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Purification of Myosin Light Chain Kinase from *Limulus* Muscle†

J. R. Sellers* and E. V. Harvey

ABSTRACT: It has previously been shown that the regulatory light chains of myosin from *Limulus*, the horseshoe crab, can be phosphorylated either by purified turkey gizzard smooth muscle myosin light chain (MLC) kinase or by a crude kinase fraction prepared from *Limulus* muscle [Sellers, J. R. (1981) *J. Biol. Chem. 256*, 9274–9278]. This phosphorylation was shown to be associated with a 20-fold increase in the actin-activated MgATPase activity of the myosin. We have now purified the Ca^{2+}–calmodulin-dependent MLC kinase from *Limulus* muscle to near homogeneity by using a combination of low ionic strength extraction, ammonium sulfate fractionation, and chromatography on Sephacryl S-300 and DEAE-Sephacel. The final purification was achieved by affinity chromatography on a calmodulin–Sepharose 4B column. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis showed 95% of the protein to be comprised of a doublet with M_r = 39 000 and 37 000. Electrophoresis of the kinase fraction under nondenaturing conditions resulted in a partial separation of the two major bands and demonstrated that each had catalytic activity. An SDS–polyacrylamide gel overlayed with ^{125}I-calmodulin demonstrated that both the M_r 39K and the M_r 37K proteins bind calmodulin. Neither of the bands could be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. With *Limulus* myosin light chains as a substrate, the V_{max} was 15.4 μmol min^{-1} mg^{-1}, and the K_m was 15.6 μM. The K_D for calmodulin was determined to be 6 nM. The enzyme did not phosphorylate histones, casein, actin, or tropomyosin.

Although the contraction of all types of muscle seems to be regulated by the free calcium level in the sarcoplasm, the site of direct calcium action is variable (Adelstein & Eisenberg, 1980). There are three distinct calcium regulatory systems which have been described: (1) Thin filament linked regulation works via the troponin–tropomyosin system in which a subunit of troponin reversibly binds calcium ions (Adelstein & Eisenberg, 1980). This appears to be the dominant or sole regulatory system in vertebrate skeletal muscle. (2) In certain invertebrate muscles, notably those of the mollusks, there is a myosin-linked regulation in which the myosin itself is directly regulated by reversible calcium binding (Szent-Györgyi et al., 1973; Chantler et al., 1981). In the absence of calcium, this type of myosin has a low actin-activated MgATPase activity, but when calcium is bound, the MgATPase activity of the myosin is greatly enhanced by actin (Szent-Györgyi et al., 1973). (3) Studies of scallop myosin have established that this type of regulation is mediated by a particular class of myosin

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light chains, termed regulatory light chains (Kendrick-Jones et al., 1976; Chantler & Szent-Györgyi, 1980; Sellers et al., 1980). The third regulatory system to be described is a myosin-linked regulation in which the MgATPase activity of myosin is controlled by calcium-dependent phosphorylation of the regulatory light chains. This type of system was first described for myosin from human platelets and from vertebrate smooth muscle (Adelstein & Conti, 1975; Sobieszek & Small, 1977; Chacko et al., 1977; Sherry et al., 1978; Ikebe et al., 1978; Sellers et al., 1981). In these systems, the enzyme responsible for phosphorylation, myosin light chain (MLC)[1] kinase, has been shown to be calcium and calmodulin dependent and is very specific for myosin or isolated myosin light chains (Dabrowska et al., 1977; Adelstein & Klee, 1981). Phosphatases isolated from smooth muscle can dephosphorylate the myosin and return it to a low activity state (Sellers et al., 1981; Pato & Adelstein, 1980).

Myosin from the striated muscle of *Limulus*, the horseshoe crab, is also regulated by calcium–calmodulin-dependent phosphorylation of its regulatory light chains (Sellers, 1981). This regulation apparently works in concert with troponin–tropomyosin-dependent thin filament linked regulation (Lehman, 1975). In the initial study of *Limulus* myosin phosphorylation, an impure kinase fraction was obtained which phosphorylated the regulatory light chains of the myosin (Sellers, 1981). We have now purified the kinase responsible for this phosphorylation to near homogeneity and have characterized its enzymatic properties. This is the first purification of a myosin light chain kinase from an invertebrate muscle. A preliminary account of this work has been reported (Sellers et al., 1984).

