Phosphorylation of Mammalian Myosin Light Chain Kinases by the Catalytic Subunit of Cyclic AMP-dependent Protein Kinase and by Cyclic GMP-dependent Protein Kinase*

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The phosphorylation of the calmodulin-dependent enzyme myosin light chain kinase, purified from bovine tracheal smooth muscle and human blood platelets, by the catalytic subunit of cAMP-dependent protein kinase and by cGMP-dependent protein kinase was investigated. When myosin light chain kinase which has calmodulin bound is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, 1 mol of phosphate is incorporated per mol of tracheal myosin light chain kinase or platelet myosin light chain kinase, with no effect on the catalytic activity. Phosphorylation when calmodulin is not bound results in the incorporation of 2 mol of phosphate and significantly decreases the activity. The decrease in myosin light chain kinase activity is due to a 5 to 7-fold increase in the amount of calmodulin required for half-maximal activation of both tracheal and platelet myosin light chain kinase.

In contrast to the results with the catalytic subunit of cAMP-dependent protein kinase, cGMP-dependent protein kinase cannot phosphorylate tracheal myosin light chain kinase in the presence of bound calmodulin. When calmodulin is not bound to tracheal myosin light chain kinase, cGMP-dependent protein kinase phosphorylates only one site, and this phosphorylation has no effect on myosin light chain kinase activity. On the other hand, cGMP-dependent protein kinase incorporates phosphate into two sites in platelet myosin light chain kinase when calmodulin is not bound.

The sites phosphorylated by the two cyclic nucleotide-dependent protein kinases were compared by two-dimensional peptide mapping following extensive tryptic digestion of the phosphorylated myosin light chain kinases. With respect to the tracheal myosin light chain kinase, the single site phosphorylated by cGMP-dependent protein kinase when calmodulin is not bound appears to be the same site phosphorylated in the tracheal enzyme by the catalytic subunit of cAMP-dependent protein kinase when calmodulin is bound. With respect to the platelet myosin light chain kinase, the additional site that was phosphorylated by cGMP-dependent protein kinase when calmodulin was not bound was different from that phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

The phosphorylation of myosin light chain is thought to play a major role in the regulation of contractile proteins in vertebrate smooth muscle and nonmuscle cells (1, 2). The phosphorylation of myosin is catalyzed by the substrate-specific enzyme myosin light chain kinase (3). This enzyme has been purified from a variety of sources including various types of smooth muscle (3-7), skeletal muscle (8-11), platelets (12, 13), brain (13, 14), fibroblasts (15), and cardiac muscle (16). MLC kinase is completely dependent on the presence of calcium and calmodulin for activity (3, 12, 13).

The catalytic subunit of cAMP-dependent protein kinase has been shown to phosphorylate turkey gizzard smooth muscle MLC kinase whether or not calmodulin is bound (17). When gizzard smooth muscle MLC kinase with calmodulin bound to it was phosphorylated by cAMP-dependent protein kinase, phosphate was incorporated into a single site and there was no effect on the ability of MLC kinase to bind calmodulin. When the catalytic subunit of cAMP-dependent protein kinase phosphorylated MLC kinase in the absence of bound calmodulin, 2 mol of phosphate were incorporated into MLC kinase and the amount of calmodulin required for half-maximal activation of MLC kinase increased by 10 to 20-fold (17). Although MLC kinase isolated from skeletal muscle can serve as a substrate for cAMP-dependent protein kinase, phosphorylation does not alter its activity (11). In addition to MLC kinases isolated from a variety of smooth muscles (6, 7, 17), phosphorylation of platelet MLC kinase by the catalytic subunit of cAMP-dependent protein kinase has been shown to result in a decrease in human platelet MLC kinase activity (18). However, the latter report did not demonstrate any difference in phosphate incorporation into platelet MLC kinase in the presence or absence of bound calmodulin.

To date, there is no report in the literature on the phosphorylation of MLC kinase by cGMP-dependent protein kinase. The mechanism of action of cGMP is thought to involve activation of its dependent protein kinase and the phosphorylation of various proteins, in a manner analogous to the cAMP system (19-22). Moreover, cyclic nucleotide-dependent protein phosphorylation reactions regulate a broad spectrum of enzyme activities, and cAMP- and cGMP-dependent protein kinases have many similarities in their physiological and kinetic properties (19, 21, 22). The similarities between the cyclic nucleotide protein kinases raised the possibility that MLC kinase might be a substrate for cGMP-dependent protein kinase and that phosphorylation by this kinase could constitute an additional modulatory mechanism.

