10 min, and the pH of the supernatant fraction was lowered to 5.7 with 20% H_3PO_4. The resulting precipitate was removed by centrifugation as described above. The supernatant fraction was then applied to a DEAE-cellulose (DE-52) column (5 × 40 cm) equilibrated with 50 mM KH_2PO_4/KOH buffer, pH 5.7, 5 mM EDTA, and 1 mM DTT. After the column was washed with one bed volume of equilibrating buffer, the enzyme was eluted by a linear KCl gradient (0–0.75 M; 2 × 1 L). The enzyme activity eluted in a conductivity range of 2.5 to 7.0 mS, with approximately 14% of the enzymatic activity lost in the wash. The fractions containing MLCK activity were pooled and subjected to a 30–50% $(NH_4)_2SO_4$ fractionation step at pH 6.8, and the pellet was resuspended in a minimum volume of 1 mM DTT and 1 mM EDTA, pH 7.0.

The sample was then applied directly to a Sephadex G-150 column (2.2 × 100 cm) equilibrated with 200 mM KCl and 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT and 1 mM EDTA, pH 7.5. The flow rate was 16–18 mL/h, and 5-mL fractions were collected. The MLCK peak (see Figure 1A) was then subjected to calmodulin affinitiy chromatography.

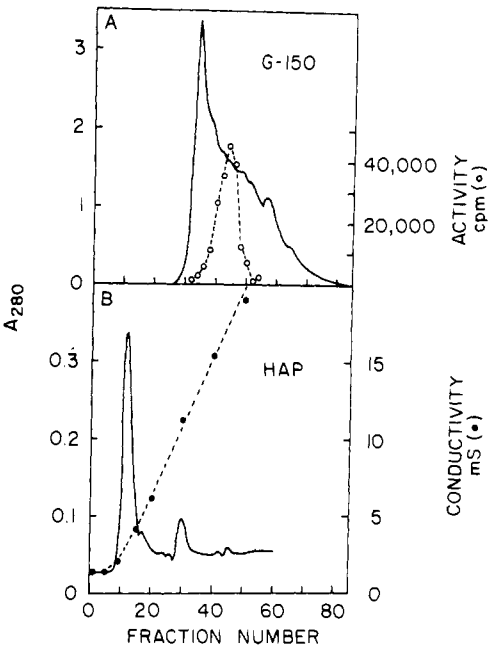

FIGURE 1: (A) Sephadex chromatography of MLCK preparation after ammonium sulfate fractionation. The fraction sizes were 5 mL. The MLCK activity migrates behind a leading high molecular weight peak. (B) Hydroxylapatite chromatography of MLCK after calmodulin affinity chromatography. The leading peak contained all the MLCK activity.

The sample was applied to a calmodulin–Sepharose (data not shown) column (2 × 10 cm; 1 mg of calmodulin/mL of Sepharose 4B) equilibrated with 50 mM Tris-HCl, 50 mM KCl, 0.2 mM $CaCl_2$, and 1 mM DTT, pH 7.5. The column was washed with 3 column volumes of this buffer and eluted with the same buffer brought to 2 mM EDTA. The eluate fractions containing MLCK were then dialyzed against 10 mM KH_2PO_4/KOH buffer, pH 6.8, containing 10 mM $MgCl_2$ and 1 mM DTT, pH 6.8. The MLCK was then subjected to hydroxylapatite chromatography. Hydroxylapatite was prepared by the method of Mazin et al. (1974), except K_2HPO_4 was used instead of Na_2HPO_4. The sample was applied to a column (1.5 × 30 cm) that had been equilibrated with 10 mM potassium phosphate and 1 mM DTT, pH 6.8, washed in with 50 mL of buffer, and eluted with a linear potassium phosphate gradient (0.01–0.5 M; 2 × 300 mL). The leading peak was found to contain the kinase activity (see Figure 1B). $NaDodSO_4$–polyacrylamide gel electrophoresis (12.5%) using the Laemmli system (1970) showed one heavy band corresponding to a molecular weight of 81 000 (Figure 2). The sample was concentrated by dialyzing against 20 mM Tris-HCl, pH 7.5, buffer containing 1 mM DTT and 1 mM EDTA, applying it to a DE-52 column (0.5 × 4 cm), and eluting it with the above buffer containing 0.5 M KCl. The homogeneity at this point was generally greater than 99% (Figure 2). However, occasionally there was contamination with lower molecular weight components. These were removed when necessary by chromatography on a Sephadex G-150 column (1.5 × 100 cm), 140 mM KCl and 20 mM Mops/KOH buffer, pH 7.0, containing 1 mM DTT and 1 mM EDTA. The MLCK collected after this step showed greater than 99% purity as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Protein Determinations. Calmodulin and myosin light chain concentrations were determined by the method of Lowry et al. (1951). MLCK concentrations were determined by absorbance by using an extinction coefficient E_{278}^{1cm} of 0.955 mL/mg as determined by protein nitrogen determination