Materials and Methods

Preparation of MLC Kinase. Live *Limulus* were purchased from Gulf Specimens (Panacea, FL). All procedures were carried out at 4 °C. Freshly dissected *Limulus* telson muscle (100–200 g) was homogenized in 4 volumes of buffer A [50 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 3 mM NaN_3 containing 0.1 mM PMSF, 2 mg/L leupeptin, 2 mg/L pepstatin A, 10 mg/L N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1 mg/L aprotinin, 5 mg/L chymostatin, and 10 mg/L antipain]. The homogenization buffer also contained freshly added 0.1 mM diisopropyl fluorophosphate and 100 mg/L soybean trypsin inhibitor. The homogenate was sedimented at 10000*g* for 15 min to remove insoluble myofibrillar material; the supernatants were collected and were then sedimented at 40000*g* for 30 min. Solid ammonium sulfate was added to 30% of saturation, the solution was sedimented at 40000*g* for 15 min, and the supernatants were brought to 60% of saturation with solid ammonium sulfate. The precipitate was collected by centrifugation, dissolved in a minimal volume of buffer A, and dialyzed against this buffer for 3 h. The sample was clarified by centrifugation at 40000*g* for 30 min, applied to a 5 × 90 cm column of Sephacryl S-300, and eluted with buffer A. The column fractions were assayed for MLC kinase activity by using isolated light chains (either from turkey gizzard or from *Limulus* muscle) or sometimes intact gizzard myosin as a substrate. The active fractions were pooled and were applied to a DEAE-Sephacel column (2.5 × 7 cm) equilibrated in the same buffer. After the column was washed, it was eluted with a 50–600 mM NaCl gradient in buffer A (total volume 600 mL). The fractions containing MLC kinase activity were pooled and were dialyzed against 40 mM Tris·HCl (pH 7.5), 0.1 mM EGTA, 2 mM dithiothreitol, and 2 mM NaN_3 plus the same cocktail of inhibitors described in buffer A. The next day $CaCl_2$ was added to a final concentration of 0.5 mM, and the sample was applied to a calmodulin–Sepharose 4B column (Adelstein & Klee, 1981) equilibrated in 40 mM Tris·HCl (pH 7.5), 0.5 mM $CaCl_2$, 2 mM dithiothreitol, and 2 mM NaN_3 plus the same cocktail of protease inhibitors described in buffer A. After the unbound protein was washed out, the MLC kinase activity was eluted with 40 mM Tris·HCl (pH 7.5), 2 mM EGTA, 2 mM NaN_3, and 2 mM dithiothreitol plus the same cocktail of protease inhibitors described in buffer A. The active fractions were concentrated by chromatography over a 0.5-mL column of DEAE-Sephacel equilibrated in the last buffer. The MLC kinase was eluted by washing the column with the same buffer but containing 0.40 M NaCl, and the active fractions were pooled and stored in this buffer at −70 °C.

Gizzard or *Limulus* total myosin light chains were prepared basically as described by Perrie & Perry (1970). Gizzard myosin light chains were separated from calmodulin by DEAE-Sephacel chromatography (Adelstein & Klee, 1981). The catalytic subunit of cAMP-dependent protein kinase (type II) from bovine heart was a gift of Dr. Edwin G. Krebs, University of Washington, Seattle, WA. Calmodulin prepared from bovine testis (Yazawa et al., 1980) was a gift of Dr. Masakatsu Nishikawa of this laboratory.

Assay of MLC Kinase Activity. Unless otherwise indicated, MLC kinase activity was assayed in a solution (0.1 mL) containing 50 mM Tris·HCl (pH 7.5), 4 mM magnesium acetate, 0.2 mM $CaCl_2$, 0.2 mM ATP (0.5 Ci/mmol), 0.1 μM calmodulin, and 10 μM myosin light chains (either from turkey gizzard or from *Limulus* muscle) at 25 °C. Four 20-μL time points were taken usually at 1-, 2-, 3-, and 4-min intervals and were applied to Whatman 3MM filter disks, which were immediately immersed in 10% trichloroacetic acid containing 2% sodium pyrophosphate (0 °C) and then washed as described by Corbin & Reimann (1975) to ensure linearity.

