Cleavage points of rabbit skeletal myosin light chains selectively modified in situ by limited proteolysis: structural characteristics of the neoformed isozymes

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Abstract **The functional significance of myosin light chains in vertebrate striated muscle is an issue of interest and myosin species selectivity modified by papain or trypsin in their LC1 and LC2 light chains are potentially useful for further investigation. We therefore determined the cleavage sites resulting in the (T)-LC1′, (P)-LC1′ and (T)-LC2′ species. Sequence analysis of (T)-LC1′ indicated that the cleavage point in LC1 is at Lys^7. Under appropriate conditions papain rapidly cleaves a short N-terminal segment from myosin light chain 1 and produces a new isozyme specifically modified in its essential light chain 1. The cleavage occurred at either Ala^{11}, Ala^{12} or Ala^{13}, the Ala^{11} cleavage being the most frequent. Trypsin was used to produce a myosin species with a regulatory light chain 2 specifically truncated of a short N-terminal segment. The cleavage was specific at Arg^8 with no indication of other significant cleavage sites in this LC2. The effects of trypsin and papain on myosin light chains are different, indicating different proteolytic specificities. None of these modifications, including (CT)-LC2″ cleavage at Phe^{19}, changed the K^+-EDTA- and Ca^{2+}-ATPase activities of monomeric myosin significantly, indicating that LC1 and LC2 N-termini have little or no direct influence on the active site. An electric birefringence study also showed that these modified species retained their average shape and flexibility. These observations are essential in showing that the role of light chain extremities is expressed only in the presence of a minimum of structural organization (filament or acto–myosin complex).**

Key words: Myosin light chain (rabbit fast skeletal); Artificial myosin isozyme; Limited proteolysis; N-terminal sequence; LC1 variability; ATPase; Electric birefringence

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Abbreviations: HMM, heavy meromyosin; S1, myosin subfragment 1; EDTA, ethylenediamine tetraacetic acid; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); DTT, 1,4-dithiothreitol; Gdn-HCl, guanidine hydrochloride; PVDF, polyvinilidene difluoride; tmA, *N*-trimethyl alanyl; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CT, α-chymotrypsin; P, papain; T, trypsin. Because of the influence of preparative methods on the structure of such subunits as LC1 and LC2, these subunits are annotated in the following way: information given in parentheses before the name of the subunit refers to the origin of the subunit, i.e. summarizes conditions under which it was obtained, e.g. (T)-LC1′ defines a myosin light chain 1 modified by trypsin. Trypsin (bovine pancreas) (EC 3.4.21.4); papain (Papaya latex) (EC 3.4.22.2); α-chymotrypsin (EC 3.4.21.1). LC1 is also called ELC1f or A1; LC2: RLCf, DTNB-, pseudo-regulatory or P-light chain; LC3: ELC3f or A2.

1. Introduction

During proteolytic digestion of myosin (rabbit fast skeletal in this study) to prepare HMM or S1 subfragments, myosin light chains are affected variously according to the experimental conditions. Chymotryptic cleavage under conditions producing S1 ((CT)-S1; millimolar EDTA, low ionic strength) degrades LC2 completely such that it is absent from the resulting S1 preparations [1]. LC1 and LC3 are left intact. In tryptic S1 preparations ((T)-S1), a number of observations have strongly suggested that a short fragment is cleaved from the N-terminus [2,3]. This has been confirmed (RC unpublished results) by ^{1}H NMR study of the characteristic methyl resonance of the N-trimethylalanine N-terminal residue at 3.23 ppm. The two S1 species obtained from papain proteolysis ((EDTA,P)-S1 and (Mg,P)-S1) also have a residual LC1′ resulting from the loss of a small N-terminal fragment. Whereas (EDTA,P)-S1 is very similar to (T)-S1 and comprises no LC2, (Mg,P)-S1 retains LC2 [4], a variable proportion of it being cleaved in its N-terminal region. These features are also found in the various HMM species.

These three proteolytic enzymes attack the various sites with significantly different rates such that the effect of each is nearly specific: thus from a practical point of view, they can be used for the preparation of various 'artificial' myosin isozymes [5], namely: myosin-[(P)-LC1′], myosin-[(T)-LC2′], myosin-[(CT)-LC2″] and variants.

