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Fluorescence Studies of the Interaction of Calmodulin with Myosin Light Chain Kinase*

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The interaction of calmodulin with myosin light chain kinase produces an ~30% increase in myosin light chain kinase tryptophan fluorescence. This represents the first report of calmodulin-induced structural changes in a protein which it activates. We find that the calmodulin-myosin light chain kinase interaction is: 1) dependent on [Ca^{2+}] (half-maximal binding at pCa 6.2) and essentially independent of [Mg^{2+}], 2) occurs before saturation of all four reported Ca^{2+}-specific sites on calmodulin. 3) saturates with 1 mol of calmodulin bound per mol of kinase with an apparent affinity of ~$2.0 \times 10^7 M^{-1}$, 4) is specific for calmodulin over troponin-C, 5) is directly related to the activation of myosin light chain kinase for phosphorylation of myosin light chain. Fluorescence stopped flow studies of these calmodulin-induced fluorescence changes in myosin light chain kinase indicate that Ca^{2+} binding to calmodulin occurs very rapidly and is not rate-limiting while the calmodulin-induced fluorescence increase in myosin light chain kinase occurs as a biphasic process with rates of ~65 s^{-1} and 6 s^{-1}. The fluorescence increase produced by calmodulin binding to myosin light chain kinase is completely reversed by ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid at a rate of ~2 s^{-1}.

The Ca^{2+} binding protein calmodulin bestows Ca^{2+} sensitivity and regulates many enzymatic processes in both muscle and non-muscle systems (for review, see Refs. 1-4). Its function in smooth muscle is quite well understood. Ca^{2+} binds to calmodulin which then binds to and activates myosin light chain kinase. This activated kinase phosphorylates the P light chain of myosin, allowing the interaction of actin and phosphomyosin, myosin ATPase activity, and contraction (see Ref. 5). During relaxation, [Ca^{2+}] is lowered and the kinase is inactivated allowing Ca^{2+}-independent phosphatases to dephosphorylate myosin, decreasing its capacity for interaction with actin.

Even though phosphomyosin was first discovered in skeletal muscle, less is known concerning the role of myosin phosphorylation in thin filament-regulated skeletal muscle contraction. While Morgan *et al.* (6) have reported that myosin phosphorylation has no effect on the actomyosin ATPase activity of skeletal muscle, Pemrick (7) has presented evidence (using a different species of myosin) that phosphorylation of the P light chain of myosin may increase actomyosin ATPase activity. In addition, it has been demonstrated that activation of muscle results in an increase in the phosphate content of myosin (8, 9). Both calmodulin (10, 11) and myosin light chain kinase (12-14) have been isolated from skeletal muscle and it has been reported that in the presence of Ca^{2+}, calmodulin complexes with myosin light chain kinase to increase its affinity for the P light chain of myosin (15).

In this report, we present evidence for a Ca^{2+}-dependent calmodulin-induced increase in myosin light chain kinase intrinsic tryptophan fluorescence. This fluorescence increase in myosin light chain kinase provides an accurate means of monitoring the CDR-MLCK[1] interaction and has allowed us to characterize the Ca^{2+} dependence, stoichiometry, affinity, and specificity of the CDR-MLCK interaction as well as the rates of the structural changes in myosin light chain kinase associated with the interaction of these proteins.

MATERIALS AND METHODS

Fluorescence studies were conducted on a Perkin-Elmer MPF 44A or 650-10S ratio recording spectrofluorometer operated in the ratio mode. Temperature was maintained at 23 °C with a Lauda K2R circulating water bath. Tryptophan emission was monitored at 340 nm with an excitation wavelength of 285 nm. Ca^{2+} titrations were conducted in a 2 mM EGTA, 10 mM Mops, 150 mM KCl buffer and free Ca^{2+} was calculated as described previously (16).