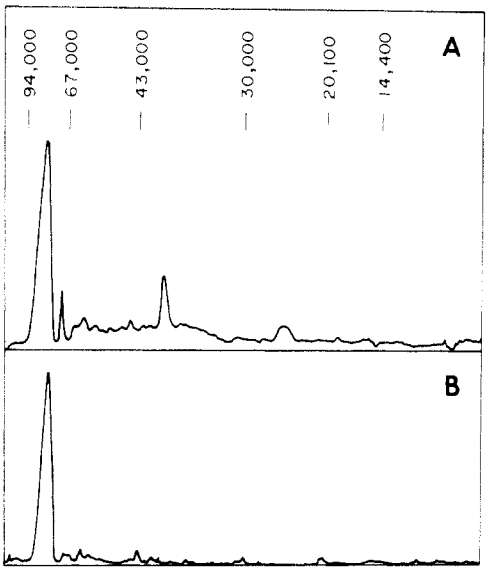

FIGURE 2: Densitometric scans of $NaDodSO_4$–polyacrylamide gels. (A) MLCK after affinity chromatography; (B) MLCK after hydroxylapatite chromatography. The MLCK peaks correspond to a molecular weight of 81 000.

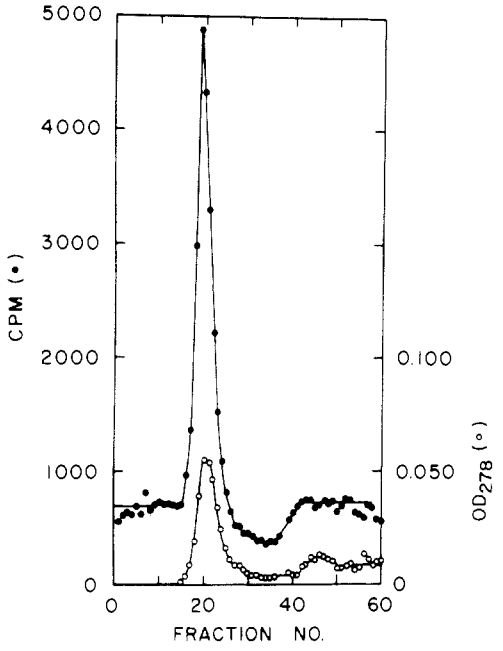

FIGURE 3: Typical Hummel–Dryer (Ackers, 1973) profile of [^{3}H]-calmodulin binding to MLCK. The concentration of calmodulin was 4.20×10^{-8} M with a specific activity of 3.163×10^{10} cpm/mol of calmodulin.

(Solaro et al., 1976), amino acid composition, and the method of Lowry et al. (1951).

Ultracentrifugation. Sedimentation velocity and meniscus depletion sedimentation equilibrium experiments (Yphantis, 1964) were performed with a Beckman Spinco Model E ultracentrifuge equipped with ultraviolet photoelectric scanning optics (Schachman & Edelstein, 1966) and charcoal–epon double-sector centerpieces at 20 °C. The buffer used was 150 mM KCl, 10 mM Mops/KOH, 0.1 mM DTT, and 0.1 mM EGTA, pH 7.0. The MLCK was dialyzed exhaustively against this buffer. The enzyme solution (400 μL) with an optical density of 0.100 at 278 nm was placed in one sector and the dialysate in the other sector. The sample was spun at 48 000 rpm for 3 h to simultaneously obtain sedimentation velocity data and deplete the meniscus to allow for rapid equilibrium. Scans were taken every 9 min. The rotor was slowed to 10 000 rpm and allowed to spin for 36 h, with scans taken every 12 h. Analysis of the last two scans showed that 24 h were sufficient to reach equilibrium and that no degradation had taken place during that time as verified by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Preparation of Calmodulin. Calmodulin was isolated from beef brain in the following manner: 2.0 kg of frozen beef brain were homogenized in 2 volumes of 50 mM KH_2PO_4/KOH, pH 5.7, containing 5 mM EDTA, and 1.5 mL of 5% PMSF in Me_2SO per L. The homogenate was centrifuged at 10960g for 30 min. The pellet was extracted in 2 volumes of the above buffer and centrifuged. Both supernatants were pooled, the pH was raised to 7.1, and the supernatants were saturated to 50% $(NH_4)_2SO_4$ without pH adjustment and centrifuged as above. The supernatant was brought to 80% saturation with $(NH_4)_2SO_4$, the pH was lowered to 4.5, and the supernatant was centrifuged as above. The pellet was resuspended in a minimum volume of distilled water and the pH raised to 7.1 with 1 M Tris base. The sample was dialyzed twice against 1 mM EDTA, pH 7.0 (6 volumes). The sample was then poured through 200 mL of DEAE-Sephadex A-50 equilibrated with 0.1 M potassium phosphate, 0.05 M NaCl, and 1 mM EDTA, pH 7.1, and washed with 2 bed volumes of this buffer. The resin was washed with 2 bed volumes of this buffer made 0.22 M in NaCl, then with 1.8 bed volumes of this buffer made 0.300 M in NaCl, and finally with 2.0 bed volumes of this buffer made 0.70 M in NaCl. The 0.30–0.70 M eluate was then dialyzed against 0.02 M Tris-HCl, pH 7.5, 0.05 M NaCl, and 1 mM $MgCl_2$ 3 times (10 volumes) and applied to a hydroxylapatite column with a volume of 0.18 mL of hydroxylapatite per optical density unit observed at 280 nM. The sample was washed with 20 mM Tris-HCl, 100 mM NaCl, and 1 mM imidazole, pH 7.5 (0.5 bed volume). The calmodulin was eluted with a linear gradient made with 6 bed volumes of this buffer and 6 bed volumes of this buffer containing 0.8 M $(NH_4)_2SO_4$. The calmodulin sample was diluted to a conductivity of 8.7 mS with 1 mM EDTA and applied to a DEAE-Sephadex A-50 column with a volume of 1.0 mL of resin per optical density unit measured at 280 nm. The column was equilibrated with 0.1 M potassium phosphate, 1.0 mM EDTA, and 50 mM NaCl, pH 7.1. The sample was washed in with 3 column volumes of this buffer made 0.22 M in NaCl and eluted with a linear gradient made with 4 column bed volumes of this buffer and the same volume of this buffer made 0.70 M in NaCl. The single peak contained homogeneous calmodulin, as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis, using the Laemmli (1970) system (15% gel), and the UV spectrum. The calmodulin was dialyzed exhaustively against 50 mM NH_4HCO_3 and lyophilized several times to remove the salt. The lyophilized calmodulin was stored at −20 °C until further use.