The N-terminus of LC1 (in a chymotryptic S1) interacts with the actin C-terminus [6–8], a property which suggested a modulatory function in actin-myosin interaction during contraction. Similarly there is evidence that LC2 is involved in the affinity of myosin for actin and thereby modulates the mechanical performance of the fiber (for a short review see Solaro [9]). The proteolytic breakdown of LC2 in situ observed in idiopathic dilated cardiomyopathic hearts has been suggested as a major cause of heart failure [10]. The whole LC2 has been removed either chemically or by proteolysis. ^{1}H NMR and proteolytic investigations suggested that the N-terminus particularly was involved in the movement of the heads to and away from the filament backbone [11]. A recent study showing functional effects of LC1 reassociation with cardiac (papain-Mg)-S1 suggested that intact, uncleaved LC1 is required for the stability of S1 heavy chains and correct Ca^{2+} regulation [12].

There is sustained interest in the functional significance of myosin light chains in vertebrate striated muscle, and myosin species selectively modified in their LC1 and LC2 light chains

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are potentially useful for further investigations. We therefore characterized in detail the cleavage sites of the (P)-LC1′, (T)-LC1′, and (T)-LC2′ species.

The neoformed myosin species were previously shown to retain full K^+-EDTA- and Ca^{2+}-ATPase activity with a slightly less favorable stability as compared to native samples. The flexibility of myosin is an essential feature in the cross-bridge mechanism. A study of the electric birefringence of myosin solutions at high ionic strength showed that cleavage of either LC1 or LC2 does not alter this characteristics.

2. Materials and methods

2.1. Light chain preparations

The modified light chains were extracted from the artificial isoforms myosin-[(T)-LC1′], myosin-[(P)-LC1′] and myosin-[(T)-LC2′] prepared as described previously [5,13] except that the selective proteolysis was continued for slightly longer for myosin-[(P)-LC1′] and myosin-[(T)-LC2′] and five times as long for the preparation of myosin-[(T)-LC1′]. Modified myosin was made up to about 20 mg/ml and denatured in the presence of 5 M Gdn-HCl, 10 mM EDTA and 5 mM DTT (the pH was adjusted to 8.0 with 1 M Tris). The sample was left to stand for 1 h at room temperature and then cooled to 4°C. The cooled solution was diluted 2-fold with 10 mM EDTA, pH 6.75 at 4°C and then 0.82 vol. of a saturated ammonium sulfate solution (containing 10 mM EDTA and adjusted to pH 6.75) was added dropwise. A slight excess of ammonium sulfate was sometimes necessary for complete precipitation of the heavy chains. The mixture was left to stand for 20 min at 4°C, then the heavy chains were pelleted by centrifugation at $70{,}000 \times g$ for 30 min at 4°C. The supernatant was dialyzed exhaustively against a solution 5 mM mercaptoethanol to eliminate salts. The solvant was then eliminated by freeze-drying. The mixture of light chains was stored frozen at −25°C until further processing.

2.2. Separation of light chains

To obtain a fine separation and in particular avoid contamination by uncleaved light chains and heavy chain fragments, light chain fragments were purified by semi-preparative disc gel electrophoresis (SDS-PAGE). To avoid possible blocking of N-termini the gels were left to polymerize overnight and a long pre-electrophoresis step (45 min) was carried out in the presence of 20 μM thioglycolate. Samples containing about 1–2 nmol of each light chain were deposited on each gel (5 mm diameter).

The gels were rinsed briefly then cut longitudinally and the proteins were transferred onto two superposed PVDF membranes (Millipore, Immobilon-P) in a Bio-Rad MiniTrans Blot apparatus (35 V constant, 25–30 min) with 50 mM Tris-H_3BO_3, pH 8.3 as the transfer solution. The poor affinity of light chains for the membrane led to a reduced yield (60–70% on the first membrane). The membranes were stained with Ponceau Red (0.5% w/v) to localize the light chains. The selected bands were cut out and extracted with acetonitrile (2 vol. in 8 vol. double-distilled water). The resulting solution was concentrated and about 100–200 pmol protein was deposited on a PVDF membrane for sequencing.