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1 The abbreviations used are: MLC, myosin light chain; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.
for the regulation of contractile proteins in smooth muscle and nonmuscle cells.

Therefore, we undertook a study of the phosphorylation of bovine tracheal smooth muscle MLC kinase (M₉ = 160,000) and human platelet MLC kinase (M₉ = 100,000) using the catalytic subunit of cAMP-dependent protein kinase and cGMP-dependent protein kinase. We were particularly interested in whether these protein kinases phosphorylated the catalytic subunit of CAMP-dependent protein kinase and platelet and bovine tracheal MLC kinase are substrates for cGMP-dependent protein kinase.²

**RESULTS**

**Incorporation of Phosphate into MLC Kinases**—Fig. 1 shows an SDS-polyacrylamide gel following phosphorylation of MLC kinase by the catalytic subunit of cAMP-dependent protein kinase or by cGMP-dependent protein kinase using [γ-³²P]ATP. Fig. 1A illustrates the phosphorylation of bovine tracheal smooth muscle MLC kinase (160,000 Da), and Fig. 1B illustrates the phosphorylation of human platelet MLC kinase (100,000 Da) in the presence and absence of bound calmodulin. Autoradiographs of the ³²P-labeled MLC kinases show that essentially all the radioactivity co-electrophoresed with the major Coomassie blue-stained band and that there appears to be very little phosphorylation of tracheal MLC kinase by cGMP-dependent protein kinase in the presence of bound calmodulin (Fig. 1A, lane 1⁴).

Figs. 2 and 3 show time courses of phosphorylation of bovine tracheal MLC kinase and human platelet MLC kinase by the catalytic subunit of CAMP-dependent protein kinase (Figs. 2A and 3A) and by cGMP-dependent protein kinase (Figs. 2B and 3B). The experiments were carried out in the presence (0.2 mM CaCl₂ and 1 μM calmodulin) or absence (2 mM EGTA and 1 μM calmodulin) of bound calmodulin. When calmodulin was not bound, 1.9 ± 0.15 mol of phosphate were incorporated into tracheal MLC kinase or platelet MLC kinase by the catalytic subunit of CAMP-dependent protein kinase in five separate experiments. When calmodulin was bound to MLC kinase, 0.85 ± 0.13 mol of phosphate were incorporated into both MLC kinases using the catalytic subunit in five separate experiments. Bovine tracheal and human platelet MLC kinase underwent a very slow rate of phosphorylation in the absence of an added protein kinase (autophosphorylation). The rate and the extent of this phosphorylation were unaffected by the addition of 1 μM cAMP and 1 μM cGMP or by the addition of the inhibitory subunit of CAMP-dependent protein kinase to the reaction mixture, indicating that the phosphorylation of MLC kinase in the absence of the protein kinase was not due to contamination of the holoenzyme of CAMP-dependent protein kinase or cGMP-dependent protein kinase. The extent of this autophosphorylation, accounting for less than 0.3 mol of PO₄/mol of MLC kinase (Figs. 2A and 3A), was not affected by the binding of calmodulin to MLC kinase.

² A preliminary report on this work was presented at the Meeting of the Biophysical Society, February 1983 at San Diego, CA.

³ Portions of this paper (including “Materials and Methods,” Figs. 2, 3, and 5–7, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2070, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

⁴ In contrast to the results with the catalytic subunit of CAMP-dependent protein kinase, cGMP-dependent protein kinase phosphorylated 0.90 ± 0.11 mol (n = 3) of PO₄/mol tracheal MLC kinase in the absence of bound calmodulin (Fig. 2B). In the presence of bound calmodulin, cGMP-dependent protein kinase incorporated the same amount of phosphate (0.2 mol) as was incorporated by autophosphorylation of tracheal MLC kinase (Fig. 2B). These data suggest that cGMP-dependent protein kinase cannot phosphorylate tracheal MLC kinase in the presence of bound calmodulin. On the other hand, when calmodulin was not bound to human platelet MLC kinase, cGMP-dependent protein kinase incorporated 0.4 mol of phosphate into platelet MLC kinase (Fig. 3B). When the platelet MLC kinase was incubated with a higher ratio of cGMP-dependent protein kinase, the MLC...
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kinase was phosphorylated much more rapidly to a final level of 2.1 ± 0.2 mol (n = 3)/mol of MLC kinase in the absence of bound calmodulin. In the presence of bound calmodulin, 0.8 ± 0.15 mol (n = 5) of phosphate was incorporated by cGMP-dependent protein kinase into the platelet enzyme (Fig. 3B).