Isolation of Rabbit Skeletal LC_2. Rabbit skeletal myosin was prepared as described by the procedure of Kielley & Harrington (1959) using the following extraction buffer:20 mM Mops/KOH, pH 7.0, 0.6 M KCl, 1 mM DTT, and 1 mM PMSF (100 mM in ethanol diluted 100-fold in the extract). The whole light chain fraction was obtained from purified myosin dissolved in 5 M urea, 0.3 M KCl, 20 mM Mops/KOH, pH 7.0, and 1 mM DTT by 50% ethanol fractionation. After removal of the heavy chain precipitate by low-speed centrifugation, the light chain supernatant was exhaustively dialyzed against 0.02% NaN_3 and 10 mM mercaptoethanol and then lyophilized.

The whole light chain fraction was dialyzed against 5 M urea, 50 mM Tris-HCl, and 1 mM DTT and then applied to

Table I: Purification of Rabbit Skeletal Muscle MLCK

	volume (mL)	A_{278} units	activity (μmol of $^{32}P_i$ incorporated min^{-1} L^{-1})	activity X vol	activity (A_{278})	recovery (%)	rel purification
extract	3800	105184	0.230	874	0.0083	100	1.0
DE-52	590	4236	1.27	749	0.177	86	21
$(NH_4)_2SO_4$	15	357	ND [b]	ND	ND	ND	ND
G-150	59	105	7.33	432	4.12	49	500
CaM-Sepharose	54	16.9	7.22	390	23	45	2800
Hap/DE-52 [a]	9.0	8.21	20.81	187	23	21.4	2800

[a] Total yield was 8.6 mg of MLCK with a specific activity of 17 μmol of $^{32}P_i$ incorporated min^{-1} (mg of protein)$^{-1}$ at an ATP concentration of 1 mM. The recovery usually ranges from 14% to 24%, with specific activities ranging from 16 to 24 μmol of ^{32}P incorporated min^{-1} (mg of enzyme)$^{-1}$. [b] ND, not determined.

a DEAE-Sephadex A-25 column (2.0 × 100 cm) equilibrated in the same buffer. Elution was achieved by a gradient made with 500 mL of this buffer and 500 mL of this buffer containing 0.5 M KCl. Flow rate was 30 mL/h, and 10-mL fractions were collected (Nairn & Perry, 1979a,b). The LC_2 fraction was located by $NaDodSO_4$–polyacrylamide gel electrophoresis.

The enriched LC_2 preparation was dialyzed against 5 mM Mops/KOH, pH 7.0, 0.02% NaN_3, and 1 mM DTT applied to a column (2.5 × 20 cm) of Cibacron Blue–Sepharose with a linear gradient made with 300 mL of this buffer and 300 mL of this buffer containing 1.0 M KCl. The LC_2–containing fractions were located by $NaDodSO_4$–polyacrylamide gel electrophoresis, and these fractions showed a single band corresponding to pure LC_2. The LC_2 was exhaustively dialyzed against 50 mM NH_4HCO_3 and 1 mM β-mercaptoethanol and lyophilized.

