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Proteolysis rates of a myosin heavy chain site with papain

Evidence for a combined LC2-filament-mediated mechanism

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In striated muscle myosin, a proteolysis site at the 25–50 kDa junction, susceptible in the filament and efficiently protected by nucleotides, is similarly protected when myosin is monomeric. Kinetic studies at low ionic strength show a close relationship between LC2 cleavage or degradation rate and cleavage of the 25–50 kDa heavy chain site. The myosin-[(T)-LC2'] species forms normal reconstituted filaments but its 25–50 kDa site susceptibility is closer to that of monomeric myosin, thus becoming practically ionic strength-independent. In this species the absence of the LC2 N-terminal segment induces a significantly greater susceptibility of the papain-sensitive site in LC1. In an LC2-depleted myosin the 25–50 kDa site susceptibility also becomes ionic strength-independent, however, the cleavage rates are then closer to that of filaments. Susceptibility in HMM and S1 is also much less dependent on ionic strength with rates intermediary between those of filament and monomer. These observations show that the maximum susceptibility to papain of the 25–50 kDa site requires both the integrity of the LC2 light chain and the filament structure and furthermore provide evidence that: (i) the LC2 N-terminus interacts specifically with some part of the filament; (ii) this interaction induces a specific transconformation in a region close to the ATPase active site; (iii) there is an interrelationship between LC1 and LC2 light chain N-terminal extremities, at least in the filament structure.

Proteolytic susceptibility; Myosin-LC2 interaction; Structure effect; Ionic strength effect; Light-chain-specific modification; LC2-ATPase site communication

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Enzymes: trypsin (T) (EC 3.4.4.4), from bovine pancreas (Boehringer); α-chymotrypsin (CT) (EC 3.4.4.5) (Worthington); papain (P) (EC 3.4.4.10) from *Carica papaya* (Boehringer); trypsin inhibitor from soybean (type IS, Sigma)

Nomenclature: Modified myosin species or subfragments are described as indicated in [20]; for instance, myosin-[(T)-LC2'] is a myosin species in which LC2 has been converted to LC2' (deprived of its *N*-Arg^8 segment) by appropriate trypsin proteolysis. 25 and 50 kDa proteolytic fragments in S1 heavy chain are referred to as N and M segments, respectively

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1. INTRODUCTION

In striated muscle the regulatory (or P-) light chain is suspected of having a modulatory function but despite a number of biochemical and physiological observations [1–6] (mostly based on phosphorylation of Ser-15) clear evidence for this is still lacking.

Recent structural studies specify more precisely the location of LC1 and LC2 on the head close to the neck region [7], the C-terminal part being in interaction with a limited 100-amino-acid-residue segment in the 20 kDa (C) region of S1 [8], while the N-terminus exhibiting a high degree of segmental mobility [9,10] as observed by ^{1}H-NMR spectroscopy is suggested to have the possibility of interacting with elements foreign to the head itself [10,11]. In a general study of the effect of biologically relevant ligands (Me(II) and nucleotides)

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on the proteolytic susceptibility of various cleavage points in myosin heavy and light chains, a striking ionic strength effect was observed whereby two very fast cleavages in LC1 and LC2 (at Lys-7 and Arg-8, respectively) were even faster in the filament when a slower cleavage would be expected [11].

A tentative explanation based on this observation and ^{1}H NMR analysis of the N-terminal residue (*N*-trimethylalanine) in LC1 and LC2 [11] is to assume that these light chain extremities find in the filament structure (maybe the shaft) an interacting zone resulting in the LC1 and LC2 N-terminal segment being drawn off an S1 'resting' position on the monomer to an extended conformation in the filament. This observation is correlated to a particular cleavage obtained with papain and trypsin at the 25–50 kDa junction (referred to here as N-M junction) which is of great interest since it is responsive to ionic strength and nucleotides in S1 [12–14]. Recent studies have shown that besides the 25 kDa segment, the 50 kDa region also contains essential residues [15,16], suggesting that this cleavage point could be close to the ATPase active site. Experiments not reported here show that with papain this cleavage is much faster in the filament. When trypsin is used instead of papain the low ionic strength effect is considerably reduced when it is also observed that LC2 is rapidly converted to LC2′, suggesting that the structural effect requires the expression of the LC2 *N*-Arg-8 segment. Moreover, in one of the control experiments performed with HMM, a non-filament-forming species, the ionic strength (structural) effect is also considerably reduced if not altogether suppressed, an observation attributed to either or both of two possible reasons: (i) the necessary structure (filament) is not formed; (ii) the necessary LC2 N-terminus interaction is not possible because in chymotryptic HMM most LC2 is converted to LC2′′ (cleavage at Phe-19 eliminating the corresponding N-terminal segment).