In addition to the difference in the extent of incorporation of phosphate into MLC kinase in the presence or absence of calmodulin, there was also a difference in the rate of phosphorylation as summarized in Table I. In the presence of bound calmodulin, the rate of phosphorylation of MLC kinase by the catalytic subunit of cAMP-dependent protein kinase was at least two times slower than that in the absence of bound calmodulin under the conditions employed. Both the catalytic subunit of cAMP-dependent protein kinase and cGMP-dependent protein kinase appeared to catalyze the phosphorylation of both MLC kinases at the same rate when assayed with 1 μM tracheal MLC kinase or 0.7 μM platelet MLC kinase. However, it is very likely that a nonsaturating concentration of the MLC kinase was used, and the table is presented only for comparison of the two cyclic nucleotide-dependent protein kinases.

Two-dimensional Analysis of Phosphopeptides—After MLC kinase was maximally phosphorylated by protein kinase, MLC kinase was fractionated on SDS-polyacrylamide gels, eluted, and digested extensively with 1-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin. The tryptic fragments were analyzed by two-dimensional peptide mapping using electrophoresis in the first dimension and ascending chromatography in the second dimension. When either tracheal or platelet MLC kinase, which was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase in the presence of bound calmodulin, was digested, a single major radioactive peptide (peptide 1) was found (Fig. 4, A and B, 1). Phosphorylation of MLC kinases by the catalytic subunit of cAMP-dependent protein kinase in the absence of bound calmodulin, followed by tryptic digestion, resulted in the appearance of two major radioactive peptides (Fig. 4, A and B, 2). One of these peptides appeared to be the same peptide as that (peptide 1) found following digestion of MLC kinase which had been phosphorylated in the presence of bound calmodulin. The second 32P-labeled peptide (peptide 2) appeared only when MLC kinase was phosphorylated in the absence of bound calmodulin. Fig. 4 also shows that both peptides 1 and 2 migrate with the same apparent mobility whether the source of MLC kinase is bovine tracheal smooth muscle or human platelets. These results are similar to those previously reported for turkey gizzard MLC kinase (17).

As expected, tryptic digestion of tracheal MLC kinase phosphorylated by cGMP-dependent protein kinase in the presence of bound calmodulin resulted in no phosphopeptide (Fig. 4A, 3). When tracheal MLC kinase which was phosphorylated by cGMP-dependent protein kinase in the absence of bound calmodulin was digested, one major phosphorylated peptide was found following two-dimensional peptide mapping (Fig. 4A, 4). A map of a 1:1 mixture of the digests of tracheal MLC kinase phosphorylated by the catalytic subunit of cAMP-dependent protein kinase in the presence of bound calmodulin or by cGMP-dependent protein kinase in the absence of bound calmodulin showed only one major phosphopeptide (Fig. 4A, 5), suggesting that cGMP-dependent protein kinase phosphorylates the site located in peptide 1. Platelet MLC kinase which was phosphorylated by cGMP-dependent protein kinase in the presence of calmodulin yielded a single major radioactive spot which co-migrated with peptide 1 (Fig. 4B, 3). However, phosphorylation of platelet MLC kinase by cGMP-dependent protein kinase, when calmodulin was not bound, yielded two phosphopeptides, and the new peptide (peptide 3) was clearly different from that found following phosphorylation by the catalytic subunit of cAMP-dependent protein kinase (Fig. 4B, compare 4 to 2 and 5).

