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CONVERSION OF A Ca^{2+}-DEPENDENT MYOSIN LIGHT CHAIN KINASE FROM SKELETAL MUSCLE TO A Ca^{2+}-INDEPENDENT FORM

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SUMMARY: The Ca^{2+}- and calmodulin-dependent myosin light chain kinase of rabbit skeletal muscle was converted to a Ca^{2+}-independent form by limited proteolysis with α-chymotrypsin. The conditions prevailing during proteolysis are important and the loss of Ca^{2+}-dependence was achieved best by hydrolysis of the Ca^{2+}-calmodulin-kinase complex. The lack of Ca^{2+}- and calmodulin-dependence was found using both myosin and isolated light chains as substrates. The specific activity of the Ca^{2+}-independent form (M_r approximately 65,000) was similar to that of the native enzyme, i.e., 2 to 5 μmol phosphate transferred min^{-1} mg^{-1} kinase. The 65,000-dalton fragment was phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase and approximately 0.8 moles phosphate were incorporated per fragment.

Myosin light chain kinase (MLCK) phosphorylates one class of myosin light chains and has been identified in all muscle types and in several non-muscle tissues (1). Initially it was thought (2) that the Ca^{2+}-dependent enzyme isolated from skeletal muscle was monomeric (M_r ~77,000). However, it was subsequently shown for the MLCKs of skeletal (3) and smooth (4) muscle that the Ca^{2+}-dependence was due to the presence of a second protein, namely calmodulin. The dependence on calmodulin has since been claimed for MLCKs isolated from a variety of tissues (1). A comparison of the many forms of MLCK indicate a considerable variation in the size of the apoenzyme and molecular weights range from 77,000 to 155,000 (1). For the skeletal muscle apoenzyme the M_r values are clustered around either 80,000 (2,5-8) or 90,000 (9,10).

Abbreviations: MLCK: myosin light chain kinase; MOPS: morpholinopropane sulfonic acid; EGTA: ethylene glycol-bis-(β-amino ethyl ether) N,N'-tetra-acetic acid; DTT: dithiothreitol; SDS: sodium dodecyl sulfate

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Recently it was shown (11) that the Ca^{2+}- and calmodulin-dependent MLCK from turkey gizzards could be converted to a Ca^{2+}- and calmodulin-independent form by limited proteolysis. The Ca^{2+}-independent form of the gizzard MLCK was interesting for two reasons: it was useful as an aid in investigating the role of myosin phosphorylation in the regulation of smooth muscle activity; and it was also useful in providing information on the structure and properties of the MLCK molecule. In the hope that a Ca^{2+}- independent form of the skeletal muscle MLCK might offer similar advantages this study was undertaken. Our intent was to determine if the conditions of proteolysis which were used successfully with the smooth muscle MLCK also applied to the skeletal muscle kinase, and if so, to define and compare the products of proteolysis. An earlier study by Tanaka et al. (12) was encouraging in that it was reported that tryptic hydrolysis of partially purified forms of MLCK from skeletal and smooth muscle resulted in a loss of Ca^{2+}-dependence. However, in these studies the levels of trypsin necessary to cause activiation in the absence of Ca^{2+} were high, and the products of proteolysis were not described.

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP was purchased from New England Nuclear, α-chymotrypsin and lima bean trypsin inhibitor were purchased from Millipore Corporation, (Freehold, NJ).

The method used for MLCK purification was adopted from Walsh et al. (13) with a few modifications: (1) extraction was done in 20 mM MOPS, pH 6.5 containing 0.2 mM DTT, 0.1 M KCl, 2 mM EDTA for 30 min; (2) ammonium sulfate fractionation was excluded. The MLCK obtained following affinity chromatography on calmodulin-Sepharose 4B was approximately 85% homogeneous as determined from scans of SDS polyacrylamide gradient gels. (The details of the purification procedure will be described elsewhere.)

Unphosphorylated myosin was extracted using the muscle residue after kinase extraction according to the method of Perry (14) with the following modifications: (1) extraction was done in 0.3 M KCl, buffered to pH 7.0 by sodium bicarbonate; (2) pellets after the second water precipitation were dissolved in 0.5 M KCl, 2 mM ATP, 2 mM $MgCl_2$ and the pH adjusted to 6.5; (3) the resultant supernatant was fractionated with saturated ammonium sulfate, the fraction obtained at 33 to 45% saturation was collected. This myosin was almost completely unphosphorylated as indicated by urea-gel electrophoresis (15). Whole light chain fractions were prepared according to the method of Perrie et al. (16). The amount of 18,000 M_r light chain was determined by densitometry of gels of the whole light chain fraction and varied from 30-40% of the total protein. Calmodulin was purified from frozen bull testis (17). Catalytic subunit of cAMP-dependent kinase was purified from bovine heart (18) and stored as described by Peters et al. (19).

