Skeletal muscle myosin light chain kinase

A refined structural model

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A hydrodynamic, enzymatic and CD spectroscopic study of skeletal muscle myosin light chain kinase, three proteolytic fragments and corresponding complexes with calmodulin was performed. A refined shape model was built for the enzyme. It was shown that a head-and-tail structure is formed from two major fragments which are aligned end-to-end. The one fragment (M_r 36000) is compact, of high α-helix content and contains the catalytic center with the light chain and the calmodulin binding domains. The other fragment (M_r 33000) with unknown function is asymmetric ($a/b > 10$), of low α-helix and of unusually high proline content.

Myosin light chain kinase *Calmodulin* *Proteolytic fragment* *Structural model*

1. INTRODUCTION

Myosin light chain kinase (MLCK) has been shown to be present in all types of muscle and also in non-muscle tissues. Most of these kinases are regulated by calmodulin (CM), the multifunctional Ca^{2+}-dependent regulator. For a review see [1]. In contrast to other known protein kinases (e.g., cyclic AMP-dependent kinases) this enzyme seems to be highly specialized showing high substrate specificity only for light chains 2 (LC2) of myosin [2]. In contrast to the large number of reports dealing with kinetic properties of myosin light chain kinase from different sources, only few amount data are available on structural details of this enzyme [3,4], although perhaps important for the high substrate specificity.

We have started therefore a detailed study of the shape and substructure of the skeletal muscle enzyme mainly by hydrodynamic and CD spectroscopic techniques using the native protein and three well characterized proteolytic fragments. From these data, a model could be developed which directs towards a head-and-tail structure of the enzyme with the head containing the enzymatically relevant domains and a proline-rich tail of unknown function.

2. MATERIALS AND METHODS

2.1. *Detailed isolation procedures*

Isolation of bovine brain CM, highly active (>70 U/mg) MLCK, LC2 from rabbit skeletal muscle and of the proteolytic fragments of MLCK termed FH_s, FH_t and FT are described in detail in [Biochemistry (1983) in press].

2.2. *Analytical ultracentrifugation experiments and subsequent data processing*

These are also described in detail in the above paper. Buffer conditions for the sedimentation equilibrium runs were: 0.5 M KCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.2 mM DTE. For sedimentation velocity studies a buffer of the composition: 0.1 M KCl, 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM DTE was employed containing either 0.2 mM $CaCl_2$ or 0.5 mM EGTA in addition.

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2.3. *Activity measurements and reconstitution experiments*

Activities were measured at 30°C in 40 mM Tris–HCl (pH 8.0), 8 mM $MgAc_2$, 100 μM $CaCl_2$, 1 mM DTE, 1 mM [γ-^{32}P]ATP (0.2 mCi/μmol), 1 μM CM, 70 μM LC2 at appropriate dilutions of the enzyme. Aliquots were analysed as in [5]. CM-independent activity was determined by including 0.5 mM EGTA in the above solution. K_m values for LC2 were determined at the same conditions as above. Reconstitution experiments using fragments FH and FT were performed by incubating equimolar amounts of both fragments at appropriate concentrations in the above assay solution without ATP for 30 min. Then the activity measurement was started by adding ATP.

2.4. *Hydrodynamic data treatment and model building*

For the native enzyme, the three fragments and corresponding calmodulin complexes, axial ratios for prolate and oblate ellipsoids of revolution were calculated by a combination of sedimentation coefficients and molecular masses and a reasonable degree of hydration. First, Stokes' radii were calculated from M and the s-value, with density ϱ, viscosity of solvent η (in centipoise) and N as Avogadro's constant, as:

$$R_{S,Sed} = M(1 - \bar{v}\varrho)/(6\pi\eta N s)$$

Frictional ratios $f/f_o = R_{S,Sed}/R_o$ were obtained using $R_o = [3\bar{v}M/(4\pi N)]^{1/3}$. Using hydration values δ estimated as in [6], axial ratios for ellipsoids of revolution now were determined via the Perrin shape factor F using $f/f_o = F[1 + \delta/(\varrho\bar{v})]^{1/3}$. Dimensions of the hydrated species were estimated via the hydrated radius $R_h = R_o \cdot [1 + \delta/(\varrho\bar{v})]^{1/3}$ by the equations given in [7].