Electrophoresis. Microslab gels of 12.5% SDS–polyacrylamide were used as described by Matsudaira & Burgess (1978). The kinase was also electrophoresed on 3.5% polyacrylamide gels in the absence of SDS as described by Pato & Adelstein (1980). The gel was sliced into 2-mm slices, eluted with 50 mM Tris·HCl (pH 7.4), 0.1 M NaCl, 1 mM $MgCl_2$, 10% glycerol, 0.05 mg/mL β-lactoglobulin, and 2 mM dithiothreitol, and assayed for kinase activity.

Calmodulin Overlay. The ^{125}I-labeled calmodulin overlay technique of Van Eldik & Burgess (1983) was used. *Limulus* MLC kinase was first electrophoresed on a 12.5% SDS–polyacrylamide microslab gel.

Results

The purification of MLC kinase from *Limulus* muscle was based on that used for other myosin light chain kinases (Adelstein & Klee, 1981; Blumenthall & Stull, 1980). All buffers contained a cocktail of protease inhibitors in order to minimize proteolysis. Greater than 50% of the kinase activity was solubilized by a single low ionic strength extraction. The kinase was then further purified by ammonium sulfate fractionation and chromatography on Sephacryl S-300 and DEAE-Sephacel where the kinase eluted at an NaCl concentration of 0.37 M. The final purification was achieved by chromatography on a calmodulin–Sepharose 4B affinity

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MLC, myosin light chain; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; Da, dalton(s).

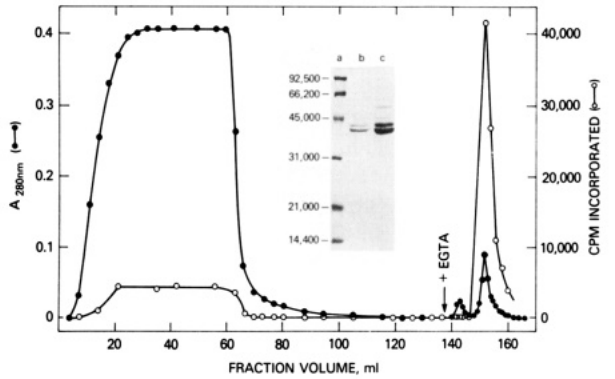

FIGURE 1: Calmodulin–Sepharose 4B chromatography of *Limulus* myosin light chain kinase. 15 mg of protein was applied in a volume of 45 mL to a 1.0-mL calmodulin–Sepharose 4B column (Adelstein & Klee, 1981) as described under Materials and Methods. The kinase activity was the cpm incorporated in 15 s. (Inset) (a) Protein standards: phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400); (b) 1 μg of *Limulus* myosin light chain kinase; (c) 2.5 μg of *Limulus* myosin light chain kinase.

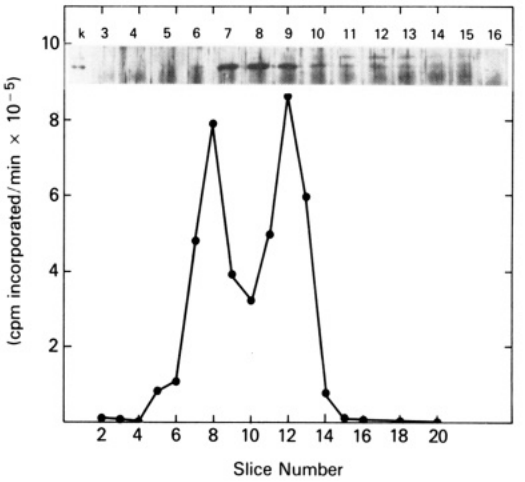

FIGURE 2: Nondissociating polyacrylamide gel electrophoresis of *Limulus* myosin light chain kinase. Following electrophoresis under nondissociating conditions as described by Pato & Adelstein (1980), the gel was sliced into 2-mm pieces which were extracted and assayed for kinase activity. The inset shows a silver-stained 12.5% SDS–polyacrylamide gel of the fractions around the peak of enzymatic activity. Lane k is the material which was applied to the gel.