Preparation of [^3H]Calmodulin. Calmodulin labeled with ^3H was prepared with C^3H_3I by the method of Jones et al. (1975). Lyophilized calmodulin was dissolved in 0.1 M sodium acetate and 0.5 mM $CaCl_2$, pH 5.2, at a concentration of 0.5 mM in a total volume of 4.0 mL. This solution was then added to 11.5 μg of C^3H_3I with a specific activity of 300 Ci/mol. The solution was stirred in the dark under a well-ventilated hood at room temperature for 24 h. The solution was then dialyzed twice against 1 L of the above buffer for 9 h at room temperature. The sample was then dialyzed against 500 mL of 0.1 M Caps, and 0.5 mM $CaCl_2$, pH 10.5, for 12 h, then 3 times against 100 mL of this buffer containing 20 mM DTE, and then against 0.1 M Tris-HCl, pH 7.5 (1 L for 12 h). The final specific activity was 4.4×10^7 cpm/mg of calmodulin, as determined in a Beckman LS155 liquid scintillation spectrometer and by Lowry protein determinations. The sample was stored in 0.1 M Tris-HCl, pH 7.5, at −20 °C until used. The apparent K_m of [^{3}H]calmodulin for MLCK was identical with that for unreacted calmodulin, as judged by its ability to activate MLCK.

For examination of the specificity of labeling, calmodulin was reacted as above with nonradioactive CH_3I at a 100-fold excess. Prior to reduction with DTE, the reacted calmodulin was subjected to amino acid analysis. There was an apparent increase in the lysine content of seven residues per mole, with a corresponding decrease in methionine content, indicating that an average of seven of the nine methionine residues are available for reaction. The apparent increase in lysine arises from *S*-methylmethionine, which cannot be resolved from lysine on the amino acid analyzer.

Binding of [^3H]Calmodulin to MLCK. The binding of [^{3}H]calmodulin to MLCK was analyzed by a modification of the procedure of Hummel and Dryer as described by Ackers (1973). A column of Sephadex G-150 (0.7 × 40 cm) was saturated with 150 mM KCl, 10 mM Mops/KOH, 0.5 mM $CaCl_2$, and 1 mM DTT, pH 7.0, containing the appropriate concentration of [^{3}H]calmodulin. The column was run at a flow rate of 4.0 mL/h and monitored by a Gilson column monitor at a wavelength of 278 nm, and 0.4-mL fractions were collected. The MLCK sample was loaded on the column in the equilibrating buffer containing 10% glycerol in a volume in 150 μL. A typical elution profile is shown in Figure 3. The first absorbance peak represents MLCK while the second represents glycerol and corresponds to the internal volume of the column. The plot of counts per minute vs. fraction number shows the characteristic peak and trough of a Hummel–Dryer type measurement.

MLCK Assay. MLCK activity was measured as described by Holroyde et al. (1979). For kinetic studies on the purified enzyme, purified LC_2 was used instead of a whole light chain fraction. For the calmodulin dependence of MLCK activation, each 100-μL assay contained the following: 20 mM Mops/KOH (pH 7.0), [γ-^{32}P]ATP, 0.4 mM $MgCl_2$, 0.5 mM $CaCl_2$, 150 mM KCl, 0.1 mM DTT, 56.4 or 2.4 nM MLCK, 5–1000 nM calmodulin, and 10.0 μM LC_2. The lower $MgCl_2$ concentration was required to lower the specific activity of the enzyme to approximately 900 nmol of phosphate incorporated min^{-1} (mg of enzyme)$^{-1}$. For the Ca^{2+} dependence of MLCK, each essay contained the following: 100 μM decalcified LC_2 and 150 mM Mops/KOH buffer, pH 7.0, containing 1 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$, 150 mM KCl, 2 mM EGTA, and 0.5 mM DTT (total 100 μL). $CaCl_2$ was added to each assay to obtain the desired free Ca^{2+} concentration which was calculated as described by Potter & Gergely (1975).

Results

Purification of MLCK. The MLCK purification presented here is a modification of the method of Yazawa & Yagi (1978). Table I shows a typical purification profile for the enzyme starting with 2.0 kg of tissue. The specific activity is reported per unit absorbance at 278 nm instead of per milligram of protein. The hydroxylapatite chromatography removes a component of approximately 78 000 daltons as well as some lower molecular weight components (see Figure 2). After the hydroxylapatite chromatography, the enzyme is greater than 99% homogeneous as estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis 12.5% gels (Laemmli, 1970; Figure 2). Isoelectric focusing by the method of DiSalvo et al. (1979) showed a single band with a pI of 5.0 ± 0.2, which is consistent with the excess of acidic over basic residues found in the amino acid composition. It has occasionally been found that after the hydroxylapatite step that there may be lower molecular weight components which copurify with the enzyme. These components bind to both LC_2 and calmodulin affinity columns. This suggests that these contaminants may be