Protein concentration was determined by the Biuret method (20) or by dye-binding as described by Spector (21).

Electrophoresis was carried out on 7.5 - 20% polyacrylamide gradient slab gels containing 0.1% SDS at 25 mA using the discontinuous buffer system of Laemmli.

Phosphorylation assays were carried out at 25°C in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 50 μM $CaCl_2$ or 2 mM EGTA and 1 mM [γ-^{32}P]ATP (about 3000 cpm/nmole). 0.5 ml of aliquots were withdrawn after various time intervals and were assayed for acid stable ^{32}P as described earlier (23). The time course of phosphorylation of MLCK by cAMP-dependent kinase was measured in a medium containing 15 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 0.5 mg/ml MLCK and 5 μg/ml of the catalytic subunit of cAMP-dependent kinase.

Preparation of Ca^{2+}-independent MLCK. The kinase obtained prior to and following affinity chromatography was dialyzed against 15 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 1 mM $MgCl_2$ overnight and used for α-chymotryptic digestion under various experimental conditions. The amount of α-chymotrypsin used was fixed at a weight ratio of protease to kinase of 1:100. This ratio was chosen after preliminary experiments of MLCK digestion at different concentrations of α-chymotrypsin and was based on the yield and minimum loss of enzymatic activity. Digestion at 25°C for 5 min was terminated by the addition of lima bean trypsin inhibitor at a weight ratio to α-chymotrypsin of 10:1. The chymotryptic digest was loaded immediately on a DEAE-Sephacel column (1.5x20 cm) equilibrated with 15 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 10 μM $CaCl_2$ 0.5 mM DTT. Protein was eluted with a linear gradient made from 75 ml each of the above buffer and buffer containing 0.50 M NaCl. Fractions (1 ml) were assayed for MLCK activity in the presence and absence of Ca^{2+} as described earlier and their protein contents were examined by SDS electrophoresis. Fractions containing Ca^{2+}- independent MLCK were pooled and stored in 1 ml aliquots at -20°C until use.

RESULTS AND DISCUSSION

Initially the conditions of digestion which are necessary to produce the Ca^{2+}-independent form of the MLCK were tested. As shown in Table I the Ca^{2+}-independent kinase was produced only in the presence of Ca^{2+} and calmodulin and in this respect the digestion profile is the same as that obtained for the smooth muscle MLCK (11). The digest obtained in the presence of Ca^{2+} and calmodulin was applied to a DEAE-Sephacel column (see Experimental Procedures) and the elution profile is shown in Fig. 1. The Ca^{2+}-independent MLCK was confined to a single peak which eluted at approximately 0.15 M NaCl. It was frequently found that if the partially purified MLCK (i.e., before chromatography on the calmodulin-affinity column) was digested under similar conditions the loss of Ca^{2+}-dependence was not complete. However the residual Ca^{2+}-dependent MLCK eluted from the DEAE-Sephacel column at a lower NaCl concentration than the Ca^{2+}-independent form and the two kinases could easily be separated (see Fig. 1).

Table 1

Effect of α-chymotrypsin digestion on MLCK under different experimental conditions

Experimental Conditions	Percentage MLCK activity (P_i/mol/mol light chain-2)[a]	
	+Ca	+EGTA
+Ca^{2+} and calmodulin		
α-chymotrypsin/LBTI[b]	80	75
LBTI/α-chymotrypsin	95	15
LBTI	100	12
No addition	100	10
+EGTA and calmodulin		
α-chymotrypsin/LBTI	92	8
LBTI/α-chymotrypin	98	10
LBTI	100	12
No addition	100	10

[a] Expressed as a percentage of the P_i incorporation into myosin (0.88 mol P_i/mol LC_2). MLCK activity was measured immediately after digestion under various conditions as described in text using 5 min incubation time.

[b] LBTI, lima bean trypsin inhibitor; LBTI was used to terminate digestion. Slash line defines which of the two α-chymotrypsin or LBTI was added first. Calmodulin concentration used was five-fold excess over MLCK concentration.

SDS-electrophoresis patterns of the Ca^{2+}-independent MLCK are included in Fig. 1. The major band which corresponds to the Ca^{2+}-independent MLCK had an M_r of ∿65,000. Contaminants of lower molecular weight were frequently seen, shown in Fig. 1 as a doublet, and at least one of these bands is thought to be derived from the native MLCK. (The parent MLCK, M_r ∿87,000, is also shown in Fig. 1.)