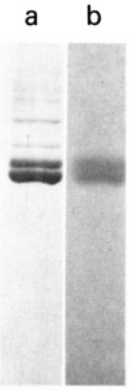

FIGURE 3: Calmodulin overlay of a 12.5% SDS–polyacrylamide gel of myosin light chain kinase. (a) 2.5 μg of myosin light chain kinase electrophoresed on a 12.5% SDS–polyacrylamide gel; (b) autoradiogram of the gel.

column (Figure 1). The majority of the kinase eluted when the column was washed with EGTA. The kinase activity seen in the flow-through fractions could subsequently be purified by reapplication of these fractions to calmodulin–Sepharose 4B, suggesting that the column was initially overloaded. We found it necessary to overload the column in order to obtain pure myosin light chain kinase. The active fractions were collected and concentrated by binding to a small (0.5 mL) DEAE-Sephacel column. The kinase was eluted by washing the column with a buffer containing 0.40 M NaCl. The kinase fractions could be collected at a concentration of 0.4–0.7 mg/mL by this procedure and were stored at −70 °C until used. Approximately 1.5 mg of kinase was isolated per 100 g of starting muscle. A 12.5% SDS–polyacrylamide gel of *Limulus* MLC kinase reveals two major bands with molecular weights of 37 000 and 39 000, respectively, that comprise 95% of the Coomassie Blue stainable material (Figure 1, inset) as well as several minor bands of higher molecular weight which comigrate with the major proteins seen in the flow-through sample of the calmodulin–Sepharose 4B column (data not shown). We found that unless the column was overloaded the relative intensity of these higher molecular weight proteins in the EGTA-elutable sample increased dramatically, suggesting that they may be adhering nonspecifically to the column.

To ascertain which protein(s) in our preparation was (were) the active MLC kinase, we employed polyacrylamide gel electrophoresis under nondenaturing conditions. A sample of *Limulus* MLC kinase was electrophoresed on a 3.5% polyacrylamide gel which was then cut into 2-mm slices and extracted overnight. An aliquot of each sample was assayed for MLC kinase activity, and the remainder of the extracted sample was concentrated by lyophilization and electrophoresed on a 12.5% SDS–polyacrylamide microslab gel (Figure 2). Two peaks of MLC kinase activity were resolved which coincided with the two major proteins present (Figure 2, inset). The 37 000-Da protein is completely separated from the 39 000-Da protein in fraction 7 and yet still shows activity indicating that a 1:1 complex of the two proteins is not required for activity. No evidence was seen for a third peak of activity even when the assay was allowed to continue for an extended period of time. Therefore, it seems likely that both the 39 000- and 37 000-Da proteins are forms of MLC kinase. That both proteins bind calmodulin was demonstrated by the use of a calmodulin overlay technique (Figure 3). None of the higher molecular weight bands bind calmodulin which is consistent with the suggestion that they may be adhering non-specifically to the calmodulin-Sepharose 4B column.

Having determined that the 37 000- and 39 000-Da proteins each possessed MLC kinase activity and bound calmodulin, we next sought to characterize some of their properties. From a double-reciprocal plot of kinase activity vs. *Limulus* myosin light chain concentration, we determined the V_{max} to be 15.6 μmol/(min·mg) and the K_m to be 15.4 μM (Figure 4). If light chains from turkey gizzard myosin were used as a substrate, a similar V_{max} was obtained, but the K_m was 5–6 times greater (data not shown). The calmodulin dependency was examined by measuring the activity at various calmodulin concentrations using calmodulin-free turkey gizzard myosin light chains as a substrate (Figure 5). A K_D of 6 nM for calmodulin was estimated.

One notable characteristic of MLC kinases from a number of species is their marked substrate specificity (Adelstein & Klee, 1981). An examination of the substrate specificity of *Limulus* MLC kinase confirmed this. Neither histones H2A, H2B, or H1 nor casein, actin, or tropomyosin served as substrates for this enzyme. *Limulus* myosin light chain kinase also phosphorylates both *Limulus* and gizzard myosins (data