Sequential Phosphorylation of Tracheal and Platelet MLC Kinase—It was of interest to ascertain whether the site in tracheal MLC kinase that was phosphorylated by cGMP-dependent protein kinase in the absence of bound calmodulin was in fact the same site phosphorylated by cAMP-dependent protein kinase in the presence of bound calmodulin. In addition, it was of interest to determine whether tracheal MLC kinase previously phosphorylated by the catalytic subunit of cAMP-

A. TRACHEA MLC KINASE

B. PLATELET MLC KINASE

FIG. 4. Autoradiography of two-dimensional tryptic peptide maps of bovine tracheal MLC kinase (A) and human platelet MLC kinase (B). 0.5 μM tracheal MLC kinase and 0.5 μM platelet MLC kinase were phosphorylated in the presence of 1 μM calmodulin and 0.2 mM CaCl₂ (1 and 3) or 1 μM calmodulin and 2 mM EGTA (2 and 4) by 4 μg/ml of the catalytic subunit of cAMP-dependent protein kinase (1 and 2) or by 5 μg/ml of cGMP-dependent protein kinase (3 and 4). Samples were processed for electrophoresis (horizontal dimension) followed by chromatography (vertical dimension) as described under "Materials and Methods." The solid circle indicates the origin. The anode is to the right and the cathode to the left side of the origin. A (5) and B (5) are maps of 1:1 mixture of the samples mapped in A (1) + A (4) and B (2) + B (4), respectively.
dependent protein kinase could serve as a substrate for cGMP-dependent protein kinase. Tracheal MLC kinase was initially phosphorylated (0.92 mol of PO_4/mol of MLC kinase) by the catalytic subunit of cAMP-dependent protein kinase in the presence of bound calmodulin and then chromatographed on DEAE-cellulose to remove the catalytic subunit of cAMP-dependent protein kinase. This monophosphorylated tracheal MLC kinase was incubated with cGMP-dependent protein kinase or with the catalytic subunit of cAMP-dependent protein kinase and [γ-32P]ATP in the presence of EGTA. Fig. 5 shows that there is no substantial increase in phosphate incorporation into monophosphorylated tracheal MLC kinase upon addition of cGMP-dependent protein kinase, although the catalytic subunit of cAMP-dependent protein kinase could increase the amount of phosphate in this tracheal MLC kinase from 0.92 mol of PO_4/mol of MLC kinase to 2.05 mol of PO_4/mol of MLC kinase. When tracheal MLC kinase was phosphorylated first by cGMP-dependent protein kinase in the absence of bound calmodulin, the addition of the catalytic subunit of the cAMP-dependent kinase resulted in an increased phosphate incorporation into MLC kinase from 0.75 mol of PO_4/mol of MLC kinase to 1.96 mol of PO_4/mol of MLC kinase (Fig. 6). These data suggest that the catalytic subunits of cAMP-dependent protein kinase and cGMP-dependent protein kinase phosphorylate the same site in peptide 1 in tracheal MLC kinase, but that only the catalytic subunit of cAMP-dependent protein kinase can phosphorylate the site present in peptide 2.

Since both cGMP-dependent protein kinase and the catalytic subunit of cAMP-dependent protein kinase incorporated 2 mol of PO_4/mol of platelet MLC kinase and since one of the sites phosphorylated appears to be different (see Fig. 4B), it was of interest to see if sequential phosphorylation by the two protein kinases could introduce a third mole of phosphate into platelet MLC kinase. Fig. 7 shows that the addition of the catalytic subunit of cAMP-dependent protein kinase after initial phosphorylation of platelet MLC kinase with cGMP-dependent protein kinase, in the absence of bound calmodulin, resulted in only a marginal increase of phosphate incorporated (from 1.4 to 1.54 mol of PO_4/mol of MLC kinase). This indicates that the phosphorylation of platelet MLC kinase by cGMP-dependent protein kinase excludes further phosphorylation by cAMP-dependent protein kinase.

The Effect of Phosphorylation on MLC Kinase Activity and Its Affinity for Calmodulin—When both tracheal and platelet MLC kinases that were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase in the presence of bound calmodulin were assayed at a concentration of 5 nM MLC kinase over a range of calmodulin concentrations (5 nM to 1 µM), there was no difference in the concentration of calmodulin required for half-maximal activation between the monophosphorylated and unphosphorylated kinase (data not shown).