The loss of Ca^{2+}-dependence for the 65,000-dalton fragment is consistent with the finding that this peptide did not bind to the calmodulin-affinity column in the presence of Ca^{2+}. This indicates that the calmodulin-binding domain of the parent MLCK is either removed or destroyed by proteolysis.

The activity of the Ca^{2+}-independent MLCK using myosin as a substrate is shown in Fig. 2. The time courses of phosphorylation in the presence and absence of Ca^{2+} are essentially the same. The activity of the Ca^{2+}-independent MLCK is similar to the Ca^{2+}-dependent form, and specific activities of the two forms (estimated from the initial rates of phosphorylation of skeletal

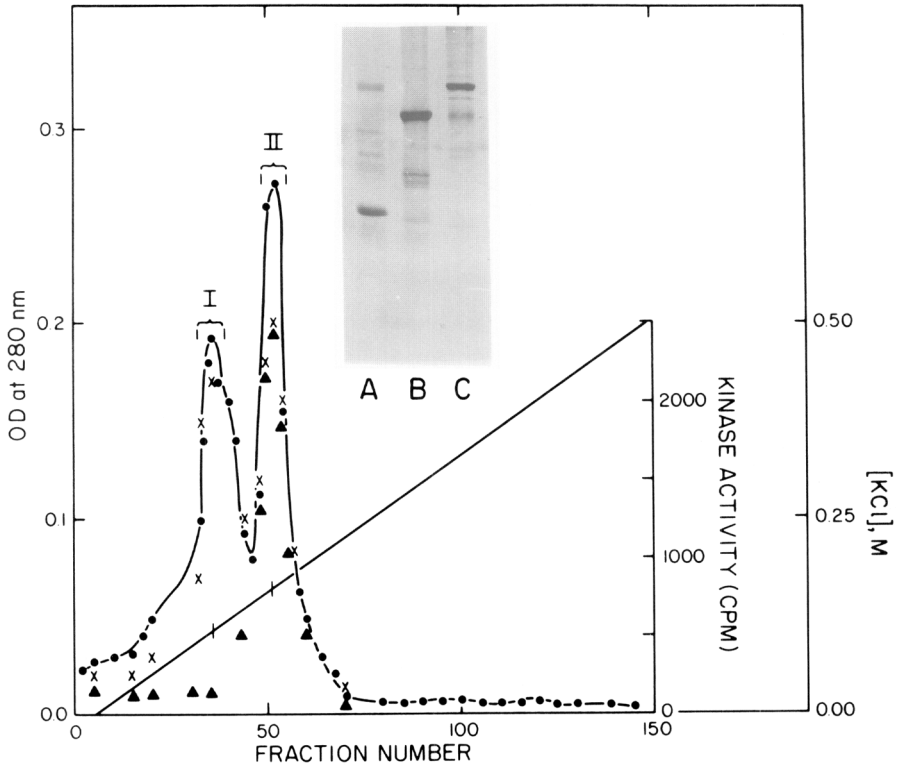

Figure 1. DEAE-Sephacel chromatography of the α-chymotryptic digest of myosin light chain kinase. 5-8 mg of digest was loaded on a column (1.5x20 cm) and eluted as described in Methods. Protein content was monitored at 280 nm (●). 20 µl of the selected fractions was used for monitoring kinase activity both in the presence (X) and absence (▲) of Ca^{2+}. Activity is expressed ^{32}P incorporated (cpm) into skeletal muscle myosin after 5 min incubation. Inset shows the SDS-gels for peak I(A), peak II(B) and original kinase (C).

muscle myosin) ranged from 3 to 5, and 2 to 5 µmol P transferred $min^{-1}mg^{-1}$ $kinase^{-1}$, respectively. Using isolated light chains as the phosphate acceptor for the Ca^{2+}-independent kinase the reaction was also Ca^{2+}-insensitive (data not shown). The activity of the Ca^{2+}-independent kinase, using either myosin or isolated light chains as substrate, was not affected by the addition of calmodulin.

It is known that the MLCK from smooth muscle is phosphorylated by the cAMP-dependent protein kinase (24) and that following proteolysis to produce the Ca^{2+}-independent form the phosphorylation sites are cleaved from the enzymatically active portion of the molecule (11). Recently it was shown (10) that the skeletal muscle MLCK is also phosphorylated by the cAMP-dependent