These observations are strongly in favour of a specific interaction of the LC2 N-terminus with some specific zone in the filament structure, resulting in a transconformation being induced in or close to the active site, rendering it more susceptible to proteolysis. Among a variety of experiments performed during the past 4 years those selected here provide some insight into the respective parts played by the LC2 N-terminal segment and the C-terminal portion.

2. MATERIALS AND METHODS

2.1. *Materials*

All reagents were of the best grade available and were used without further purification. Solutions and buffers were prepared with deionized and glass-distilled water.

2.2. *Proteins*

Rabbit skeletal myosin was prepared from back muscle as described [17]. The extract was purified by 3–4 precipitation cycles at low ionic strength. Occasionally, myosin was further purified by ammonium sulfate precipitation [18] as in [19] but omitting the final high-speed centrifugation step. Myosin-[(T)-LC2′] was prepared as described in [20] and LC2-depleted myosin was obtained according to [6]. All experiments were carried out with freshly prepared myosin less than 5 days old. Myosin was always fully dephosphorylated as checked by urea-PAGE.

2.3. *Proteolysis kinetics*

Purified myosin, modified myosin or subfragment (9–12 mg/ml, or myosin equivalent) were placed in appropriate buffers by dialysis. Reaction was started by addition of enzyme and aliquots were taken periodically and placed in a suitable quenching solution. Protease specific activity was found to vary from one preparation to another, so for valid conclusions to be drawn comparisons were made between experiments carried out on the same myosin (or modified species) and the same enzyme stock solution (freshly prepared) with a negligible zero-time shift (always less than 20 min between first and last experiment in the series).

2.4. *Analysis of reaction products*

The time course study was carried out by quantitative electrophoretic analysis of the reaction products using a highly standardized staining and destaining procedure in a device specially designed for polyacrylamide disc gels and ensuring satisfactory reproducibility (general conditions otherwise as in [17]).

3. RESULTS

With papain the LC2 → LC2′ conversion rate is reasonably slow and it was therefore possible to observe the influence of ligands in cases where the presence of the LC2 N-terminal segment is essential. A parallel study of LC2 degradation and 25–50 kDa (N-M) cleavage at low ionic strength in the presence or absence of Ca(II) is reported in figs 1a,b and 2. Rapid degradation of LC2 in the presence of EDTA is accompanied by slower N-M cleavage. Conversely, when LC2 is protected by Ca(II) and in this case LC2 → LC2′ conversion slow, N-M cleavage appears to be much faster. The shape of the degradation curves also shows the rate dependence of N-M cleavage with the presence of the LC2 N-terminus; the N-M cleavage rate decreases as intact LC2 disappears. Since the rapid LC2 alteration (when no Me(II) is present) is here complete degradation, the experiment does not specify which part of LC2 is essential for maximum susceptibility of the N-M site.

The myosin-[(T)-LC2′] species is a myosin in which the LC2 *N*-Arg-8 segment has been selectively removed by trypsin whereas heavy chain and other light chains are left practically intact. This species was shown to have a Ca(II) and K^+-ATPase activity very similar to that of the intact form [17]; it forms normal reconstituted filaments as observed by electron microscopy (un-