Fluorescence stopped flow studies were conducted on a Durrum D110 stopped flow spectrophotometer equipped with a fluorescence accessory. Excitation was at 285 nm and the tryptophan emission was monitored through a 340 nm narrow bandpass interference filter. The fluorescence signals were recorded on a Nicolet storage oscilloscope, digitized, and then transmitted to a Modcomp Computer for signal averaging, base line subtraction, logarithmic analysis, and plotting. In the fluorescence stopped flow experiments, equal volumes of two solutions, one in reaction syringe A and the other in syringe B, were rapidly mixed. All solutions contained 10 mM Mops, 150 mM KCl, and 0.1 mM dithiothreitol. In addition for reaction 1 (MLCK + CDR mixing with Ca^{2+}), syringe A contained 1 μM MLCK, 2.5 μM CDR, 0.15 mM EGTA, pH 7.0, and syringe B contained 1 mM $CaCl_2$, pH 7.0; for reaction 2 (MLCK + Ca^{2+} mixing with CDR + Ca^{2+}), syringe A contained 1.6 μM MLCK, 2 mM EGTA, 3 mM $CaCl_2$, pH 7.0, and syringe B contained 4 μM CDR, 2 mM EGTA, 3 mM $CaCl_2$, pH 7.0; for reaction 3 (MLCK·CDR·Ca^{2+} mixing with EGTA), syringe A contained 1 μM MLCK, 2.5 μM CDR, 0.155 mM EGTA, 1 mM $CaCl_2$, pH 7.0, and syringe B contained 28 mM EGTA, pH 7.26. When syringe A and B were mixed in all cases the final pH was 7.0.

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[1] The abbreviations used are: MLCK, skeletal muscle myosin light chain kinase; CDR, calmodulin; S-TnC, skeletal troponin-C; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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Calmodulin was purified from bovine brain by a modification of the method of Klee (17) as described elsewhere.[2] Skeletal myosin light chain kinase was purified from rabbit fast skeletal muscle by modification of the procedure of Yazawa and Yagi (13) to be described elsewhere.[2] Both proteins were judged to be of greater than 99% purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined by the biuret method (18) and the method of Lowry (19) standardized with bovine serum albumin. The concentrations of CDR and MLCK were determined from protein nitrogens by micro-Kjeldahl analysis and then used to calibrate the biuret and Lowry procedures. For both calmodulin and kinase all methods were within 5% of agreement.

RESULTS

Calmodulin-induced Fluorescence Changes in Myosin Light Chain Kinase—The tryptophan residues of MLCK serve as intrinsic fluorescence reporter groups for the interaction of the Ca^{2+}-dependent regulator protein, calmodulin, with myosin light chain kinase. The binding of calmodulin (which has no tryptophan residues) to myosin light chain kinase produces a 30% increase in the fluorescence intensity and a 8 nm blue shift (from 338 nm to 330 nm) in the wavelength of the emission maxima of myosin light chain kinase tryptophan fluorescence (see *inset* to Fig. 1). Fig. 1 shows the calmodulin dependence of this increase in kinase fluorescence at 340 nm in the presence of pCa 3.0. This fluorescence increase levels off with the addition of ~1.0 mol of CDR/mol of MLCK. It is strictly dependent upon Ca^{2+} and fully reversible with EGTA. In the absence of Ca^{2+}, additions of CDR, even at 5-fold molar excess, produces no increase in kinase tryptophan fluorescence. In the absence of Ca^{2+}, the CDR-induced increase in kinase fluorescence is not produced by 3 mM $MgCl_2$ and in the presence of Ca^{2+}, the CDR dependence of the increase in kinase fluorescence is not influenced by 3 mM $MgCl_2$. These CDR-induced fluorescence increases in myosin light chain kinase are specific for the CDR-MLCK interaction since skeletal troponin-C, which is quite homologous to CDR (2), will not increase kinase tryptophan fluorescence even at 5-fold molar excess over kinase in the presence (pCa = 3.0) or absence (pCa = 10.0) of Ca^{2+} (data not shown). Thus, the interaction of CDR with myosin light chain kinase is dependent upon Ca^{2+}, independent of Mg^{2+}, and may be accurately followed by these changes in kinase tryptophan fluorescence. A good fit of these data is provided by the theoretical curve in Fig. 1 generated by assuming that the fluorescence increase in myosin light chain kinase occurs as the result of a 1:1 complex formation between CDR and kinase with an association constant of $\sim 2.6 \pm 1.8 \times 10^7$ M^{-1}. Titrations of lower concentrations of enzyme (1×10^{-7} M and 4×10^{-7} M) with calmodulin produced similar increases in kinase tryptophan fluorescence which leveled near 1:1 molar ratios of calmodulin to kinase. Association constants of $1.63 \pm 0.5 \times 10^7$ M^{-1} and $1.54 \pm 0.7 \times 10^7$ M^{-1} were determined at 1×10^{-7} M and 4×10^{-7} M kinase, respectively. Thus, over the range of 1×10^{-7} M to 8×10^{-7} M myosin light chain kinase, we determined an association constant of $\sim 2.0 \times 10^7$ M^{-1} for the calmodulin·MLCK complex.