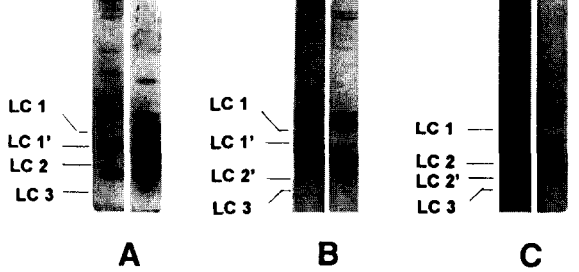

Fig. 1. SDS-PAGE of myosin and selectively modified light chains. (A) Myosin as a reference and extracted modified (P)-LC1′. (B) Myosin and modified (T)-LC1′ and (T)-LC2′; in this sample only LC1′ obtained by a prolonged proteolysis was sequenced. (C) Myosin and modified (T)-LC2′.

Table 1
(T)-LC1′ sequencing and residue yield

Cycle	Main res. (pmol)	'Contaminant'* (pmol)
1	K (54)	–
2	P (146)	–
3	A (178)	–
4	A (181)	–
5	A (188)	–
6	A (185)	–
7	A (205)	–
8	P (180)	A (41)
9	A (177)	P (35)
10	P (165)	A (51)
11	A (155)	P (39)
12	P (158)	A (60)
13	A (140)	P (55)
14	P (128)	A (72)

*The 'contaminant' is indicated when it is more than five times more abundant than the most frequent contaminants (e.g. G, E, D).

Protein sequences were determined using an Applied Biosystems microsequencer model 473 A.

2.3. ATPase activity measurements

K^+-EDTA-ATPase and Ca^{2+}-ATPase activities were determined as described previously [13].

2.4. Electric birefringence measurements

The basic equipment [15] was modified to permit the measurements in highly conducting media [16]. The fundamental principles of electric birefringence applied to macromolecular solutions have been described by Fredericq and Houssier [17]. The steady state birefringence, Δn_0 at limiting low fields E was shown to verify the Kerr law:

$$K_{sp} = \Delta n_0 / n\nu C E^2$$

with K_{sp}, specific Kerr constant; C, myosin concentration; ν, partial specific volume; n, refractive index of the solution.

The dynamic part of the study provided a relaxation value: τ according to:

$$\Delta n = \Delta n_0 \cdot e^{-t/\tau}$$

with:

$$\tau = 1/6\Theta$$

(Θ: rotary diffusion constant of the macromolecule).

3. Results and discussion

Trypsin cleaves LC1 in situ more slowly than LC2. The reverse is true for papain. However during the preparation of such fragments as (T)-S1 or (T)-HMM, cleavage in LC1 is not negligible. The amino acid sequence of LC1 was first determined by Frank and Weeds [18]. The sequence of the LC1 specific segment is (residues 1–50, corrected sequence, v.i.):

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tmAPKKDVKKPA AAAAPAPAPA
  PAPAPAPAKP KEEKIDLSAI
  KIEFSKEQQD
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Table 2
(P)-LC1′ sequencing and residue yield

Cycle	Main res. (pmol)	'Contaminant'* (pmol)
1	A (54)	
2	A (43)	P (15)
3	A (44)	P (8)
4	P (34)	A (11)
5	A (40)	P (9)
6	P (38)	A (13)
7	A (37)	P (9)
8	P (32)	A (12)
9	A (38)	P (10)
10	P (33)	A (13)
11	A (41)	P (12)

*See Table 1.

The known specificity of trypsin and the apparent molecular weight of cleavage products as estimated by SDS-PAGE strongly suggests that new N-terminus in LC1′ is at Lys^8. Early studies [2,3] concluded that the cleavage was probably on the N-terminal side of Lys^8 mainly because LC3, lacking the N-terminal 'extra-sequence', was not attacked by papain or trypsin.

3.1. N-terminal sequence of (T)-LC1′

(T)-LC1′ was separated from the mixture of light chains and purified by gel electrophoresis as described in section 2 (Fig. 1B). It can be unambiguously identified by criteria previously used to establish its origin [2]. Two N-terminal sequence determinations of two different preparations yielded respectively: KPAAAAAP and KPAAAAAPAPAPAP (Table 1). There was no evidence of other N-terminal sequences in these preparations indicating that the cleavage between Lys^7 and Lys^8 was faster than any other cleavage in the N-Lys^7 segment. This also shows that the conformation induced by Pro^9 efficiently prevents cleavage at Lys^8 as expected. In both samples a contaminating sequence was detected at the 8th cycle (Table 1). This could reflect incomplete cleavage during the five preceding Ala cycles. Alternatively, this could be due to variations: variants in the N-terminus of the homologuous chicken LC1 have been described [19] with the following sequences starting at Lys^8:

type I: Lys-Pro-$(Ala)_7$(Pro-Ala)$_6$
type II: Lys-Pro-$(Ala)_5$(Pro-Ala)$_7$
type III: Lys-Pro-$(Ala)_3$(Pro-Ala)$_7$.