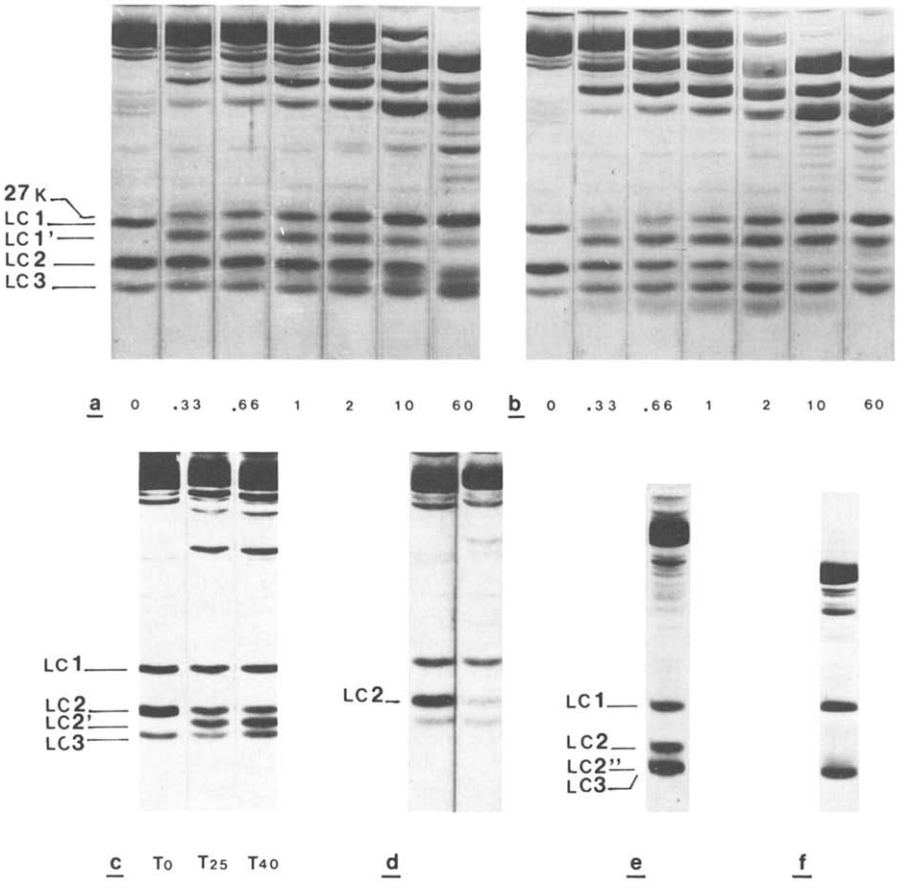

Fig.1. SDS-PAGE of various myosin and fragment species used to study the structural effect on papain proteolysis rates. (a) Proteolysis of myosin at low ionic strength (0.06 M NaCl, 0.02 M ammonium acetate) in the presence of 1 mM $CaCl_2$ (time in min). (b) As a, in the presence of 1 mM EDTA. (c) Preparation of myosin [(T)-LC2′] by brief tryptic digetion [14]; T_0, unmodified myosin; T_{25}, 25 s proteolysis with trypsin; T_{40}, 40 s protelysis with trypsin. (d) Myosin depleted of LC2 by DTNB treatment [6]. (e) HMM. (f) S1 prepared by chymotryptic digestion [32].

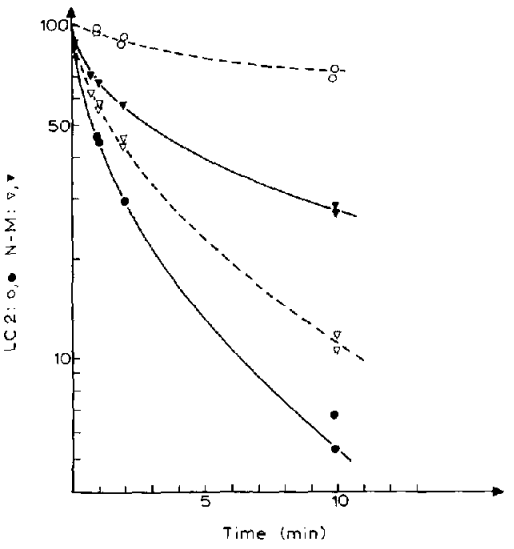

Fig.2. Proteolytic cleavage of LC2 (LC2 → LC2′ conversion) and N-M junction (50–25 kDa) by papain. Myosin, 12 mg/ml; reaction carried out at 24°C in 0.06 M NaCl, 0.02 M ammonium acetate (pH 6.85); enzyme, 0.03 mg/ml in the presence of: (——) 1 mM EDTA; 1 mM Ca(II). (●,○) LC2 → LC2′; (▼,▽) N-M cleavage. Experimental points were obtained from a densitometric analysis of SDS-PAGE of reaction products stained with Coomassie brilliant blue.

published). A kinetic study of the LC1 → LC1′ conversion is given in fig.3a. Under standard conditions (intact myosin, no nucleotide), the conversion is faster at low ionic strength. When myosin-[(T)-LC2′] is proteolyzed this conversion is systematically faster, indicating that there exists some interrelationship between LC1 and LC2 light chain extremities either in the form of a reciprocal cooperative protective interaction or simply as a proximity hindrance.

Fig.3b confirms the structural effect (monomer vs filament) as well as the protective effect of nucleotides. When myosin is replaced by the LC2 modified species the most important observation is that N-M cleavage is very significantly slower at low ionic strength in support of our LC2 interaction hypothesis. Protection by nucleotides is less efficient in modified myosin, further confirmation that the LC2 extremity influences the active-site conformation. As expected, the structural protective effect observed in the monomer is enhanced by nucleotides and the myosin modification effect is not very significant. Thus, it is confirmed that the LC2-mediated effect requires the N-terminus and a necessary interaction with a structure provided by the filament and not present in the monomer.