The Ca^{2+} Dependence of the Calmodulin-Myosin Light Chain Kinase Interaction—The Ca^{2+} dependence of the CDR-induced fluorescence increase in myosin light chain kinase is shown in Fig. 2. In these experiments, kinase in the presence of 2-fold molar excess of CDR was titrated with Ca^{2+}. The full 30% increase in kinase tryptophan fluorescence characteristic of CDR binding occurs with a midpoint at pCa 6.25. In the presence of 3 mM $MgCl_2$, the same fluorescence increase occurs at pCa ~ 6.05. In the absence of CDR, myosin light chain kinase tryptophan fluorescence is not Ca^{2+}-dependent. These studies suggest that Ca^{2+} binding to Ca^{2+}-specific site(s) on calmodulin are responsible for its binding to myosin light chain kinase and the subsequent increases in kinase tryptophan fluorescence.

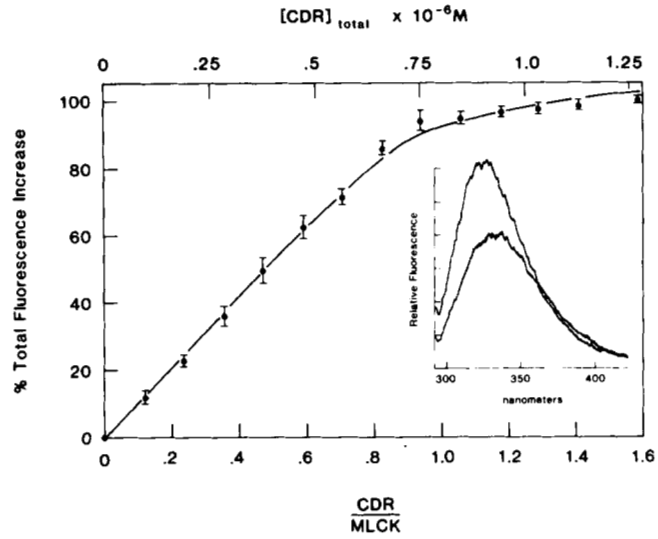

FIG. 1. **The effect of calmodulin on myosin light chain kinase tryptophan fluorescence.** Successive additions of CDR (1.43×10^{-4} M) were added to 3 ml total volume of 8×10^{-7} M kinase and the fluorescence increase at 340 nm was recorded. The per cent of the total (30%) fluorescence increase is plotted as a function of the molar ratio of [CDR] to [MLCK] and as a function of [CDR] total. Both proteins were in 10 mM Mops, 150 mM KCl, 2 mM EGTA, 3 mM $CaCl_2$ (pCa 3.0), 0.1 mM dithiothreitol at pH 7.0. The *inset* shows the fluorescence spectra of MLCK with no added CDR (*lower trace*) and with 1.6-fold molar excess of CDR to kinase. Excitation was at 285 nm. These data represent the mean of four titrations. The theoretical curve (——) was generated assuming that the fluorescence increase occurs as the result of a 1:1 interaction between CDR and kinase with a K_a of $2.6 \pm 1.8 \times 10^7$ M^{-1}. The maximal fluorescence was determined from double reciprocal plots of the fluorescence increase as a function of [CDR]. The fractional fluorescence change is assumed to be directly proportional to the ratio of [CDR·MLCK] complex to total [MLCK] and for each point a value for K_A can be calculated from the relation

$$K_A = \frac{[\text{CDR}\cdot\text{MLCK}]}{([\text{CDR}_T] - [\text{CDR}\cdot\text{MLCK}])([\text{MLCK}_T] - [\text{CDR}\cdot\text{MLCK}])}$$

and averaged.

The relationship between the calmodulin-induced increase in myosin light chain kinase fluorescence and the rate of phosphorylation of myosin light chains is shown as an *inset* in Fig. 2. It is clear that there is a direct linear relationship (r = 0.98) between calmodulin binding to myosin light chain kinase and the phosphorylation of myosin light chain.