However the presence of the type III variant is excluded since it would result in the release of Pro at the 6th cycle. In fact the amount of Pro detected in this cycle was less than two pmol. The presence of the type I variant remains questionable. In any case the trypsin cleavage of LC1 in situ appears remarkably specific. The second sequence obtained is long enough to indicate that a Lys between Pro^{17} and Ala^{18} [21] is not present in this sample. The rabbit LC1 is thus very similar to that of chicken and in particular possesses the same homogeneous (Ala-Pro)$_7$ sequence.

3.2. N-terminal sequence of (P)-LC1′

The (P)-LC1′ species was separated and purified by the method used for (T)-LC1′ (Fig. 1A). The residues sequentially cleaved are reported in Table 2. A small amount of Pro was released in the second and third cleavages, while Ala was still the predominantly released residue. Incomplete degradation of peptide Ala^{14} which would be the only species present at the start is not a satisfactory explanation in view of the quantitative analysis in Table 2. Thus the sample was a mixture of three peptides with Ala^{12}, Ala^{13}, and Ala^{14} as the respective N-termini.

pept. Ala^{12}	A	A	A	P	A	P	A	P	A	P	A
pept. Ala^{13}	A	A	P	A	P	A	P	A	P	A	P
pept. Ala^{14}	A	P	A	P	A	P	A	P	A	P	A
cycle	1	2	3	4	5	6	7	8	9	10	11

(The size of the letters are roughly proportional to the relative amount of the corresponding peptide.)

As expected no Pro was released at the first cycle. Pro was released at the second cycle and the residue yield is a measure of the amount of 'Ala^{14}' peptide while the Ala yield corresponds to peptides 'Ala^{12}' and 'Ala^{13}'. Pro released at the third cycle was a measure of the amount of peptide 'Ala^{13}', estimated to be about half that of 'Ala^{14}'. Under the conditions used the order of abundance of the three peptides was 'Ala^{12}', 'Ala^{14}', 'Ala^{13}'. Thus the papain cleavage was rapid, preferentially at Ala^{11} then at Ala^{13} and less frequently at Ala^{12} (cleavages at other points in the tmAla-Ala^{11} segment were not detected). The resulting peptide species are obviously very similar. They have not previously been detected in the (P)-LC1′ band in SDS-PAGE. As observed with papain, thermolysin was shown to produce also a ragged cleavage within the Ala_5 region of isolated LC1 [19]. Although not strictly specific the cleavage by papain produces a LC1′ species which can be considered as reasonably homogeneous as the three cleavage points are contiguous and result in fragments with the same numbers of charged residues.

The present study establishes firmly that (1) the papain cleavage site is upstream the (Ala-Pro)$_7$ repeat, (2) in this type of light chain (as in chicken) Lys is found at position 29 and not at position 17 (confirmation of Dr. A.G. Weeds' personal communication), and (3) the papain cleavage is at Ala^{11-13} and not Lys^7

Table 3
(T)-LC2′ sequencing and residue yield

Cycle	Main res. (pmol)	'Contaminant'* (pmol)
1	A (41)	
2	A (65)	
3	A (75)	
4	E (12)	A (9)
5	G (7)	E (8)
6	G (7)	E (8)

*See Table 1.

Table 4
Specific Kerr constants (in $m^2/V^2 \times 10^{15}$) at various applied electric fields E

E (V/cm)	300	400	480	560
Native myosin*	1.37	1.42	1.41	1.37
Myos-[(P)-LC1′]	1.57	1.60	1.61	1.53
Native myosin*	1.32	1.43	1.38	1.48
Myos-[(CT)-LC2′]	1.29	1.29	1.30	1.30

*Native myosin from two independent preparations each being the reference to the corresponding modified species.