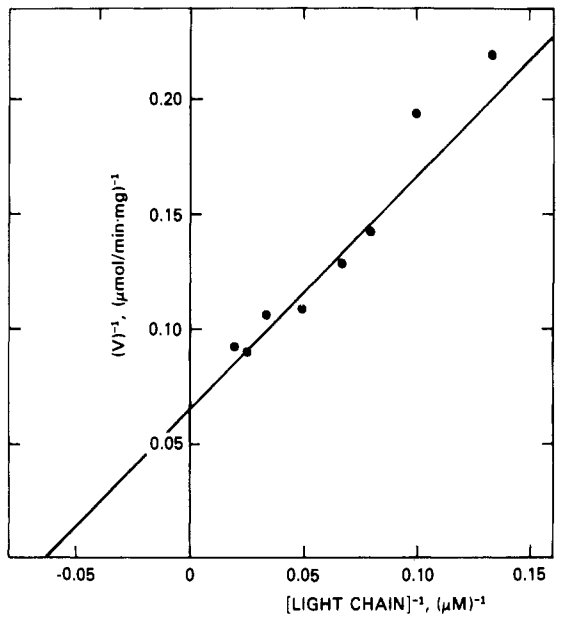

FIGURE 4: Double-reciprocal plot of kinase activity vs. light chain concentration. Conditions were as described under Materials and Methods except that 1 mg/mL bovine serum albumin was present. The *Limulus* light chain concentration was varied between 7.5 and 50 μM. The concentration of phosphorylatable light chain in the total light chain preparation was determined by measuring the maximal incorporation of ^{32}P. The data were fit by a nonlinear least-squares method (Bevington, 1969).

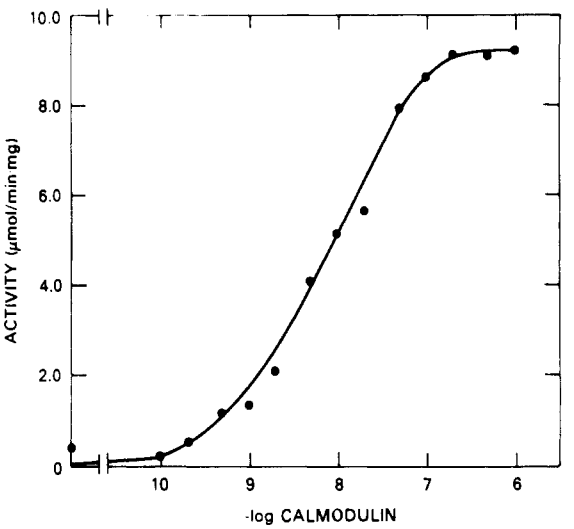

FIGURE 5: Determination of K_D for calmodulin. Conditions were as described under Materials and Methods except that the calmodulin concentration was varied between 0.1 nM and 1 μM. The data were fit by a nonlinear least-squares method (Bevington, 1969).

not shown). The maximum stoichiometry of phosphorylation for either *Limulus* or gizzard regulatory light chains was 1 mol/mol. If *Limulus* light chains are incubated with both *Limulus* myosin light chain kinase and gizzard myosin light chain kinase, only 1 mol/mol of ^{32}P is incorporated, suggesting that the same site is being phosphorylated by both enzymes.

We next attempted to phosphorylate *Limulus* MLC kinase with the cAMP-dependent protein kinase. No incorporation was seen either in the presence or absence of calcium-calmodulin under conditions in which a gizzard MLC kinase control incorporated 0.6 mol ^{32}P/mol in the presence of calcium-calmodulin and 1.4 mol/mol in the absence of calmodulin. This experiment also demonstrates that there is no autophoshorylation of *Limulus* MLC kinase.

Discussion

MLC kinase has been isolated from smooth, skeletal, and cardiac muscles from both avian and mammalian sources (Dabrowska et al., 1977; Adelstein & Klee, 1981; Blumenthal & Stull, 1980; Walsh et al., 1981; Nishikawa et al., 1984; Wolf & Hoffmann, 1980). In addition, it has been isolated from a number of nonmuscle cells (Dabrowska & Hartshorne, 1978; Yerna et al., 1979; Nishikawa et al., 1984). This is the first report of a purification of MLC kinase from an invertebrate muscle source. Although the myosin from vertebrate skeletal (Perrie et al., 1973; Manning & Stull, 1979; Pemrick, 1980; Barsotti & Butler, 1984) and cardiac muscle (Perry et al., 1979; Kopp & Barany, 1979; Westwood & Perry, 1981) is phosphorylated both in vivo and in vitro, the phosphorylation does not appear to be the dominant regulatory mechanism as it is in smooth muscle (Adelstein & Eisenberg, 1980).