Fluorescence Stopped Flow Studies of the Rate of the Calmodulin-induced Fluorescence Changes in Myosin Light Chain Kinase—The kinetics of the Ca^{2+}-regulated interactions of CDR and myosin light chain kinase may be followed using the increase in kinase tryptophan fluorescence which is characteristic of this interaction and stopped flow fluorometry. Fig. 3 shows the time course of the fluorescence increase that occurs when a Ca^{2+}-free MLCK-CDR solution (2.5 mol of CDR/mol of MLCK) is rapidly mixed with 1 mM [Ca^{2+}] (Reaction 1). In this kinetic trace we observe ~90% of the total 30% increase in kinase tryptophan fluorescence which occurs at steady state. This fluorescence increase is 90% complete with ~175 ms and is clearly not single exponential. *Inset A* to Fig. 3 shows a log plot of this fluorescence increase as a function of time. Approximately 35% of the total observed fluorescence increase occurs very rapidly with a rate of ≥51

[2] T. H. Crouch, M. J. Holroyde, J. H. Collins, R. J. Solaro, and J. D. Potter (1981) *Biochemistry*, in press.

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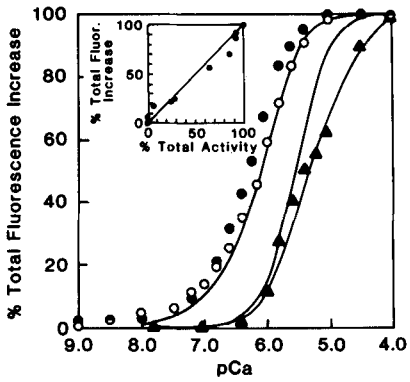

FIG. 2. **The Ca^{2+} dependence of the CDR-induced increase in myosin light chain kinase tryptophan fluorescence.** The per cent of the total (30%) increase in kinase fluorescence is plotted as a function of pCa in the absence (●) and presence (○) of 3 mM $MgCl_2$. A 3.0-ml solution of 8×10^{-7} M MLCK, 1.6×10^{-6} M CDR, 10 mM Mops, 150 mM KCl, 2 mM EGTA, 0.1 mM dithiothreitol at pH 7.0 was titrated with $CaCl_2$ as described under "Materials and Methods." Two theoretical curves were generated assuming that Ca^{2+} binding to any one (P_1, *solid curve on left*) or any two (P_2, *solid curve in middle*) of the four Ca^{2+} specific sites ($K_{Ca} = 2.2 \times 10^5$ M^{-1}) of CDR is responsible for complex formation. They were calculated from the relationship $P_1 = 1 - (1 - y)^4$ and $P_2 = 3y^4 - 8y^3 + 6y^2$, respectively, as a function of pCa where y is the fractional saturation of the Ca^{2+} sites at specific pCa: $y = K_{Ca}[Ca^{2+}]/1 + K_{Ca}[Ca^{2+}]$, and $K_{Ca} = 2.2 \times 10^5$ M^{-1} from the Ca^{2+} binding data (▲). Hill plot analysis of these data, with or without 3 mM Mg^{2+}, gave average Hill coefficients of 1.8 and 1.7, respectively. The *inset* shows the relationship of the calmodulin-induced increase in myosin light chain kinase tryptophan fluorescence to the phosphorylation of myosin light chains. The per cent of the total 30% increase in kinase tryptophan fluorescence produced by calmodulin binding as a function of pCa (data taken from Fig. 3, $+Mg^{2+}$) is plotted *versus* the per cent of the total increase in the rate of phosphorylation of myosin light chains as a function of pCa^{2+}. Preparation of myosin light chains and the Ca^{2+}·calmodulin·kinase-dependent incorporation of ^{32}P into myosin light chains are as described in Crouch *et al.*[2] The total increase in the rate of phosphorylation was from 0 to 6.2 μmol of P_i incorporated per mg per min. For the Ca^{2+} dependence of MLCK, each assay contained the following: 100 μM decalcified myosin light chain, 150 mM Mops/KOH buffer, pH 7.0, containing [γ-^{32}P] ATP, 2 mM $MgCl_2$, 150 mM KCl, 2 mM EGTA, and 0.5 mM dithiothreitol (total 100 μl). $CaCl_2$ was added to each assay to obtain the desired free Ca^{2+} concentration.

The per cent of the total increase in Ca^{2+} binding to bovine calmodulin as a function of pCa is shown (▲). Ca^{2+} binding was performed as described previously (22) except in a 10 mM Mops, 150 mM KCl, 0.1 mM EGTA, 3 mM $MgCl_2$, pH 7.0, buffer. Saturation occurred at 3.8 mol of Ca^{2+}/mol of CDR.

s^{-1} from Fig. 3. When the rapid phase of this fluorescence increase is examined during a shorter time window, only the rapid phase is observed. This rapid phase occurs as a single exponential increase with a rate of 65–70 s^{-1} (Fig. 3, *inset B*). The final 30% of the total fluorescence increase occurs much more slowly at a rate of ~5–7 s^{-1} (see Fig. 3, *inset A*).