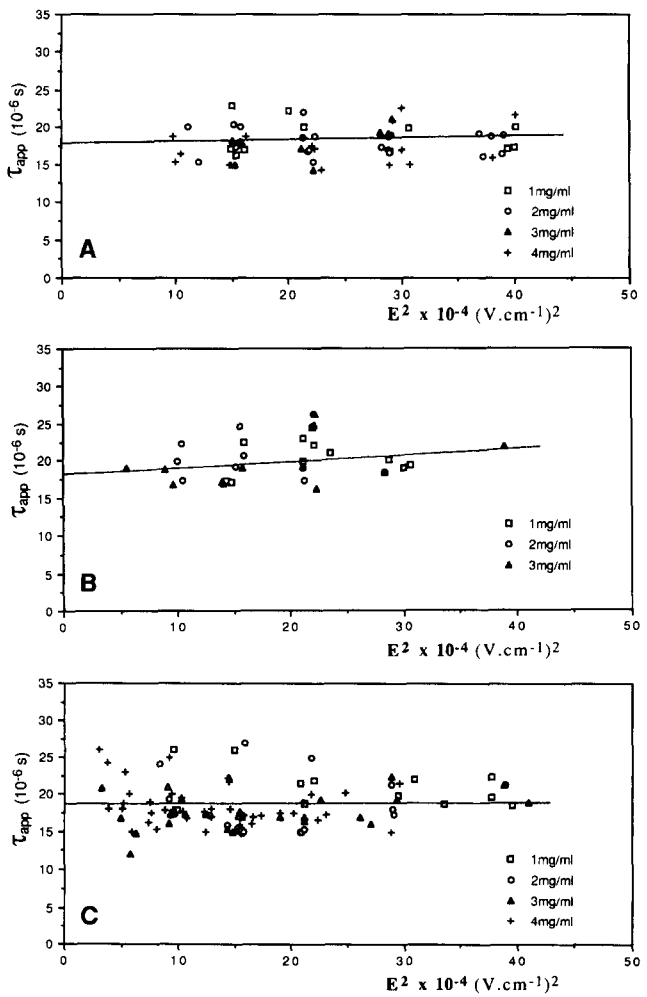

Fig. 2. Electric birefringence study of myosin solutions. Relaxation time (τ) measurements in 300 mM NaCl, 20 mM PO_4, pH 7.3. (A) Native myosin; (B) Myosin-[(P)-LC1']; (C) Myosin-[(CT)-LC2'']. Measurements were made at four different myosin concentrations: 1, 2, 3, 4 mg/ml. It was checked that τ_{app} was independent of myosin concentration.

as provisionally admitted in previous reports but not checked experimentally. Thus myosin or subfragment species with a (P)LC1' have the complete (Ala-Pro) repeat but lack the positively charged N-Ala$^{11-13}$ segment.

3.3. N-terminal sequence of (T)-LC2'

(T)-LC2' was easily identified on SDS-PAGE (Fig. 1C) by specific labeling of Ser^{16} with ^{32}P-P0$_4$ using the endogenous myosin light chain kinase and [γ-^{32}P]ATP. The N-terminal sequence of LC2 [20,21] is

tmA P K K A K R R A A A E G G S S N ...

A cleavage is therefore expected in the Lys^6-Arg-Arg^8 segment. The first three residues identified by sequence determination were Ala, Ala, Ala followed by Glu, Gly, Gly (Table 3). Thus the tryptic cleavage is between Arg^8 and Ala^9. The Arg peak in the first cycle was less than 2% of that of Ala, indicating a much slower cleavage between Arg^7 and Arg^8 and confirms a previous report [5] that Ala^9 is the neoformed N-terminus of (T)-LC2'. Again, the absence of significant amounts of Lys or Arg in the first three cycles indicates that possible cleavages at Lys^3, Lys^4 or Lys^6 are also very much slower. Thus the Arg^8 cleavage is faster than any other cleavage in the tmAla-Arg^8 segment and the resulting (T)-LC2' obtained by in situ proteolysis is a homogeneous species. This fast and specific cleavage can be used to prepare myosin-[(T)LC2'], a useful species for the study of the role of LC2 and particularly its positively-charged, N-Arg^8 segment.