Table II: Amino Acid Composition of Rabbit Skeletal Muscle MLCK [a]

residue	no. [d]	residue	no. [d]
Asp	65.3 (65)	Leu	61.8 (62)
Thr	34.7 (35)	Tyr	10.2 (10)
Ser	41.3 (41)	Phe	25.8 (26)
Glu	110 (110)	Lys	51.0 (51)
Pro	69.3 (69)	His	10.7 (11)
Gly	61.1 (61)	Arg	24.5 (24)
Ala	80.5 (80)	Met	13.0 (13)
Val	42.1 (42)	Cys [b]	10 (10)
Ile	25.5 (26)	Trp [c]	12 (12)

[a] Expressed as residues per 81 000 daltons. Numbers in parentheses are nearest integers. [b] From titration with 2-(4-maleimidylanilino)naphthalene-6-sulfonic acid. [c] From extinction coefficient (see text). [d] Total number of residues (in parentheses) = 748.

subfragments of the enzyme generated by proteolysis, which remain intact and active under nondenaturing conditions. These fragments can be removed by Sephadex G-150 gel filtration. It has also been found that if the purified enzyme is allowed to stand in the presence of 1 mM $CaCl_2$ for extended periods of time, all activity is lost. This is consistent with observations made by Pires & Perry (1977). The enzyme is stable for long periods when stored in 100 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.5, and 50% glycerol at −20 °C. It is also stable for several weeks when stored in 150 mM KCl, 20 mM Mops/KOH, 2 MM EDTA, and 1 mM DTT, pH 7.0, at 0 °C.

Amino Acid Composition. Table II shows the amino acid composition of MLCK. The results are based on a molecular weight of 81 000 as determined from $NaDodSO_4$–polyacrylamide gel electrophoresis. The number of cysteine residues was determined by fluorescence titration with 2-(4-maleimidylanilino)naphthalene-6-sulfonic acid (Gupte & Lane, 1979), and the number of tryptophan residues from the molar extinction coefficient assuming E^{1cm}_{278} for tryptophan and tryosine are 5554 and 1260 M^{-1}, respectively (Sober, 1970). The results given here are the average of four different determinations on three different preparations. All hydrolysis were carried out at 110 °C for a single time of 20 h. The overall standard error for these results is estimated to be ±8%.

The amino acid composition of MLCK, listed in Table II, is that of a typical globular protein, with no unusual features except for the high proline content of approximately 9%. A comparison of the composition of MLCK with those of other rabbit skeletal muscle proteins (see Table III) reveals some similarity with myosin light chain-1 (LC_1), but this may only be coincidental. There is some similarity between turkey gizzard and rabbit skeletal muscle MLCK. There is no apparent similarity between MLCK and any of the other muscle proteins described in Table III.

Ultracentrifugation. The sedimentation velocity data showed an s_{20} of 3.2 S in 150 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, and 10 mM Mops/KOH at pH 7.0. From the sedimentation equilibrium data, the quantity $M_W(\partial\rho/\partial C_2)_\mu$ where M_W is the molecular weight and the partial derivative represents the buoyancy term (Banks et al., 1976) is 1.92×10^4 g/mol. The plot of the ln C vs. the square of the radial distance was linear, with slight downward curvature at the outer radius indicating a monomeric species. Stokes' radius R_s can be calculated from this data (Tanford et al., 1974) where

$$R_s = \frac{M_W(\partial\rho/\partial C_2)_\mu}{6\pi\eta N_A s_{20}} \quad (1)$$

and η is the solvent viscosity in centipoise and N_A is Avagadro's number. The units of R_s thus written is in meters. Upon substitution of the appropriate values, R_s becomes 53 Å.

If the buoyancy term is estimated from the amino acid composition (Cohn & Edsall, 1943; McMeekin et al., 1949), the value of $(\partial\rho/\partial C_2)_\mu$ is 0.286, and the weight-average molecular weight becomes 67 000. However, the assumption that the $\bar{v}$ values for each amino acid are additive in the protein has been shown to be invalid (Banks et al., 1976). Assuming a value of $(\partial\rho/\partial C_2)_\mu$ to be 0.260, which assumes an hydration of 0.45 g of water and a $(\partial\rho/\partial C)_{2\mu} = 0.286$ (Cooke & Kuntz, 1974; Kupke, 1973), the molecular weight becomes 75 000, which is in good agreement with the values obtained from $NaDodSO_4$–polyacrylamide gel electrophoresis reported here of M_r 81 000 and the value M_r 77 000 described by Pires & Perry (1977). This term, however, must be independently measured before a valid molecular weight can be determined from any sedimentation equilibrium data.