If kinase (1.6 μM) at pCa 3.0 is rapidly mixed with a 2.5-fold molar excess of CDR at pCa 3.0 (Reaction 2), a ~30% increase in kinase tryptophan fluorescence is again observed. The kinetics of this fluorescence increase is essentially identical with those of Reaction 1. Approximately 35% of the fluorescence increase occurs with a rate of ~65–70 s^{-1} and the final 30% of the increase occurs much more slowly, with a rate of 5–7 s^{-1}. Thus whether MLCK + Ca^{2+} is rapidly mixed with CDR + Ca^{2+} or MLCK + CDR is rapidly mixed with Ca^{2+}, the fluorescence changes (and their rates) which occur with CDR binding to myosin light chain kinase are identical.

When a solution of kinase (1.6 μM, pCa 3.0) was rapidly mixed with less than equimolar (0.8 μM) CDR at pCa 3.0, a smaller (~13%) fluorescence increase was observed. This fluorescence increase was again biphasic with the same rate constants for the rapid and slow phases of the increase as reported above with higher [CDR]. When the calmodulin concentration was held constant (2 μM) and mixed with varying concentrations of kinase (1.6 μM, 0.8 μM, or 0.2 μM), neither the rates nor the extent of the observed 30% fluorescence increase was affected.

Thus, 5-fold variations in calmodulin concentration and 8-fold variations in myosin light chain kinase concentrations did not affect the rates of this calmodulin-induced fluorescence increase in myosin light chain kinase. This suggests that we are observing two first order reactions presumably resulting from relatively slow structural changes about the tryptophan residues of myosin light chain kinase which occur after calmodulin binds. When Ca^{2+} free kinase is rapidly mixed with CDR in the absence of Ca^{2+}, no fluorescence changes are observed.

If MLCK·CDR in the presence of Ca^{2+} is rapidly mixed with EGTA (Reaction 3), the MLCK-CDR complex dissociates and we observe a 30% decrease in fluorescence. A kinetic trace of this fluorescence decrease is shown in Fig. 4. The full 30% decrease is observed in this kinetic trace and it is complete in ~2 s. The *inset* of Fig. 4 shows a semilog plog of this decrease. It is clear that the fluorescence decays as a single exponential process with a rate of ~2 s^{-1}.

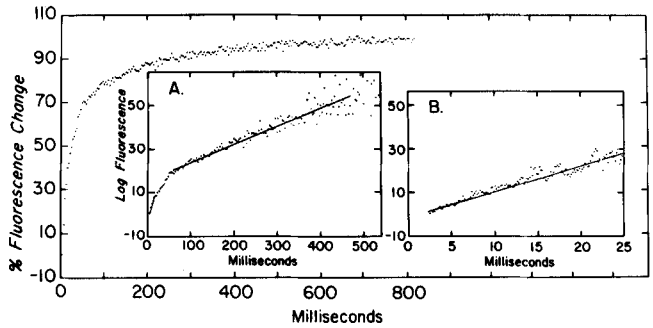

FIG. 3. **A kinetic stopped flow trace of the increase in myosin light chain kinase tryptophan fluorescence produced by the binding of CDR.** Reaction conditions are as described in the text. Excitation was at 285 and the emission was monitored through a 340 nm narrow bandpass interference filter. *Inset A* shows a log plot of this fluorescence increase as a function of time between 2.4 ms and 500 ms. *Inset B* shows a log plot of the more rapid phase of the increase over the time window 2.4 to 25 ms.

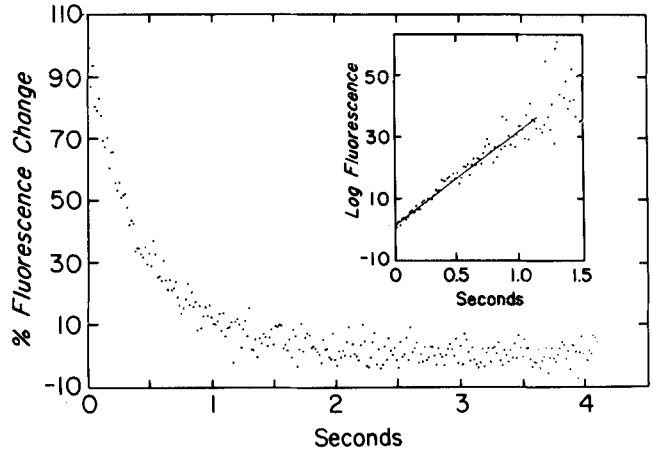

FIG. 4. **A kinetic trace of the decrease in myosin light chain kinase fluorescence produced by the EGTA-induced removal of CDR from kinase.** Reaction conditions are described in the text. The *inset* shows a log plot of this fluorescence decrease as a function of time.