Based on the hydrodynamic dimension estimates, a refined model was generated using spherical beads of fixed dimensions assembled in hexagonal dense packing in a way to simulate the suspected overall shape of the enzyme. Frictional ratios were calculated by the approach applied for macromolecules by Bloomfield et al. [8,9] using the formulation:

$$f_o = 6\pi\eta R_o$$

and

$$f = 6\pi\eta nr/(1 + \frac{r}{n}\sum_{i=1}^{n}\sum_{\substack{j=1 \\ i\neq j}}^{n} R_{ij}^{-1})$$

with a number of beads n of hydrated radius r and distances R_{ij} between beads i and j. The f/f_o ratios were compared with the corresponding ones derived from the s-values and the assembly of the beads was changed until best agreement in frictional ratios was obtained. A computer program (HP 85 program BLOOM, available upon request) was performed for calculation.

3. RESULTS AND DISCUSSION

3.1. *Isolation of proteolytic fragments of myosin light chain kinase*

Myosin light chain kinase of >99% purity and a specific activity of 70–100 U/mg was isolated by a rapid isolation procedure (section 2). Proteolysis occurred at an early purification stage by an endogenous protease allowed to isolate two distinct fragments of M_r 35000 and M_r 33000 as determined by gel electrophoresis as in [10]. The former of these fragments still bound specifically to CM and thus could be further purified by CM-Sepharose chromatography whereas the latter did not. The CM-binding fragment was termed FH_s (spontaneously proteolytic head fragment), the other fragment was termed FT (tail fragment, see below for explanation).

A slightly larger CM-binding fragment of M_r 36000, termed FH_t could be generated from native MLCK when the enzyme was adsorbed onto CM-Sepharose and digested by trypsin. After washing out the trypsin together with non-binding digestion products, two peptides of M_r ~55000 and M_r ~36000 remained adsorbed which could be specifically eluted by EGTA. The M_r 36000 CM-binding fragment termed FH_t (tryptic head fragment) was further purified to >97% purity before use for the following studies.

3.2. *Characterization of the fragments*

Table 1 summarizes the results of the enzymatic characterization of these fragments. Both CM-binding fragments FH_s and FH_t showed significant

Table 1

Enzymatic properties of MLCK and its fragments FH_s, FH_t and FT

	MLCK	FH_s	FH_t	FT	$FH_s \cdot FT$		$FH_t \cdot FT$
					4 nM[a]	40 nM[a]	40 nM[a]
% Activity (+ Ca^{2+})[b]	100	12.6	24.7	0.2	30.7	56.3	29.0
% Activity (+ EGTA)[b]	0.8	1.6	0.2	0.1	0.4	0.5	0.4
Fraction of Ca^{2+}–CM dependent activity (%)	>99	87	>99	–	99	>99	99
K_m for LC2 (μM)[c]	18	17.5	17.8	–	–	–	–

[a] Concentration of the 1:1 mixture in the assay based on the relative molecular masses of table 3

[b] Mean specific activities of the fragments are given as % values of the activity (+ Ca^{2+}) of MLCK from which they were isolated. For conditions see section 2

[c] Same conditions as for the above measurements, except that LC2 concentration was varied

Table 2

Amino acid composition of MLCK and fragments FH_s and FT given in mol%

	MLCK	FH_s	FT	FH_s + FT[a]
Asx	8.5	12.3	6.9	9.6
Thr	4.8[b]	4.6	4.0	4.3
Ser	6.6[b]	6.2	6.7	6.5
Glx	13.6	11.8	17.5	14.7
Pro	9.0	5.8	11.1	8.5
Gly	8.6	6.2	11.3	8.7
Ala	10.4	5.5	11.8	8.6
Val	5.4[c]	6.2	4.7	5.5
Cys	1.8[d]	n.d.	n.d.	–
Met	1.7[e]	3.5[e]	1.0[e]	2.3
Ile	4.1[c]	4.9	3.3	4.1
Leu	7.6	10.4	6.8	8.6
Tyr	1.2[f]	2.0[f]	1.0[f]	1.5
Phe	3.4	4.7	3.0	3.9
Lys	7.3	7.6	6.6	7.1
His	1.6	2.5	1.4	1.9
Arg	3.4	4.5	2.4	3.5
Trp	0.8[g]	1.1[h]	0.2[h]	0.7

[a] Weight average contents calculated using the relative molecular masses of table 3

[b] Obtained by extrapolation to zero hydrolysis time

[c] 72 h values

[d] Determined after performic acid oxidation as in [16] as cysteic acid

[e] From thioglycolic acid containing samples

[f] Mean of the values from amino acid analysis and those determined as in [17]