Affinity of Calmodulin for MLCK. A Scatchard plot of the binding data of [^{3}H]calmodulin to MLCK is shown in Figure 4. The stoichiometry is 1 mol of calmodulin per mol of MLCK with an affinity constant of $(1.9 \pm 0.6) \times 10^7$ M^{-1}. The affinity constant was also estimated by a large zone gel filtration experiment (Ackers, 1973). In this case a column of 2.0-mL total volume of resin was saturated with 1.4 mL of solution containing 2.63 μM MLCK and 0.604 μM [^{3}H]-

Table III: Statistical Comparison of the Amino Acid Compositions of Rabbit Skeletal Muscle MLCK and Other Proteins [a]

	source [b]											
	1	2	3	4	5	6	7	8	9	10	11	12
skeletal muscle MLCK	0	193	140	42	111	88	106	346	178	182	210	69
calmodulin		0	128	155	72	57	167	309	27	223	280	157
myosin heavy chains			0	96	165	86	201	83	119	51	57	101
myosin light chain 1				0	58	61	120	242	133	128	154	48
myosin light chain 2					0	32	104	339	70	188	250	76
myosin light chain 3						0	94	273	45	141	218	62
actin							0	498	164	242	345	84
tropomyosin								0	279	137	87	302
troponin C									0	182	240	155
troponin I										0	52	130
troponin T											0	176
turkey gizzard MLCK												0

[a] Individual differences in mole percent for each amino acid were squared and summed to obtain the tabulated values. Values below 50 indicate possible amino acid sequence homology (Weltman & Dowben, 1973). [b] Source: (1) none; (2) Cohen et al. (1978); (3) J. H. Collins, unpublished results; (4) Frank & Weeds (1974); (5) Lowey & Holt (1972); (6) Frank & Weeds (1974); (7) Collins & Elzinga (1975); (8) Stone & Smillie (1978); (9) Collins et al. (1977); (10) Wilkinson & Grand (1975); (11) Pearlstone et al. (1976); (12) R. S. Adelstein & C. B. Klee, personal communication.

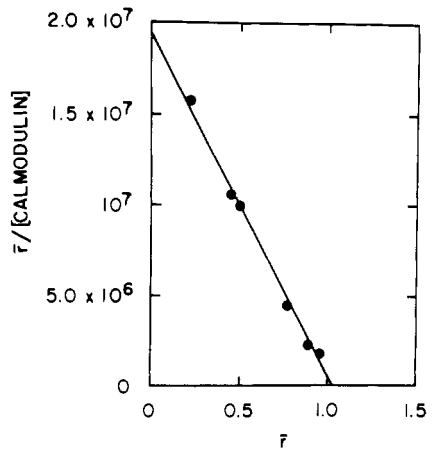

FIGURE 4: Scatchard analysis of calmodulin binding to MLCK showing a binding ratio of one calmodulin per MLCK and an affinity constant of 1.9×10^7 M^{-1}.

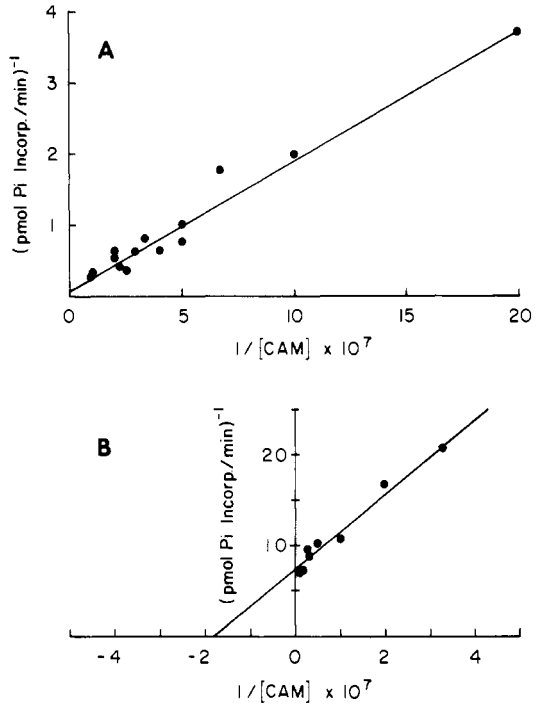

FIGURE 5: Association of calmodulin with MLCK as determined by enzymatic essay. (A) Inverse velocity plotted as a function of inverse calmodulin concentration. The total MLCK concentration was 56.4 nM. The LC_2 concentration was 10 μM, and the ATP and $MgCl_2$ concentrations were 1 and 0.4 mM, respectively. See text for the method of calculating $K_A = 1.6 \times 10^7$ M^{-1}. (B) The same type of plot as in (A) under the same conditions with the enzyme concentration being 2.4 nM. Both MLCK preparations are different, and here $K_A = 1.79 \times 10^7$ M^{-1} is given by the intercept divided by the negative slope of the linear least-squares fit of the data since the MLCK concentration is low (see text).

calmodulin in 150 mM KCl, 10 mM Mops/KOH, 0.5 mM $CaCl_2$, and 0.1 mM DTT, pH 7.0. The K_A measured by this method was estimated to be 1.7×10^7 M^{-1}.