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DISCUSSION

The enhancement in myosin light chain kinase tryptophan fluorescence with the binding of CDR provides an accurate and facile means of monitoring and characterizing the interaction of CDR with myosin light chain kinase. This fluorescence increase saturates near 1 mol of CDR per mol of kinase suggesting a 1:1 complex formation with a K_a of $\sim 2.0 \times 10^7$ M^{-1}. This study is in good agreement with the results of Yagi and Yazawa (10) and Nairn and Perry (11) who show ~90% activation of myosin light chain kinase at 1:1 molar ratios of CDR to kinase and the column chromatography studies of Nairn and Perry (15) showing that in the presence of Ca^{2+}, CDR:MLCK elutes as a 1:1 complex. The value that we obtain for the association constant of CDR for myosin light chain kinase is in excellent agreement with the value of 2×10^7 M^{-1} obtained recently from direct binding studies.[2] In addition, these studies attest to the specificity of the CDR-MLCK interaction. Skeletal troponin-C is highly homologous to calmodulin in its primary structure (20) and Ca^{2+} binding properties, yet S-TnC cannot interact with myosin light chain kinase to produce these fluorescence changes, even at 5-fold molar excess. Moreover, S-TnC does not activate myosin light chain kinase for the phosphorylation of myosin P light chain (10, 11).

This calmodulin-induced fluorescence increase in myosin light chain kinase allowed us to determine the Ca^{2+} dependence of the CDR-MLCK interaction. Their interaction was strictly Ca^{2+}-dependent being half-maximal at pCa 6.25. $MgCl_2$ could not induce complex formation and only produced a small shift (to pCa 6.05) in the Ca^{2+} dependence of these interactions. This is in good agreement with the observation that CDR has four Ca^{2+}-specific sites which do not bind Mg^{2+} competitively (21, 22). Further, Mg^{2+} cannot elicit the increase in α-helix or CDR tyrosine fluorescence (22) nor the calmodulin activation of MLCK (10, 11).

Fig. 2 also shows Ca^{2+} binding to CDR under the same conditions used to measure the Ca^{2+} dependence of the interaction of CDR with kinase. It is clear that the Ca^{2+} dependence of the CDR-MLCK interaction is shifted to higher pCa values than the actual CDR Ca^{2+} binding curve, which shows a midpoint near pCa 5.35. A very good fit to the Ca^{2+} dependence of the CDR-MLCK fluorescence change is obtained by assuming that Ca^{2+} binding to any one of four identical Ca^{2+} specific sites ($K_{Ca} = 2.2 \times 10^5$ M^{-1}) is responsible for complex formation. An average Hill coefficient of 1.8 was obtained when the data of Fig. 2 were analyzed by Hill plots.

Although alternative interpretations of these data are possible, both of these methods of analysis, with their inherent assumptions (Fig. 2 legend), would suggest that the initial steps in Ca^{2+} binding (*i.e.* Ca^{2+} binding to one of the four identical sites on calmodulin) are sufficient for CDR-MLCK complex formation. In the past, we (22) and others (23) have reported Ca^{2+}-induced structural changes in calmodulin which occur at higher pCa than the Ca^{2+} binding curve.

Pires and Perry (12) have studied the effect of Ca^{2+} on myosin light chain kinase activity and ATPase activity. They find half-maximal activation of each (in 6.25 mM $MgCl_2$) near pCa 6.1. Their activity curves as a function of pCa are very similar to the curves we present in Fig. 2 which show that the interaction of CDR with kinase (as monitored by tryptophan fluorescence) is half-maximal at pCa 6.05 in the presence of $MgCl_2$. Further, using the same conditions reported here for the CDR-MLCK interaction we have shown an almost identical pCa-MLCK activation curve.[2] The midpoint of this activation occurs at pCa 6.1 in the presence of 3 mM Mg^{2+}. The strong correlation between calmodulin-MLCK complex formation and the phosphorylation of myosin light chain suggest that the interaction of CDR with kinase is directly related to enzyme activation. Apparently, Ca^{2+} binds to CDR producing structural changes in CDR which facilitate its interaction with myosin light chain kinase. CDR binding to kinase, in turn, produces structural changes in the kinase altering the environment of some of its tryptophan residues. These Ca^{2+}-calmodulin-induced structural changes presumably activate myosin light chain kinase for the phosphorylation of the P light chains of myosin.

