FEBS Letters 346 (1994) 213–216

FEBS 14088

Heterogeneity of smooth muscle myosin light chain kinase

Characterization of isoelectric variants

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Received 21 April 1994

Abstract

Purified chicken gizzard myosin light chain kinase (MLCK) analyzed by anion-exchange high-performance liquid chromatography (HPLC) can be consistently resolved into three well separated peaks, termed α, β, γ. These peaks are shown to correspond to differently charged forms of MLCK with the charge difference between α and β twice as large as between β and γ. The isoelectric point and elution position of the peaks as well as their amplitudes are modified by phosphorylation or by autophosphorylation of MLCK suggesting that the observed charge differences are related to their different phosphate content. The three forms appear to have similar apparent affinity for both the substrates, ATP and the isolated regulatory light chain, but their specific activities are different.

Key words: Myosin light chain kinase; Smooth muscle; High-performance liquid chromatography; Isoform; Phosphorylation

1. Introduction

Ca^{2+}-calmodulin (CaM)-dependent phosphorylation of the 20-kDa regulatory light chain of myosin (LC 20) is a dominant regulatory mechanism in vertebrate smooth muscle. Myosin light chain kinase (MLCK) was identified as a Ca/CaM-dependent enzyme which catalyzes the transfer of phosphate from ATP to LC 20 [1]. Recently, factors other than Ca/CaM have been proposed to be involved in the biological regulation of MLCK activity. Among them there is the role of post-translational modification of MLCK, specifically phosphorylation, in modulating interactions between MLCK, LC 20 and CaM [2].

Previously we have described that purified MLCKase can be separated by anion-exchange HPLC into three species which retained phosphorylating activity and were characterized by similar peptide mapping [3].

In the present study the enzyme kinetic properties of these species have been elucidated in some details. In addition we show that the charge of the species was modified by MLCK phosphorylation.

2. Materials and methods

2.1. Protein and enzyme preparation

MLCK was purified essentially from chicken gizzard according to the procedure described by Sobieszek and Barylko [4]. MLCK concentration was measured from its absorption of 278 nm using the extinction coefficient $E^{1\%} = 1.1$ [5].

Turkey gizzard calmodulin and the regulatory myosin light chain, used as the kinase substrate, were purified as described previously [6,7].

2.2. High-performance liquid chromatography

The equipment used was the same described in [14]. Water used for chromatography was deionized (Milli-Q (Millipore)) and all solutions were degassed and passed through a 0.45-mm Millipore filter prior to the chromatography. All reagents used were of analytical-reagent grade.

Anion-exchange separations were performed on a Mono Q column from Pharmacia or on an analytical (75 × 7.5 mm, I.D.) Bio-Gel DEAE-5-PW column from Bio-Rad. All chromatographic runs were carried out at room temperature at a flow-rate of 1.0 ml/min. Separations on the Mono Q column were performed as described in [3]. Separations on the DEAE-5-PW column were performed as described in [8]. Peaks were collected manually and analyzed for MLCK activity.

2.3. Phosphorylation rate measurements

Phosphorylation assays were carried out as described previously [9].

2.4. Gel electrophoretic procedures

One-dimensional SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [10]. Isoelectric focusing was carried out in a pH range 7–9. Two-dimensional gel electrophoresis was performed according to O'Farrell [11].

3. Results

Standard purified MLCK from chicken gizzard subjected to anion-exchange chromatography on a Mono-Q column is normally resolved into three distinct fractions,

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Abbreviations: MLCK, myosin light chain kinase; HPLC, high-performance liquid chromatography; CaM, calmodulin; DTT, dithiothreitol; LC 20, 20-kDa regulatory light chain of myosin.

SSDI 0014-5793(94)00474-A

termed α, β and γ in order of increasing retention times, (Fig. 1a). The same profile was obtained after analysis the same type of preparation on a different ion-exchange chromatographic column (Fig. 1b). A similar chromatographic pattern was obtained for turkey gizzard MLCK (Fig. 1c), but in this latter case the relative proportion of the three peaks was different as compared to that of chicken MLCK. For turkey MLCK α and β were dominant on γ and were present in about the same amount. The separation and maxima of these three peaks varied a little from preparation to preparation, but for a given preparation the patterns wre reproducible. This variability could arise from oligomerization of the kinase [6] which depends on its concentration. However, the fact that very similar profiles were obtained for a given preparation at loading concentration ranging from 0.1 to 1.0