From the calmodulin dependence of the activation of MLCK, using LC_2 phosphorylation to report the fraction of enzyme binding calmodulin as has been done with other enzyme systems (Crouch & Kupke, 1980), the K_A of the calmodulin MLCK interaction was also estimated. In this case, the maximum velocity was estimated (Figure 5) by extrapolating a plot of inverse velocity to infinite calmodulin concentration. The quantity v/v_m (measured velocity divided by the maximum velocity) at a given calmodulin concentration was then related directly to the fraction of total MLCK with

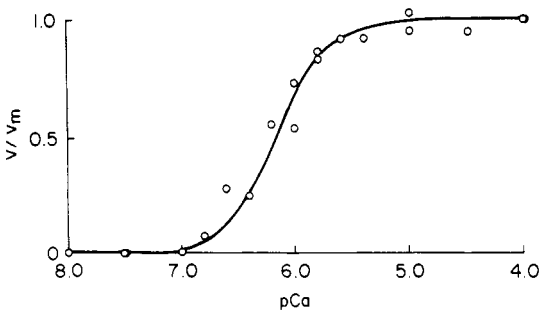

FIGURE 6: Ca^{2+} dependence of MLCK activation. The concentration of MLCK was 7.4 nM, and the calmodulin concentration was 135 nM. For details, see Materials and Methods.

calmodulin bound. Thus, for each data point obtained at MLCK concentrations of 56.4 nM, an association constant was calculated from the relation

$$K_A = \frac{(v/v_m)[MLCK_T]}{[MLCK_T](1 - v/v_m)[CDR_T] - (v/v_m)[MLCK_T]}$$

The calmodulin activation of MLCK was repeated at 2.4 nM MLCK. From the plot of inverse velocity vs. inverse calmodulin concentration, an association constant was obtained by dividing the intercept by the negative slope. This type of treatment was possible since the total MLCK concentration was much lower than that of calmodulin and the quantity $(v/v_m)[MLCK_T]$ is much less than $[CDR_T]$ so that the above relation can be written as

$$\frac{1}{v} = \left(\frac{1}{K_A v_M}\right)\frac{1}{[CDR_T]} + \left(\frac{1}{v_m}\right)$$

In both cases, these data were obtained at LC_2 concentrations of 10 μM. The substrate concentration was kept low since there have been reports that calmodulin influences the affinity of LC_2 (Nairn & Perry, 1979b) for the enzyme and LC_2 could therefore be expected to affect the affinity of calmodulin for the enzyme. The average affinity was thus estimated to be $(1.7 \pm 0.5) \times 10^7$ M^{-1} (see Figure 5).

Calcium Dependence of MLCK Activation. Figure 6 shows the calcium dependence of MLCK activation. The solid line represents the Hill relationship

$$\frac{v}{v_m} = \frac{K[Ca^{2+}]^2}{1 + K[Ca^{2+}]^2}$$

where $K = 2.29 \times 10^{12}$ M^{-2}. These data are not in agreement with those of Pires & Perry (1977) or those of Stull et al. (1980). The reason for disagreement is probably the difference in assay conditions (for example, the Mg^{2+} and/or salt concentrations) and substrate (mixed cardiac light chains) used (Blumenthal & Stull, 1980). The Ca^{2+} dependence of MLCK activation is to the left of the Ca^{2+} binding curves for calmodulin reported by Crouch & Klee (1980), Potter et al. (1977), and Dedman et al. (1977). However, the Ca^{2+} dependence of MLCK activation follows the Ca^{2+} dependence of MLCK tryptophan fluorescence changes observed as calmodulin binds to the enzyme (Johnson et al., 1981) and is consistent with Ca^{2+} binding to any one or two of the four Ca^{2+}-specific sites of calmodulin.

Discussion

Bovine brain calmodulin and rabbit skeletal MLCK can be routinely purified to homogeneity by procedures described here. Several aspects of the method for MLCK purification deserve comment. Gel filtration chromatography of the enzyme prior to affinity chromatography on a calmodulin–Sepharose matrix was required in order to maximize usage of the affinity column

which was subject to proteolytic degradation by contaminating enzymes in the DEAE-cellulose purified MLCK. Prolonged exposure of the calmodulin affinity column to partially purified MLCK preparations in the presence of Ca^{2+} was avoided as much as possible. The affinity column was stored in the presence of 2 mM EDTA and 0.02% NaN_3. Hydroxylapatite chromatography of the MLCK, following affinity chromatography, was required to remove low molecular weight contaminants of the preparation in addition to those of similar molecular weight.