Fluorescence stopped flow studies provide an indication of the rate of these CDR-induced structural changes in myosin light chain kinase. Ca^{2+} binds to CDR, CDR binds to kinase, and the structural changes about the tryptophan residues of the kinase occur to produce the observed fluorescence increase as represented below:

$$Ca^{2+} + CDR \rightleftharpoons Ca^{2+}\cdot CDR \rightleftharpoons Ca^{2+}\cdot CDR^* \quad \text{(A)}$$

$$Ca^{2+}\cdot CDR^* + MLCK \rightleftharpoons Ca^{2+}\cdot CDR^*\cdot MLCK \rightleftharpoons Ca^{2+}\cdot CDR^*\cdot MLCK^* \quad \text{(B)}$$

where $Ca^{2+}\cdot CDR^*\cdot MLCK^*$ is the complex with the increased tryptophan fluorescence and $Ca^{2+}\cdot CDR^*$ is the $Ca^{2+}\cdot CDR$ complex which is activated for binding myosin light chain kinase. This reaction occurs when Ca^{2+} is rapidly mixed with calmodulin in the presence of kinase. The observed fluorescence increase has two components. The first component comprises ~35% of the total increase with a rate of 60–70 s^{-1} while the final 30% of the increase occurs more slowly with a rate of 5–7 s^{-1}. When Ca^{2+} + calmodulin is rapidly mixed with kinase + Ca^{2+}, we observe the same fluorescence increase with the same kinetics as above. This indicates that the A part of this reaction sequence is not rate-limiting and must occur faster than 70 s^{-1}. This is consistent with the observation that CDR is similar to S-TnC (24) in that it can bind Ca^{2+} and undergo structural changes within a submillisecond time range.[3]

The kinetic processes observed may be attributed, therefore, to some components of part B in the reaction scheme: $Ca^{2+}\cdot CDR^* + MLCK \rightleftharpoons Ca^{2+}\cdot CDR^*\cdot MLCK \rightleftharpoons Ca^{2+}\cdot CDR^*\cdot MLCK^*$. Because the kinetics of this reaction did not vary when [CDR] was varied over a 5-fold range (at 1.6 μM MLCK) or when [MLCK] was varied over an 8-fold range (at 2 μM CDR), the observed rates are most likely first order[4] and associated with the rates of structural changes in myosin light chain kinase about several of its tryptophan residues. Thus, from these "on-reactions" we conclude Ca^{2+} can bind to and activate CDR and this activated CDR can bind to kinase very rapidly ($\geq$70 s^{-1}). The subsequent structural changes in myosin light chain kinase are not uniform with respect to time, however, some occur quite rapidly (60–70 s^{-1}) and others more slowly (5–7 s^{-1}). The exact role of these rapid and slow structural changes in the binding of myosin light chain kinase to P-light chain and its phosphorylation are as yet unclear.

Ca^{2+} removal from the $Ca^{2+}\cdot CDR^*\cdot MLCK^*$ complex with

[3] Calmodulin labeled with the fluorescent probe dansylaziridine by the same procedure as we have reported for S-TnC (16) will bind Ca^{2+} and undergo a greater than 2-fold, Ca^{2+}-specific, fluorescence increase within the 2.4-ms mixing time of our Durrum stopped flow instrument (J. D. Johnson *et al.*, unpublished results).

[4] The rates of these fluorescence changes are independent of protein concentration over the range tested and therefore presumably result from structural changes in myosin light chain kinase which occur after CDR·MLCK complex formation. If adequate signal to noise could be obtained at lower concentrations of CDR and kinase, then these rates could be reduced at lower reactant concentrations because they also depend on the rate of CDR binding to kinase.