Tracheal MLC kinase and platelet MLC kinase which were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase or cGMP-dependent protein kinase in the absence of bound calmodulin were also assayed for enzyme activity over a range of calmodulin concentrations of 0.1 nM to 2 µM. Data presented in Figs. 8 and 9 show that diphosphorylation of both MLC kinases by the catalytic subunit of cAMP-dependent protein kinase resulted in a significant decrease in the affinity of MLC kinase for calmodulin. Phosphorylation of tracheal MLC kinase by cGMP-dependent protein kinase, which resulted in 1 mol of phosphate being incorporated, had no effect on MLC kinase activity (Fig. 8). On the other hand, phosphorylation of platelet MLC kinase by cGMP-dependent protein kinase in the absence of bound calmodulin, which resulted in 2 mol of phosphate being incorporated, did have an effect on the affinity of MLC kinase for calmodulin, but a substantially smaller one than phosphorylation by the cAMP-dependent protein kinase catalytic subunit (Fig. 9).

When these data were analyzed by generating double reciprocal plots (insets in Figs. 8 and 9), the calmodulin concentra-
Phosphorylations required for half-maximal activation of the tracheal MLC kinase were 5.35 ± 0.51 nM \((n = 3)\) for the diphosphorylated enzyme and 1.07 ± 0.22 nM \((n = 3)\) for the unphosphorylated or monophosphorylated enzyme. A similar analysis of the data presented in Fig. 9 showed that the calmodulin concentrations required for half-maximal activation of the platelet MLC kinase were as follows: 10.0 ± 2.8 nM \((n = 3)\) following diphosphorylation by the catalytic subunit of cAMP-dependent protein kinase; 4.06 ± 0.38 nM \((n = 3)\) following diphosphorylation by cGMP-dependent protein kinase; and 2.55 ± 0.26 nM \((n = 3)\) for the unphosphorylated platelet MLC kinase. In contrast to the effect of phosphorylation on the binding of calmodulin, there was no effect of phosphorylation on the \(V_{max}\) of MLC kinase activity ( insets in Figs. 8 and 9), although a previous report has shown that phosphorylation of platelet MLC kinase decreases both the ability of the enzyme to bind calmodulin and \(V_{max}\). This difference from the previous report most likely reflects an improved method for preparation of the platelet enzyme.

**DISCUSSION**

The work presented here on the phosphorylation of bovine tracheal and human platelet MLC kinase, using the catalytic subunit of cAMP-dependent protein kinase, complements previous works reported on MLC kinases isolated from turkey gizzard \((17)\), blood platelets \((18)\), bovine stomach \((6)\), and bovine trachea \((7)\). In each case, phosphorylation by the catalytic subunit of cAMP-dependent protein kinase resulted in a decrease in MLC kinase activity due to a decrease in the affinity for calmodulin. However, this reduction in MLC kinase activity is critically dependent on the incorporation of 2 mol of phosphate/mol of MLC kinase. This suggests that there is something unique about the site phosphorylated in peptide 2. First, it appears to be phosphorylated only by the catalytic subunit when calmodulin is not bound to MLC kinase. Second, the tryptic phosphopeptides labeled 1 and 2 (see Fig. 4, A and B) generated from bovine tracheal smooth muscle, human platelet, and turkey gizzard MLC kinase (data for turkey gizzard not shown) have the same apparent mobility when analyzed by two-dimensional peptide mapping on cellulose sheets. This suggests that the primary structure in this part of the molecule is relatively conserved. Finally, the finding that cGMP-dependent protein kinase phosphorylates a different site on platelet MLC kinase (peptide 3, Fig. 4B) without substantially altering the activity of MLC kinase suggests that the site in peptide 2 plays a unique role in affecting the binding of calmodulin to MLC kinase. We do not, at present, know whether phosphorylation of the site in peptide 2 alone is sufficient to decrease calmodulin binding or whether both site 1 and site 2 must be phosphorylated to alter the calmodulin binding characteristics. However, as shown above, phosphorylation of a site in peptide 1 alone, whether by cGMP-dependent protein kinase or by the catalytic subunit of cAMP-dependent protein kinase, has no effect on the activity of tracheal or platelet MLC kinase under the conditions assayed.