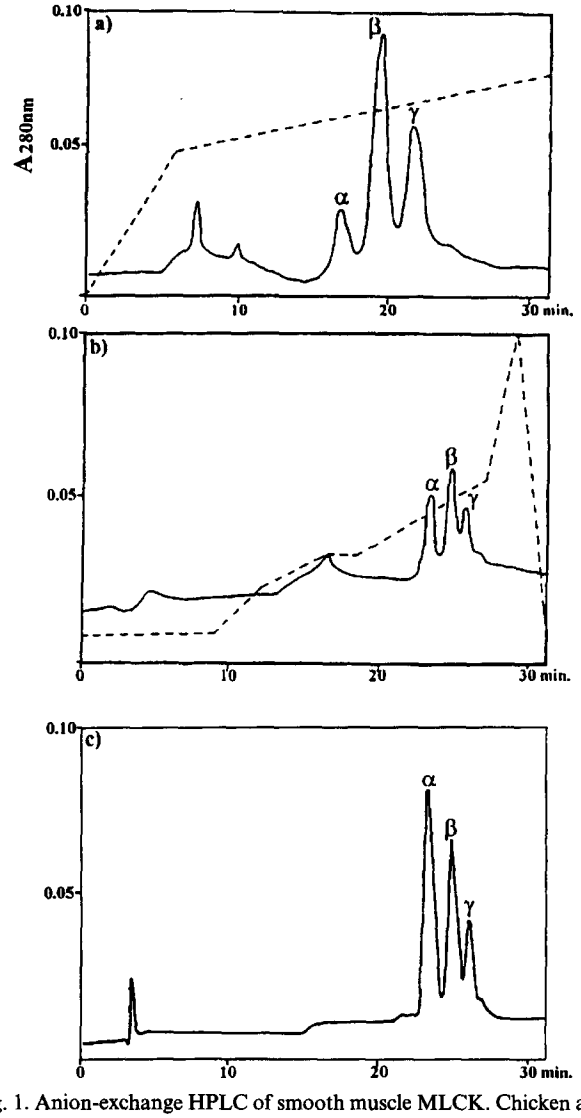

Fig. 1. Anion-exchange HPLC of smooth muscle MLCK. Chicken and turkey MLCK (250 μg), purified by standard techniques, were analyzed with two different columns. (a) Chicken gizzard MLCK on a Mono Q column; (b) chicken gizzard MLCK on a DEAE-5-PW column; (c) turkey gizzard MLCK on a DEAE-5-PW column. The relative elution conditions are described in section 2.

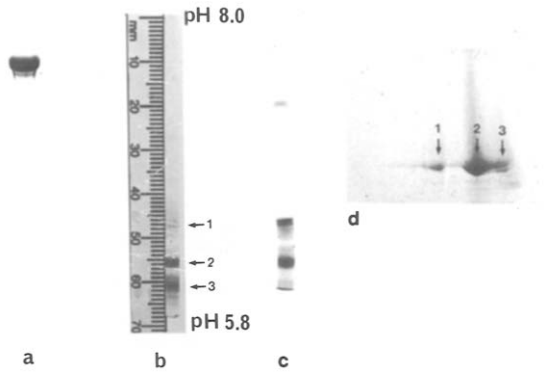

Fig. 2. One- and two-dimensional gel electrophoretic analysis of MLCK. (a) Chicken gizzard MLCK (4 μg) purified by standard techniques was analyzed by 10% SDS-polyacrylamide gel electrophoresis to check its purity; (b) isoelectric focusing of the same MLCK preparation (10 μg) was performed on a pH gradient ranging from 5 to 8; (c) isoelectric focusing of turkey gizzard MLCK (10 μg) was performed as in (b); (d) a gel parallel to that shown in (b) was run in the second dimension on a 6% SDS-polyacrylamide slab gel. Arrows and numbers in (b) and (d) indicate MLCK variants. All gels were stained with Coomassie brilliant blue.

milligram (results not shown) indicates that the observed differences were of different type and more likely reflected isoelectric variants. This conclusion was confirmed by a two-dimensional analysis of MLCK where the three forms were found to correspond to differently charged species of MLCK. From the results it is evident that a pure chicken gizzard MLCK preparation (Fig. 2a) gave origin to an isoelectric focusing pattern characterized by three bands (Fig. 2b, arrows), the isoelectric point values of which were 6.6, 6.3 and 6.0, respectively. For turkey MLCK the same basic pattern was found (Fig. 2c), except that the band corresponding to isoelectric point of 6.6 was present in major amount as compared with chicken. When analyzed in the second dimension, i.e. according to the molecular weight, the three proteins displayed the same electrophoretic mobility typical to MLCK, thus negating a possibility that the charge differences resulted from a simple proteolysis of MLCK (Fig. 2d).