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EGTA results in a complete reversal of these increases in kinase fluorescence at a rate of ~2 s^{-1}. This is presumably too slow to be the rate of Ca^{2+} removal from CDR[5] and could represent a slower structural change associated with Ca^{2+} removal from CDR or CDR removal from the kinase. Again, it is difficult to assign this kinetic process to any specific step in the deactivation of kinase or the dissociation of the Ca^{2+}·CDR*·MLCK* complex. At present we can only say that Ca^{2+} removal from Ca^{2+}·CDR*·MLCK* produces structural changes in myosin light chain kinase which occur at least as fast as 2 s^{-1}. These structural changes appear to be directly related to the activation of kinase and yet they occur and are reversed at a relatively slow rate compared to the activation and relaxation of muscle. This would indicate that phosphorylation of myosin by this mechanism may be too slow to be directly involved in the contraction-relaxation cycle of skeletal muscle. They would occur sufficiently fast to be involved in post-tenatic potentiation. Manning and Stull have suggested a role for myosin phosphorylation in this latter process (25).

Finally, this calmodulin-induced increase in the tryptophan fluorescence of its receptor protein (myosin light chain kinase) has provided us with a very convenient means of monitoring their interaction and relating it to the activation of this kinase.

It is now becoming clear that calmodulin regulates a wide variety of cellular functions by binding to specific receptor proteins in a Ca^{2+}-dependent manner (1-4). If calmodulin produces similar changes in the tryptophan fluorescence of its various receptors, then these fluorescence changes may provide a valuable means of monitoring calmodulin's interactions and regulation of its various receptor proteins.

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[5] Assuming a diffusion-limited "on rate" for Ca^{2+} of 1×10^8 $M^{-1} \cdot s^{-1}$ and using the equilibrium binding constant of Ca^{2+} to CDR of 4×10^5 M^{-1} (22), we calculate the "off rate" of Ca^{2+} from CDR to be ~250 s^{-1}. This is similar to the off-rate of Ca^{2+} from S-TnC that we have reported (24).

REFERENCES

1. Marx, J. L. (1980) *Science* **208,** 274–276
2. Cheung, W. Y. (1980) *Science* **207,** 19–27
3. Means, A. R., and Dedman, J. R. (1980) *Nature* **285,** 73–77
4. Klee, C., and Crouch, T. H. (1981) *Annu. Rev. Biochem.* **49,** 489–515
5. Adelstein, R. S. (1978) *Trends Biochem. Sci.* **3,** 27–29
6. Morgan, M., Perry, S. V., and Ottaway, J. (1976) *Biochem. J.* **157,** 687–697
7. Pemrick, S. M. (1981) *J. Biol. Chem.* **255,** 8836–8841
8. Barany, K., and Barany, M. (1977) *J. Biol. Chem.* **252,** 4752–4754
9. Stull, J. T., and High, C. W. (1977) *Biochem. Biophys. Res. Commun.* **77,** 1078–1083
10. Yagi, K., and Yazawa, M. (1978) *J. Biol. Chem.* **253,** 1338–1340
11. Nairn, A. C., and Perry, S. V. (1979) *Biochem. J.* **179,** 89–97
12. Pires, E., Perry, S. V., and Thomas, M. A. W. (1974) *FEBS Lett.* **41,** 292–296
13. Yazawa, M., and Yagi, K. (1977) *Biochem. J.* **82,** 287–289
14. Pires, E., and Perry, S. V. (1977) *Biochem. J.* **167,** 137–146
15. Nairn, A. C., and Perry, S. V. (1979) *Biochem. Soc. Trans.* **7,** 966–977
16. Johnson, J. D., Collins, J. H., and Potter, J. D. (1978) *J. Biol. Chem.* **253,** 6451–6458
17. Klee, C. B. (1977) *Biochemistry* **16,** 1017–1024
18. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **191,** 751–766
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
20. Dedman, J. R., Jackson, R. L., Schreiber, W. E., and Means, A. R. (1978) *J. Biol. Chem.* **253,** 343–346
21. Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L., and Means, A. R. (1977) in *Calcium-Binding Proteins and Cell Function* (Wasserman, R. H., *et al.*, eds) Elsevier/North Holland, Amsterdam
22. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., and Means, A. R. (1977) *J. Biol. Chem.* **252,** 8415–8522
23. Wolff, D. J., Poirier, P. G., Brostrom, C. O., and Brostrom, M. A. (1977) *J. Biol. Chem.* **252,** 4108–4117
24. Johnson, J. D., Charlton, S. C., and Potter, J. D. (1979) *J. Biol. Chem.* **254,** 3497–3502
25. Manning, D. R., and Stull, J. T. (1979) *Biochem. Biophys. Res. Commun.* 164–170

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