Lehman (1975) demonstrated the presence of a functional troponin–tropomyosin system in *Limulus* muscle, and Sellers (1981) showed that the MgATPase activity of *Limulus* myosin in the presence of pure actin was regulated by phosphorylation of the myosin light chains. Kerrick & Bolles (1981), using skinned *Limulus* muscle fibers, have shown that the myosin light chains of *Limulus* are phosphorylated in a calcium-dependent manner. These authors also showed that skinned fibers containing thiophosphorylated myosin could still be relaxed upon removal of calcium, and they interpreted this as evidence against this phosphorylation being a primary regulatory mechanism. We feel that this is not contrary evidence since it has been shown that the troponin–tropomyosin complex can regulate the actin-activated MgATPase activity of phosphorylated platelet myosin (Adelstein & Conti, 1972). We have recently confirmed that troponin–tropomyosin also regulates phosphorylated gizzard heavy meromyosin. Furthermore, the myosin in the skinned fibers was always partially phosphorylated, and therefore, the authors could not determine whether contraction could occur in the absence of phosphorylation. Therefore, we feel that phosphorylation of *Limulus* myosin is likely to be involved in regulation but that the relative dominance of the two systems remains to be established.

Not all species possess myosin light chain kinase activity in their muscles. Several groups have failed to demonstrate either myosin light chain kinase activity or phosphorylation of the myosin light chains in scallop adductor muscle (Frearson et al., 1976; Kendrick-Jones & Jakes, 1977; Sellers, 1980).

We now report the purification of *Limulus* MLC kinase to near homogeneity. It is similar to MLC kinases from other sources in the following respects: (1) it is very specific for myosin light chains (Adelstein & Klee, 1981); (2) its activity is dependent upon calcium and calmodulin, with a K_D of about 6 nM for calmodulin which is within the range reported for other MLC kinase (Adelstein & Klee, 1981; Edelman & Krebs, 1982; Nishikawa et al., 1984); (3) the V_{max} and K_m for phosphorylation of isolated *Limulus* myosin light chains are also similar to the values reported for other MLC kinases (Adelstein & Klee, 1981; Nishikawa et al., 1984).

The major difference between *Limulus* MLC kinase and the others is its low molecular weight. On SDS–polyacrylamide gels, it is seen as a pair of bands with molecular weights of 39 000 and 37 000. Both proteins have activity and can bind calmodulin. It is interesting that calmodulin-dependent activity can be obtained in such a small protein. In this regard, it should be noted that the relative proximity between the catalytic site and the calmodulin binding site has also been shown for MLC kinases from both avian smooth muscle (Foyt et al., 1983) and rabbit skeletal muscle (Mayr & Heilmeyer, 1983).

Mayer & Heilmyer (1983) have recently reported that skeletal muscle MLC kinase can be subdivided proteolytically to yield a 33 000-Da inactive fragment and a 36 000-Da fragment which binds calmodulin and has catalytic activity. The specific activity of the 36 000-Da fragment can be increased in the presence of the 33 000-Da fragment.

It is possible that *Limulus* MLC kinase may be a proteolytic breakdown product of a larger enzyme which exists in intact cells. To attempt to minimize this possiblity, we have employed a large number of proteolytic inhibitors throughout the preparation. It was also noted that the activity eluted at a position consistent with this low molecular weight during the Sephacryl S-300 chromatography which was usually initiated within 6–8 h of the start of the preparation. More work will be required to ascertain the true molecular weight of this enzyme.

MLC kinases from vertebrate smooth, skeletal, and cardiac muscles as well as that from vertebrate nonmuscle cells can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. The activity of smooth muscle and nonmuscle MLC kinase is inhibited by this phosphorylation due to a decrease in the affinity of the phosphorylated enzyme for calmodulin (Conti & Adelstein, 1981). Although cardiac muscle (Wolf & Hoffman, 1980) and skeletal muscle (Edelman & Krebs, 1982) MLC kinases are phosphorylated, neither their activity nor their calmodulin affinity is affected. *Limulus* MLC kinase is not phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

The existence of a phosphorylation-dependent–myosin-linked regulatory system has also been proposed for the striated muscle of the locust (Winkleman & Bullard, 1977). It would be interesting to examine the properties of the MLC kinase from this tissue as well as from other invertebrate sources. Such studies may provide information not only for the evolution of MLC kinase but also on the structure–function relationships of the enzyme.

Acknowledgments

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Registry No. MLC kinase, 51845-53-5.