[g] Mean of spectrophotometric determinations [17] and colorimetric ones [18]

[h] Determined only as in [17]

Samples were hydrolyzed in 6 N HCl at 110°C for 24, 48 and 72 h for MLCK and for 36 h for the fragments

activity which still was CM-dependent by 87% and 99%, respectively. Their K_m values for LC2 were identical to that of native MLCK. The relative molecular masses of FH_s and FT closely added up to that of native MLCK. As only these two fragments were detected after spontaneous proteolysis of native MLCK we tried to find out whether they are complementary; i.e., generated by restricted cleavage in the middle of the polypeptide chain. Three criteria indeed point towards such a complementarity:

(i) The amino acid contents of the fragments FH_s and FT were quite different (table 2); however, taken together they add up closely to that of native MLCK, suggesting that, if any, only a small polypeptide region may be missing;

(ii) Mixing equimolar amounts of the active fragment FH_s with the inactive fragment FT increased the specific activity (as based only on the M_r of FH_s) although FT was not active itself (table 1). In addition the degree of CM dependence of this activity increased from 87–99%. Both findings suggest that partial reconstitution has led to a conformation of FH_s closer to that in the native enzyme which should have been possible if no segment of the polypeptide chain was missing or present in duplicate. In contrast, combination of FH_t, the active tryptic fragment, with FT did not significantly change the activity.

(iii) Complementarity is also evident from the CD spectra shown in fig.1. Fragment FH_s showed a CD spectrum similar to that of other intracellular

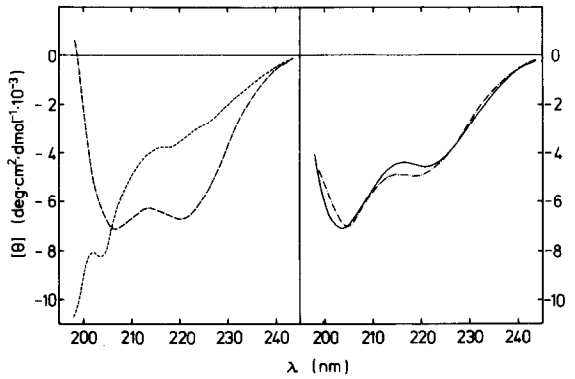

Fig.1. Far-UV CD spectra of proteolytic fragments FH_s (– – –) and FT (- - -), of native MLCK (——) and the residue number average spectrum calculated for an equimolar mixture of FH_s and FT (-----). CD spectra were recorded under the buffer conditions in section 2.2 for the sedimentation velocity studies in quartz cells of 0.2–1 mm pathlength at 20°C. A Dichrograph Mark III (CNRS–Roussel Jouan/Jobin Yvon) was employed.

globular enzymes with relatively high α-helix content. In contrast, fragment FT had a quite unusual CD spectrum suggesting very little α-helix and a high amount of non-helical structure. The residue number average spectrum calculated for a 1:1 molar mixture of both fragments however was very similar to the spectrum measured with native MLCK; so the two fragments obviously represent most of the secondary structure of the whole enzyme. Fragment FH_t showed a CD spectrum very similar to that of FH_s.

3.3. *Hydrodynamic properties of the native apo- and holoenzyme and of the fragments*

Isolation of two large complementary and obviously conformationally intact fragments, FH_s and FT, and of a third fragment, FH_t, which was very similar in size and characteristics to fragment FH_s allowed for a significant expansion of the number of hydrodynamic data which could be incorporated into a refined shape model of the native enzyme. These data are compiled in table 3. Identification of fragments FH_s and FH_t as CM-binding also allowed for a rough positioning of CM in the holoenzyme. An analysis of the 1:1 molar Ca^{2+}–CM complexes of these fragments and of native MLCK was possible since complex formation was shown by sedimentation equilibrium and the sedimentation profiles (no CM boundary detectable in presence of Ca^{2+}) to be almost complete at the concentrations employed (0.2–1 mg/ml of complex).