Consistent with this observation the chromatographic pattern was different for MLCK preparations which underwent autophosphorylation or when the kinase was phosphorylated by catalytic subunit of cAMP dependent protein kinase. As is evident from Fig. 3 the relative amounts of α species appeared to remain unchanged while the amounts of β and γ species become markedly reduced. At the same time a new peak termed α' appeared between α and β. These results clearly show that the presence of MLCK variants was related to covalent modification (phosphorylation) of the enzyme.

Characterization of the enzyme kinetic properties involved initial rate measurements and were carried out over a range of ATP and LC 20 concentration of 14–350 μM and 12–300 μM, respectively. Using a common ap-

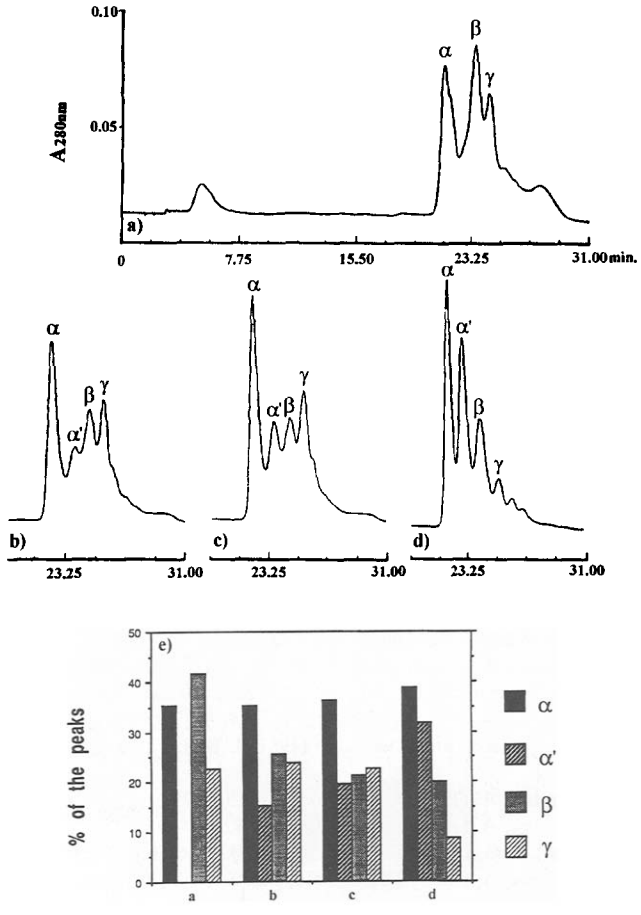

Fig. 3. Effect of phosphorylation of MLCK on its HPLC profile. MLCK was either autophosphorylated or phosphorylated by the catalytic subunit of cyclic AMP dependent protein kinase and then chromatographed on a DEAE-5-PW column as described in the legend to Fig. 1. (a) Control MCLK (250 μg); (b) autophosphorylated MLCK (MLCK, 5 μM; CaM, 200 nM; $CaCl_2$, 0.1 mM; ATP, 1 mM; total volume 700 μl, incubated on ice for 60 min; (c) MLCK phosphorylated by catalytic subunit of cyclic dependent protein kinase in the presence of calcium (MLCK, 5 μM; CaM, 200 nM; $CaCl_2$, 0.1 mM; cAMP subunit 5 μg; ATP, 1 mM; total volume 700 μl, incubated on ice for 60 min); (d) MLCK phosphorylated by catalytic subunit of cyclic dependent protein kinase in the absence of calcium (MLCK, 5 μM; CaM, 200 nM; EGTA 1 mM; cAMP subunit 5 μg; ATP, 1 mM; total volume 700 μl, incubated on ice for 60 min); (e) graphic illustrating the relative percentage of peaks in chromatograms of panels a, b, c and d. For panel b, c and d only the region corresponding to peaks is shown.

proach based on the reciprocal plots we obtained the curves shown in Fig. 4. The three isoelectric variants exhibited a similar apparent affinity for both ATP and LC 20, but the V_{max} was higher for α and β species as compared with that of γ.

4. Discussion

In a previous paper we have presented evidence, based on anion-exchange HPLC, about a possible existence of different charged species of MLCK in chicken gizzard smooth muscle MLCK [3]. In the present report we extend those observations and demonstrate that these species arise from the presence of different isoelectric variants as well as from the kinase being phosphorylated to a different extent. Furthermore we characterized the enzyme kinetic properties of the three variants.