cGMP-dependent protein kinase cannot phosphorylate bovine tracheal MLC kinase in the presence of bound calmodulin. When calmodulin is not bound to the tracheal enzyme, cGMP-dependent protein kinase incorporates 1 mol of phosphate into a site located in peptide 1, whereas cAMP-dependent protein kinase incorporates 2 mol of phosphate into tracheal MLC kinase in the absence of bound calmodulin. This difference between the two nucleotide-dependent protein kinases may be related to the larger size of the cGMP-dependent protein kinase, as well as to enzyme substrate specificity. Thus, part of the tracheal MLC kinase, which has a molecular weight of 160,000, may sterically hinder phosphorylation by the larger cGMP-dependent protein kinase, but not by the catalytic subunit of cAMP-dependent protein kinase. This steric hindrance, however, may not be present in the case of the platelet MLC kinase. Platelet MLC kinase, which is considerably smaller than tracheal MLC kinase \((100,000 \text{ Da versus } 160,000 \text{ Da})\), can incorporate 1 mol of phosphate catalyzed by cGMP-dependent protein kinase when calmodulin is bound to it and two mol of phosphate when calmodulin is not bound. Surprisingly, however, this second mole of phosphate is incorporated into a different site (located in peptide 3) than that phosphorylated by the catalytic subunit of the cAMP-dependent enzyme.

As noted above, the molecular weight of platelet MLC kinase is lower than that of bovine tracheal MLC kinase. This raises the possibility that the purified enzyme represents a proteolytic fragment of a native enzyme with a higher molecular weight and the limited proteolysis accounts for the difference in the phosphorylation properties by cGMP-dependent protein kinase. However, precautions were taken to minimize proteolytic degradation \((i)\) by using fresh unfrozen platelets and \((ii)\) by using a number of proteolytic inhibitors throughout purification steps of platelet MLC kinase. Furthermore, one would anticipate more than one peak of MLC kinase activity, during the column chromatography steps of the purification procedure, if the native enzyme was undergoing proteolysis. However, there was always only one sharp peak of the enzyme activity during each step of the chromatography. Moreover, a 35,000-Da proteolytic fragment of tracheal MLC kinase, which was produced by digestion with the staphylococcal V-8 protease, retained Ca\(^{2+}\)-calmodulin-dependent MLC kinase activity and incorporated only 1 mol of phosphate by cGMP-dependent protein kinase in the absence of bound calmodulin.\(^4\) Taken together, these data suggest that platelet MLC kinase is not a proteolytic product and that the differences in the phosphorylation properties of the two MLC kinases may reflect differences between bovine tracheal smooth muscle and human platelets.

The results of the sequential phosphorylation of platelet MLC kinase suggest an interaction between peptide 2 and peptide 3 since the phosphorylation of peptide 2 prevents the phosphorylation of peptide 3. However, this cannot be taken as evidence for a catalytic role for peptide 2, since it was not possible to demonstrate this interaction by chromatography. However, it is clear that the phosphorylation of peptide 2 is required for the activation of platelet MLC kinase, since neither CTP nor cGMP can activate these enzymes in the absence of Ca\(^{2+}\)-calmodulin.

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\(^4\) M. Nishikawa, P. de Lanerolle, and R. S. Adelstein, unpublished observation.
REFERENCES

Supplement to: Phosphorylation of Human Myosin Light Chain Kinases
by the Catalytic Subunit of CTP-dependent Protein Kinase and by CTP-dependent Protein Kinase by Nakatani and S. Adarstein

MATERIALS AND METHODS

Preparation of Proteins: All procedures were carried out at 4°C. Myosin kinase from human smooth muscle was isolated and purified as previously described (1). The procedure employed ammonium sulfate fractionation followed by sequential heparin, EGTA, and calcium-dependent chromatography. To minimize proteolytic degradation, a large number of proteolytic inhibitors such as phenylmethylsulfonyl fluoride (PMSF), leupeptin (100 µg/ml), pepstatin A (10 µM), chymostatin (10 µM), Antipain (10 µM), and PMSF (10 µM) were used during dialysis and also during chromatography stages of purification. Both Myosin kinase preparations were free from phosphatases and ATPase activity. The specific activities of the purified human smooth muscle Myosin kinase and human skeletal Myosin kinase were 20 µmol/gain and 15 µmol/gain, respectively, when assayed with skeletal light chain isolated from turkey pate. Substrate specific activity (pS) was determined by the method of Nakatani et al. (13). The specific activity of the skeletal light chain myosin kinase was prepared according to the method of Nakatani et al. (13) and purified by heparin-Sepharose chromatography. The catalytic subunit of CTP-dependent protein kinase (type I) from bovine heart was a gift of Dr. Robert N. Burke (University of Edinburgh). The preparation of CTP-dependent protein kinase was purified as described (15). The specific activities of the catalytic subunit of CTP-dependent protein kinase and the bovine CTP-dependent protein kinase employed for the determination of the rate of phosphorylation of Myosin kinase were 15 µmol/gain and 10 µmol/gain, respectively. CTP (purified) was used as a substrate at 37°C.