A low M_r for native MLCK of 70300 in contrast to ~80000 as estimated from gel electrophoresis as in [10] was established by a large number of sedimentation equilibrium experiments performed at different speeds and loading concentrations. As also the buoyancy term has been measured, this M_r of MLCK was used for further calculation. For the

Table 3

Hydrodynamic parameters of MLCK, proteolytic fragments and of the calmodulin complexes

Parameters	MLCK	MLCK·CM	FH_s	FH_s·CM	FH_t	FH_t·CM	FT	Ca^{2+}–CM
$\bar{v}_{20}$ (ml/g)	0.732[a]	0.721[a]	0.735[b]	0.726[b]	0.735[b]	0.726[b]	0.726[b]	0.707[c]
M	70300	87200[d]	36100	53000[d]	36500	53400[d]	32600	16720[e]
$s_{20,w}$ (s)	3.03	3.63	3.11	3.73	3.08	3.56	1.81	1.83
$R_{S,Sed}$ (Å)	54.5	58.8	27.3	34.5	27.8	36.3	43.6	23.8
f/f_o	2.00	2.02	1.25	1.40	1.27	1.46	2.07	1.42
δ_{calc} (g/g)	0.42	0.43	0.42	0.43	0.42	0.43	0.47	0.46
$a/b_{prolate}$	13	13.2	2.0	3.7	2.3	4.6	13.5	3.9
a/b_{oblate}	16.9[f]	17.1[f]	2.0	4.0	2.3	5.1	17.6[f]	4.1

[a] Measured values after normalization
[b] Calculated from amino acid composition
[c] From [19]
[d] Relative molecular mass obtained by addition of 16720 to the one of the 'apoenzyme'
[e] Calculated from primary structure
[f] Unlikely values, given only for completeness

fragments analyzed, means of the M_r values determined by electrophoresis as in [10] and the relative molecular masses estimated by sedimentation equilibrium experiments are given. In these cases, partial specific volumes have been calculated from the amino acid composition as in [11]. The s and $\bar{v}$ values of table 3 are given as values normalized to water and 20°C, the frictional ratios however were calculated directly from the values measured at 4°C to avoid normalization errors. For the correction of $\bar{v}$ values, the relationship $d\bar{v}/dT$ = 0.0004 ml/(g·K) was employed according to [12].

The high frictional ratios found for MLCK and MLCK–CM may be the result of a high degree of asymmetry and/or hydration. Hydration alone however may not have caused these high values as then a hydration of >3 g/g had to be assumed, a value quite unusual for native proteins. Therefore a significant amount of asymmetry seems to be present in the native enzyme with and without CM.

Both head fragments in presence and absence of CM showed relatively high s-values and low frictional ratios as compared with the native enzyme directing towards a relatively compact, not more than normally hydrated structure. This finding also was the reason for the corresponding terminology for these 'head fragments'. In both cases frictional ratios increased upon CM binding, perhaps caused by an increase of asymmetry.

The fragment FT sedimented appreciably more slowly than the FH fragments and correspondingly showed a very high frictional ratio. This can only be due to significant asymmetry if a reasonable hydration between 0.5–1 g/g is assumed. The name FT, tail fragment, therefore was chosen.

3.4. *Building a shape model of the native apo- and holoenzyme*

For each of the fragments and their calmodulin complexes axial ratios for prolate and oblate ellipsoids of revolution were calculated using the hydration values given in table 3. In order to construct a bead model according to [8,9] based on these data, a decision was necessary on the optimal size of a hydrated model sphere which should act as the unit for building up all the fragments and calmodulin complexes. As fragment FT showed the greatest deviation in axial ratio from unity and therefore only a prolate ellipsoid seemed reasonable, the model sphere size was chosen so that, based on the hydration in table 3, the dimensions calculated for the prolate ellipsoid representing FT could also be approximated by a linear array of these spherical beads. With 12 beads of a hydrated radius of ~11 Å a good approximation of the frictional ratio of the model to that in table 3 was obtained. Further small changes in the dimension of the model sphere were made in order to get, for a certain number of beads, the closest agreement to the hydrated volumes of all species calculated from relative molecular masses and hydrations in table 3. A bead of 5760 Å^3 of hydrated volume was finally used. Numbers of beads and resulting hydrated volumes chosen for the single species of table 3 are given in fig.2. The beads were rearranged for each species until the best approximation to the frictional ratios of table 3 was achieved (fig.2). Next, the complementary fragments with and without CM were arranged in a way that again best agreement of the calculated frictional ratios to the ones of the native apo- and holoenzyme given in table 3 was obtained (fig.2). Only one type of arrangement, namely an end-to-end arrangement proved to yield this close agreement.