The purified preparation of MLCK was judged homogeneous by the criteria of isoelectric focusing and NaDodSO$_4$–polyacrylamide gel electrophoresis as well as sedimentation velocity and equilibria measurements. MLCK activity and protein isoelectrically focused together with an isoelectric point of 5. NaDodSO$_4$–polyacrylamide gel electrophoresis of the MLCK preparations routinely gave a single protein band of an apparent molecular weight of 80 000–84 000 on 7.5%, 10%, and 12.5% gels. A value estimated by osmotic pressure or ultracentrifugation in conjunction with good density data would thus be highly desirable. In any case the calmodulin binding data, which is based on a MLCK molecular weight of 81 000, tend to confirm a molecular weight between 72 000 and 89 000. A value for the buoyancy term $(\partial \rho/\partial C_2)_\mu$ can be estimated from the sedimentation, equilibrium, and electrophoresis data. This value is 0.24, which corresponds to a V' (Kupke, 1973; Banks et al., 1976) of 0.76 mL/g, which is in the range normally reported for proteins. An apparent Stokes radius for the enzyme can be estimated independently of the buoyancy term by using eq 1 and the value for M_w-$(\partial \rho/\partial C_2)$ obtained from sedimentation equilibrium measurements. The value of 53 Å for the Stokes radius thus determined is greater than the value of 29 Å one would expect for a globular protein of M_r 81 000. This observation could indicate that the molecule is highly asymmetric or that it has a flexible polypeptide chain attached to a single globular structure. In Figure 1 the enzyme activity migrated as a protein of greater than 100 000 daltons on Sephadex G-150; however, in the Hummel–Dryer studies, the enzyme was nearly excluded from the gel (G-150), indicating a much larger molecular size. This change cannot be accounted for simply by the binding of calmodulin. It is interesting that the proline content of the MLCK is approximately 9%. This would indicate that any helical structure would be greatly perturbed, giving rise to the low sedimentation coefficient and large apparent Stokes radius. These aspects of molecular shape and size need further investigation by other hydrodynamic techniques such as viscosity.

An MLCK molecular weight of 81 000 was used to calculate the stoichiometry of calmodulin binding to the enzyme. The extrapolation of the Scatchard plot showing a 1:1 stoichiometry ±10% confirms by direct physical measurement the observations of Nairn & Perry (1979a), Stull et al. (1980), and Yazawa & Yagi (1978).

The association constant measured by both the Hummel–Dryer techniques and enzyme activity showed good agreement. An apparent K_m of 5.6×10^{-8} M of the enzyme for calmodulin at 0.5 mM $CaCl_2$ showed agreement with the data of Yagi et al. (1978). Preliminary experiments indicated that the low Mg^{2+} or the substrate concentrations used in the assays here may be responsible for the higher K_m observed here than by Stull et al. (1980) (Blumenthal & Stull, 1980). Thus, when the enzymes are assumed to be identical, the possibilities for the discrepancy in these data are the differences in Mg^{2+} concentrations, substrate concentrations, and ionic strength. We find the apparent K_m of calmodulin (data not shown) for MLCK at 50 μM LC_2 to be 8 nM. This supports the observation by Nairn & Perry (1979b) that calmodulin enhances the affinity of MLCK for LC_2, because if this observation is true, LC_2 will enhance the affinity of calmodulin for MLCK. Stull has shown (personal communication) that under conditions of higher ionic strength (150 mM KCl), this value is 5 nM. Thus the assay conditions are extremely important, the interaction of calmodulin with MLCK is both LC_2 and ionic strength dependent, and one can expect apparent K_m values for calmodulin interaction with MLCK to vary from 1 to 70 nM.

Another interesting observation is the Ca^{2+} dependence of MLCK activation in the presence of calmodulin. As in other systems we have studied (Potter et al., 1977; Dedman et al., 1977; Piascik et al., 1980; Potter et al., 1980), the Ca^{2+} dependence of the enzymatic activation is clearly to the left (lower Ca^{2+} concentrations) than the direct binding of Ca^{2+} to calmodulin. These data are consistent with Ca^{2+} binding to any one or possibly two of the four Ca^{2+}-specific sites of calmodulin (Potter et al. 1977; Dedman et al., 1977) being responsible for the activation. Other factors such as alterations in the Ca^{2+} affinity of calmodulin upon binding to MLCK or in cooperative Ca^{2+} binding to calmodulin (Crouch & Klee, 1980) may also influence the apparent results.

In conclusion, an association constant of 1.93×10^7 M^{-1} of calmodulin to MLCK is the first such direct measurement of the association of calmodulin to any of its activatable enzymes. The use of the Hummel–Dryer and large zone techniques (Ackers, 1973) and [^{3}H]calmodulin should prove useful in the study of other such systems where one subunit of an associating system is much smaller than the other and the two interact in a reversible manner. There is no reason that the specific activity of the [^{3}H]calmodulin cannot be increased by using the labeling technique outlined here, where systems with association constants on the order of 10^9–10^{10} M^{-1} can be readily studied.

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