Phosphorylation of Myosin Kinase: Myosin kinase (10-12.5 µg final concentration) was phosphorylated by incubation with the catalytic subunit of the CTP-dependent protein kinase or CTP-dependent protein kinase in S-20 Triton X-100 extract (5 µl, 3.6 or 8 µl [110-126 µM] ATP, 100-150 µM NAD, 15 [150 µM] MgCl₂, 10 [150 µM] DTT, 10 [100 µM] NaF, or 10 [100 µM] NaF, with or without 10 [100 µM] sodium fluoroacetate) at 37°C for various times. When Myosin kinase was phosphorylated, 10 [100 µM] ATP was added to the reaction mixture. Nonspecific phosphorylation of Myosin kinase was determined under the same conditions except that the catalytic subunit of CTP-dependent protein kinase was omitted. Incorporation was determined by overnight electrophoresis of a 48% polyacrylamide gel (12.5 µl) on a slab gel using the method of Kornblum et al. (27). This gel was stained with Coomassie Blue R-250 and the lower bands corresponding to Myosin kinase were excised from the gel and radioactivity was determined. The specific activity was estimated by the method of Kornblum et al. (27).

Analysis of Myosin Kinase Activity: Myosin kinase activity was assayed in a solution (50 µl) containing 40 mM Tris·HCl (pH 7.6), 60 [60 µM] MgCl₂, 60 [100 µM] NaF, 10 [150 µM] DTT, 0.6 [0.6%] Triton X-100, 2 [100 µM] ATP, 10 [100 µM] NaF, and 0.1 [0.1%] bovine serum albumin in a total reaction volume of 32 µl. Phosphate activities were assayed in a solution (100 µl) containing 50 [50 mM] Tris·HCl, 0.3 [30 µM] ATP, and 2% (2% v/v) bovine serum albumin in a total reaction volume of 75 µl. Activities (μmol/gain) of the reaction mixture were determined at 30°C. Phosphate values (determined by the method of Kornblum et al.) were corrected by the method of Kornblum et al. (27).

Other Methods: Myosin kinase was analyzed by 12.5% polyacrylamide-10% SDS gel electrophoresis (18). The gel was stained with Coomassie Brilliant Blue and the upper bands corresponding to Myosin kinase were excised from the gel and radioactivity was determined by the method of Kornblum et al. (27). The specific activity was estimated by the method of Kornblum et al. (27). The gel was stained with Coomassie Brilliant Blue and the lower bands corresponding to Myosin kinase were excised from the gel and radioactivity was determined by the method of Kornblum et al. (27). The specific activity was estimated by the method of Kornblum et al. (27).

Figure 2. Time course of phosphorylation of human smooth muscle Myosin kinase by the catalytic subunit of CTP-dependent protein kinase (C) or by CTP-dependent protein kinase (D) in the presence (closed circles) or absence (open circles) of bound calmodulin.
Phosphorylation of Myosin Light Chain Kinases

**Figure 1:** Sequential phosphorylation of bovine thymus MLCK kinase by the catalytic subunit of cAMP-dependent protein kinase and CDP-dependent protein kinase.

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**Figure 2:** Sequential phosphorylation of platelet MLCK kinase by cAMP-dependent protein kinase and by the catalytic subunit of CDP-dependent protein kinase.

**Table 1:** Rates of Phosphorylation of MLCK Kinases and Histone 2B by Cyclic Nucleotide-Dependent Kinases

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<th>Substrate</th>
<th>cAMP-Dependent (Catalytic Subunit)</th>
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<td>MLCK</td>
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N.D. = Not determined
MLCK = Myosin Light Chain Kinase