By the bead modelling results it was also shown that binding of CM to the head fragments and to

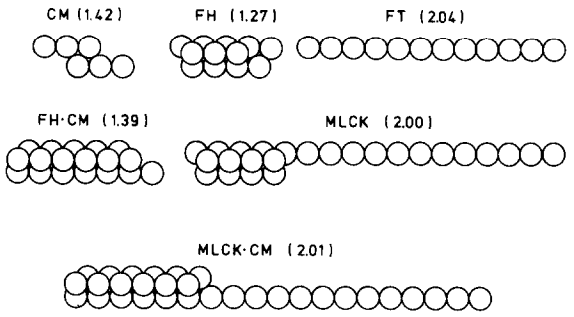

Fig.2. Final bead models of MLCK, the proteolytic fragments FH_s and FT and the corresponding complexes with CM obtained as discussed in the text. Hydrated volumes (in cubic Å) for the single species shown are 34560, 144000, 178560, 69120, 103680, and 69120 for CM, MLCK, MLCK·CM, FH_s, FH_s·CM and FT, respectively. Frictional ratios calculated as in section 2.4 are indicated. Hydrations for all species shown are close to the values of table 3 (0.44–0.55 g/g). An oblate shape of FH_s and FH_s·CM was excluded since alignment to FT always resulted in f/f_o values which were too low. For CM alone, several shapes were possible.

the apoenzyme obviously leads to an increase in asymmetry of the head part of the enzyme. As the LC2 binding domain obviously resides totally on this fragment (see unchanged K_m values above), the elongated shape of the head may somehow be a prerequisite for the binding of this substrate.

3.5. *Further structural details evidenced*

Reconstitution of FH_s with FT has led to an increased CM dependence of enzymatic activity, whereas the slightly larger fragment FH_t alone already showed this stronger CM dependence. This might be possible if a part of the CM binding site conferring CM dependence to the activity of the enzyme resides in a region of FT close to the cleavage site, which may not have been split off in FH_t. In smooth muscle MLCK [13,14] some structural differences to the skeletal muscle enzyme seem to exist, since the smallest proteolytically produced still active fragment showed M_r ~50000 and was no longer CM-dependent, and the major CM-binding fragment no longer expressed activity. However as no certain criteria of a nonoverlap or a consecutive array of the fragments isolated were provided, it may also apply for the smooth muscle enzyme, that the activity center and most of the CM-binding domain reside on a fragment of the enzyme of M_r not much larger than 35000.

The head fragment contains most of the α-helix present in whole MLCK. UV-spectra (not shown) and the amino acid compositions show, that most of the aromatic residues also reside in this catalytic and CM-binding fragment. The amino acid composition of FT shows an unusually high content of the small nonpolar amino acids Pro, Gly, Ala together with a high amount of Glx as compared with FH_s whereas the major fraction of Met, Arg and of the branched chain amino acids is contained in fragment FH_s. This high degree of imbalance in amino acid distribution also points to quite different structures and functions of these fragments. In the foregoing sections it was shown, that FT obviously is highly asymmetric with average dimensions of ~18 Å of diameter and ~270 Å of length. On the other hand, it was shown by the CD-spectrum in fig.1 that FT contains only a very low amount of α-helix. The asymmetric structure of this fragment therefore must be made up by any other kind of extended secondary structure. A simple calculation based on the number of residues of this fragment (~300) showed, that the length of the fragment (see above) could only be spanned by 2 or 3 chains of any secondary structure with 3 or 2 Å rise per residue, respectively. Only two kinds of extended secondary structure come into account: the β-sheet (3.3 Å rise/residue) and the collagen-like or polyproline-like extended helix (2.9 Å rise/residue) [15].

The high contents of the above mentioned amino acids indeed suggest the presence of the latter extended helical structure but from the amount of these residues and from the CD-spectrum it may be assumed, that this is not the only structural component responsible for asymmetry.

4. CONCLUSIONS

We could demonstrate by the data presented, that skeletal muscle myosin light chain kinase is built up from two major fragments, termed head fragment FH and tail fragment FT, both aligned end-to-end. The head fragment is compact and shows a high content of α-helix; it contains the catalytic center with the whole LC2 binding domain and the CM binding domain. The tail fragment though low in α-helix content is highly asymmetric. Its unusual amino acid composition suggests a special type of extended secondary structure responsible for the asymmetry. The function of this fragment is unknown; however, it may be speculated that a kind of 'anchoring' of the protein to myofilaments might necessitate this part of the enzyme.

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