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Studies on Structure and Function of Rhodopsin by Use of Cyclopentatrienylidene 11-*cis*-Locked-rhodopsin†

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ABSTRACT: The photochemical reaction of cyclopentatrienylidene 11-*cis*-locked-rhodopsin derived from cyclopentatrienylidene 11-*cis*-locked-retinal and cattle opsin was spectrophotometrically studied. The difference absorption spectrum between the cyclopentatrienylidene 11-*cis*-locked-rhodopsin and its retinal oxime had its maximum at 495 nm (P-495). Irradiation of P-495 at −196 °C with either blue light or orange light caused no spectral change, supporting the cis–trans isomerization hypothesis for formation of bathorhodopsin. Upon irradiation of P-495 at 0 °C with orange light, however, its absorption spectrum shifted to a shorter wavelength owing to formation of a hypsochromic product. The difference absorption spectrum between this product (P-466) and its retinal oxime showed its maximum at 466 nm. Analysis of retinal isomers by high-performance liquid chromatography showed that this spectral shift was not accompanied by photoisomerization of the chromophore. P-466 could almost completely be photoconverted to the original pigment (P-495) by irradiation at 0 °C with blue light with little formation of the other isomeric form of its chromophore. The α-band of the circular dichroism spectrum of P-495 was very small in comparison with that of rhodopsin, while that of P-466 was comparable to it. These facts suggest that P-495 has a planar conformation in the side chain of the chromophore and that P-466 has a twisted one, probably at the C_8–C_9 single bond. Cyclic-GMP phosphodiesterase in frog rod outer segment was activated by neither P-495 nor P-466. This result suggests that the isomerization of the retinylidene chromophore of rhodopsin is indispensable in the phototransduction process.

It has been proposed that a photon absorbed by rhodopsin isomerizes the 11-*cis*-retinylidene chromophore to an all-trans form (Hubbard & Kropf, 1958). Later, one of us (Yoshizawa & Wald, 1963) suggested that an early photoproduct of rhodopsin photolysis, bathorhodopsin (formerly called prelumirhodopsin), might possess a twisted form of *all-trans*-retinal as its chromophore and extended the cis–trans photoisomerization hypothesis. In order to obtain direct evidence for this hypothesis, two kinds of retinal analogues have recently been synthesized in which rotation around the C_{11}–C_{12} double bond is blocked because of a fixed chemical bond: one is locked in the 11-cis conformation with cycloheptene (seven-membered ring; Akita et al., 1980) and the other with cyclopentene (five-membered ring; Ito et al., 1982). Both analogues bind with cattle opsin to produce rhodopsin analogues having their λ_{max}s at 490 (Akita et al., 1980) and 498 nm (Ito et al., 1982), respectively. Low-temperature spectrophotometric study of an artificial pigment derived from the former analogue and cattle opsin (Mao et al., 1981) showed the lack of a bathointermediate. This paper also shows that cyclopentatrienylidene 11-*cis*-locked-rhodopsin[1] (we tentatively call this analogue, having a five-membered ring in the side chain of the chromophore, Rh5) prepared from cyclopentatrienylidene 11-*cis*-locked-retinal (Ret5; Figure 1) and cattle opsin yields no bathointermediate and supports the cis–trans isomerization hypothesis as to the formation of bathorhodopsin.

Another aim of this study using this rhodopsin analogue is to demonstrate the origin of induced circular dichroism (CD) of rhodopsin. 11-*cis*-Retinal in free form has its absorption maximum in the near-ultraviolet spectral region but displays no optical activity. Binding of it to opsin induces optical activity in the visible and near-ultraviolet spectral regions with a large red shift of absorption maximum from 370 to 498 nm (Hubbard et al., 1965; Crescitelli et al., 1966). So far, three different explanations for the origin of the induced CD of rhodopsin have been proposed (Ebrey & Yoshizawa, 1973; Honig et al., 1973; Kropf et al., 1973). One of them attributed it to a twisted 11-*cis*-retinylidene chromophore, because calculations showed that 11-*cis*-retinal has at least two twisted single bonds at C_6–C_7 and at C_{12}–C_{13} due to steric hindrances (Honig et al., 1973) between the H atom of C_8 and the methyl group of C_5 and between the H atom of C_{10} and the methyl group of C_{13}, respectively. Supposedly, one of the conformers of 11-*cis*-retinal may selectively bind to the retinal-binding site of opsin. Thus, the optical activity of rhodopsin may be ex-

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[1] Cyclopentatrienylidene is a term used loosely, rather than rigorously, to indicate the presence of a 3,5-cyclopentenediylidene group. Thus, cyclopentatrienylidene 11-*cis*-locked-retinal is not retinal with a cyclopentatrienylidene substituent but rather is retinal containing this system.

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