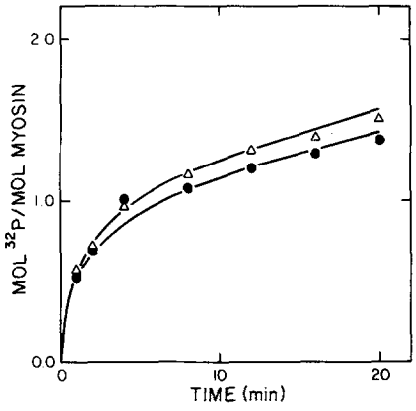

Figure 2. Time course of phosphorylation of skeletal myosin with Ca^{2+}-independent MLCK in the presence (△) and absence (●) of Ca^{2+}. Phosphorylation was carried out in a medium containing 20 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$ or 2 mM EDTA, 1-2 μM myosin, 1 mM ATP and 5 μg/ml kinase.

protein kinase and slightly greater than 1 mol ^{32}P was incorporated per mol MLCK. Using our preparation of MLCK from skeletal muscle we wished to determine if the native enzyme is phosphorylated by the cAMP-dependent protein kinase, and if so, to follow the fate of the phosphorylation site(s) on proteolysis to form the Ca^{2+}-independent kinase. These results are presented in Fig. 3. Approximately 1.2 mol ^{32}P/mol MLCK (assuming an M_r of 87,000) was incorporated into the parent enzyme. The Ca^{2+}-independent form was also phosphorylated, although the extent of the phosphorylation was lower than that of native kinase (Fig. 3).

In order to identify the peptide upon which the phosphorylation site is located the parent MLCK was phosphorylated using [γ-^{32}P]ATP with cAMP-dependent protein kinase and the digestion carried out to produce the Ca^{2+}-independent kinase. Following SDS-electrophoresis, excision and counting of each protein band the ^{32}P was located only in a peptide of M_r approximately 65,000. Autoradiography confirmed this location. It is therefore suggested that the hydrolysis to generate the Ca^{2+}-independent form liberates a peptide of M_r ~65,000 which contains both the catalytic site and the site of phosphorylation for the cAMP-dependent protein kinase. Studies are underway to determine if the phosphorylation site can be separated from the catalytic site without losing enzymatic activity.

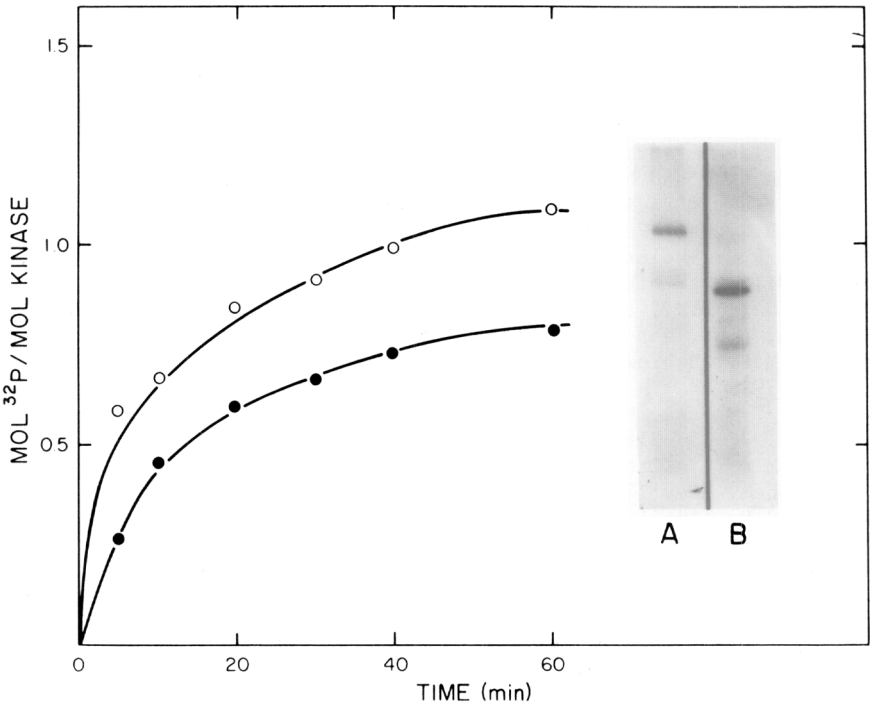

Figure 3. Time-course of phosphorylation of Ca^{2+}-dependent (O) and Ca^{2+}-independent (●) kinase. Inset shows autoradiographs of phosphorylated Ca^{2+}-dependent (A) and Ca^{2+}-independent (B) kinase.

The above results show that the Ca^{2+}-dependent form of the MLCK from skeletal muscle can be converted into a Ca^{2+}-independent form on limited proteolysis. During this conversion the site of phosphorylation by the cAMP-dependent protein kinase is retained, and in this respect the skeletal enzyme is different from the smooth muscle MLCK (11). The size of the active fragment had an M_r of about 65,000, whereas for the smooth muscle kinase the fragment was about 80,000. Further experiments are necessary to allow a more detailed comparison of these two enzymes.

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