The fourth cycle revealed a major contaminant (Ala). This Ala may result from incomplete cleavage during the preceding cycles as it is also present in the fifth cycle in addition to Glu. A fall in yield after the third cycle illustrates the low affinity of LC2 for the PVDF membrane.

The cleavage at Arg^8 had been suggested by default as complementary to a cleavage at Lys^{166} [3]. Similarly it had been remarked that trypsin, like chymotrypsin released a small peptide from LC2. The chymotryptic cleavage is at Phe^{19} [22] indicating the susceptibility of the N-terminal segment, it was likely therefore that trypsin also cleaved LC2 on the N-terminus [23]. The persistence of radioactivity on LC2 ([^{3}H]NEM labeling of Cys^{157}) indicated that the only possible cleavage at the C-terminal end was at Lys^{166} releasing a segment too short to account for the observed difference in apparent molecular weights between LC2 and LC2'.

A myosin-[(CT)LC2''] can also be prepared [13]. In this species the LC2'' was cleaved at Phe^{19} [22]. Myosin-[(CT)LC2''] is an interesting complement to myosin-[(T)LC2'] because it lacks Ser^{16} and therefore is refractory to phosphorylation.

Our previous observations on the proteolytic susceptibility of myosin indicated that either (or both) LC1 and LC2 light chains N-terminal segment contribute in the conformation of a region close to the ATPase active site at low ionic strength, only when myosin was in the form of filaments. K^+-EDTA- and Ca^{2+}-ATPase activity measurements showed that (P)-LC1' and (T)-LC2' cleavages did not significantly change the initial specific ATPase activity (24 h after proteolytic action) of myosin solutions at high ionic strength [13]. The activity decrease with time was also very similar in modified and native myosin. These results were confirmed with the following values: V (μmol Pi/min/mg) for K^+-EDTA-ATPase: native myosin: 1.2; myosin-[(P)-LC1']: 1.4; myosin-[(CT)-LC2'']: 0.9; for Ca^{2+}-ATPase: native myosin: 0.19; myosin-[(P)-LC1']: 0.16; myosin-[(CT)-LC2'']: 0.15. The average activity decrease was less than 10% over a period of 7 days in solutions kept undisturbed at 4°C.

The location of the two light chains on the C-terminal part of the long α-helix of the 20K fragment [24] has suggested that they might play a structural role. To distinguish between a general structural role attributed to the part 'common' in all light chains and a specific modulatory function of the N-terminal segment it is important to check that the structural role is not affected when the characteristic N-terminal segment has been removed. To obtain this information we measured the electric birefringence of modified and native myosin solutions. This technique is highly sensitive to the shape of the molecule and was previously used to show that the flexibility of myosin is significantly influenced by the medium composition [25].

Electric birefringence measurements were carried out in 20 mM NaH_2PO_4, 300 mM NaCl, pH 7.3 at 7.1°C in myosin solutions between 0.8 and 4 mg/ml. Under these conditions recorded values of Δn_0 with applied electric fields between 200

and 600 V/cm were found in good agreement with the Kerr law (Table 4). The removal of the (P)-LC1′ N-terminal segment affected slightly the myosin dipolar moment whereas no significant variation was observed with myosin-[(CT)-LC2″]. Apparent relaxation times recorded at different fields were extrapolated to $E^2 = 0$ (Fig. 2). The modified species had apparent relaxation times at zero field very close to that of native myosin and reduced values ($\tau_{20,w} = 13 \pm 2\,\mu s$) were those expected from a myosin species presenting the shape and flexibility of native myosin. The present myosin isoforms assume structural and mechanical characteristics identical to that of native myosin in the same medium (300 mM NaCl, 20 mM PO_4, pH 7.3), i.e. a bent configuration with an average angle of 110°. For comparison it could be shown that the relaxation time of native myosin in pyrophosphate solutions (10 mM, pH 9.0, 24.4°C) was 35 μs indicating a quasi straight configuration [25]. Both specific Kerr constant and apparent relaxation time were found independent of myosin concentration, an indication that no aggregation, oligomerization or interaction occurred in the 1 to 4 mg/ml concentration range as discussed previously [16]. Thus monomeric myosins modified specifically in their LC1 or LC2 N-termini exhibit no alteration in their basic structural and enzymatic characteristics. This clearly shows that the role of these light chains is to be found in systems with a higher order of structural organization such as the filament or the acto–myosin complex.

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