By means of two different HPLC columns we were consistently able to fractionate chicken and turkey MLCK into three peaks, α, β and γ. Both in the HPLC and in isoelectric focusing experiments we can see that

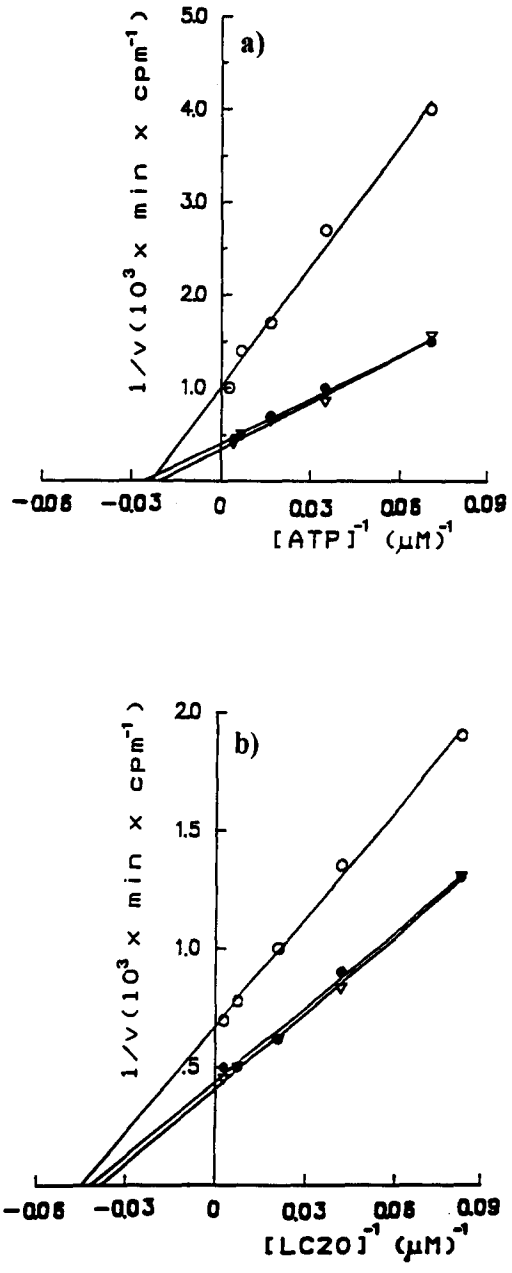

Fig. 4. Initial velocity relationship pattern for MLCK isoelectric variants as a function of ATP (a) and LC 20 (b) concentration. MLCK was chromatographed on a Mono Q column as described in Fig. 1. The three peaks were collected manually and the rates of ^{32}P incorporation into LC 20 were determined as indicated in section 2. Key: peak α (●); peak β (▽); peak γ (○).

the charge difference between α and β is twice as large as between β and γ.

The amplitude of the peaks varied for chicken and turkey MLCK, suggesting the presence of a true isomer distribution different for the two avian species. This is in agreement with the already observed structural differences between the two MLCKs [12]. In addition different HPLC patterns were already obtained for newborn and adult chicken MLCK [8]. The three MLCK species can have origin from amino acid sequence differences and/or from post-translational modifications of the protein. MLCK is known to exists as several distinct molecular species in skeletal muscle, heart, and smooth muscle or non-muscle tissue [13]. Moreover for rabbit skeletal muscle MLCK sequence analysis indicates the presence of approximately equal quantities of two isoforms differing in a single amino acid replacement [14]. Isoforms were also found for cAMP-dependent protein kinase (cAPK) and protein kinase C (PKC) (see [15] for a review).

As far as smooth muscle MLCK is concerned only one full-length cDNA clone has been identified. This has allowed the definition of the complete amino acid sequence of the protein [16], suggesting that one type only of MLCK is expressed in this muscle. In agreement with this observation we have shown that peptide maps of the three forms are very similar [3]. In the present report we demonstrate that both the amplitude and the elution position of the three forms were affected by phosphorylation of MLCK. Therefore we conclude that the observed charge differences were due to different phosphate content as well as could result from small differences in the amino acid sequence.

Enzymatic properties of MLCK isoforms have been characterized by determination of specific activity (V_{max}), apparent Michaelis–Menten constants (K_m) and of their apparent affinity for Ca-CaM complex. Some differences were observed for the three forms, α and β appearing to have higher specific activity than γ, even though the K_m value for both ATP and LC 20 is similar for all the three forms.

Studies are in progress to understand the relationship, if any, between MLCK forms and their autophosphorylation levels in the regulation of smooth muscle contraction.

Acknowledgements: These studies were supported by institutional funds of the Consiglio Nazionale delle Ricerche to L.D.L. and by the Austrian Science Foundation to A.S. Part of the work was carried out by L.D.L. in A.S. laboratories thanks to a short-term fellowship from EMBO. We thank the slaughter-house 'Tre Valli' for providing chicken and turkey gizzards and Mr. V. Gobbo for technical assistance.

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