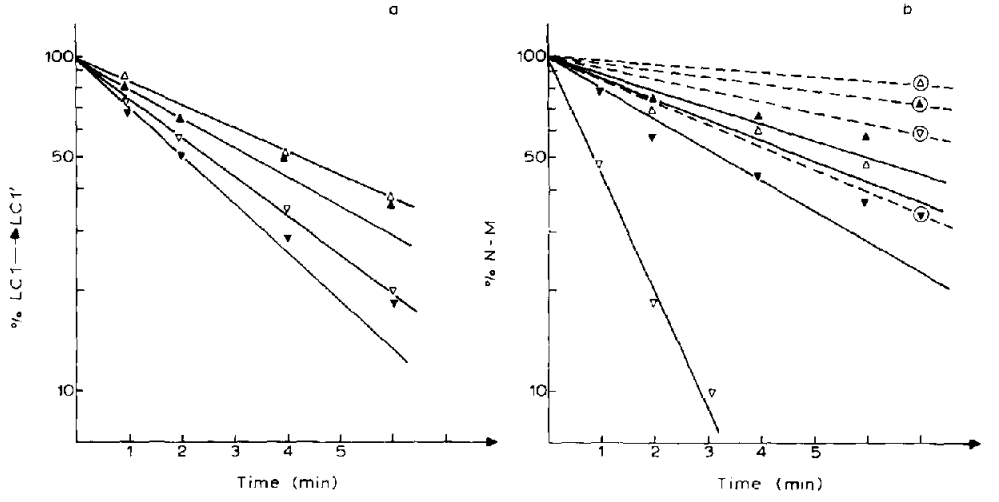

Fig.3. Proteolytic cleavage of LC1 (LC1 → LC1′ conversion) and N-M junction with papain. A parallel between intact myosin and myosin-[(T)-LC2′], an LC2-modified myosin species prepared as described in [14] with the characteristics: LC2′ percentage conversion, 65%; intact heavy chain, 85%; reaction carried out at 24°C in: (△,▲) 0.6 M NaCl or (▽,▼) 0.06 M NaCl, 0.02 ammonium acetate (pH 6.8), 0.2 mM $CaCl_2$, 7.5 mM $MgCl_2$; enzyme, 0.03 mg/ml. Filled symbols refer to the myosin-[(T)-LC2′] species; broken lines are reaction curves in the presence of 3.06 mM AMP-PNP identified by the corresponding encircled symbols; experimental points not shown. (a) LC1 → LC1′ conversion; (b) N-M cleavage.

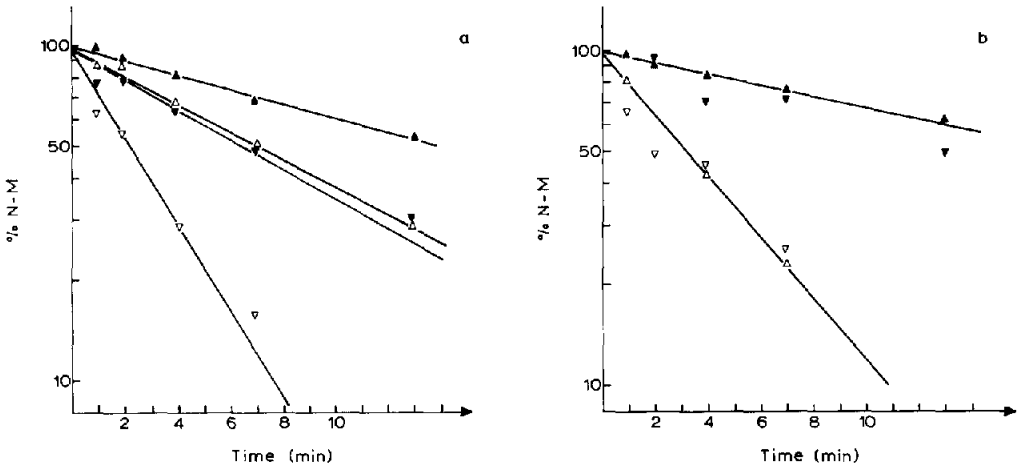

Fig.4. Proteolytic cleavage of the N-M junction in intact myosin and LC2-depleted myosin prepared as in [6] (present sample characteristics: LC2 content, 50%). Reaction conditions as in fig.3 (▽) 0.06 M NaCl; (△) 0.6 M NaCl. Filled symbols: 3.06 mM AMP-PNP. (a) Reference with intact myosin; (b) LC2-depleted myosin.

In the LC2-modification experiment 80–85% myosin molecules have their heavy chains intact and the observed effect can be attributed reasonably to LC2 modification. However, in order to check that this is indeed the case, a parallel study was carried out with a myosin in which the whole LC2 was removed (by DTNB treatment as in [6] with no concomitant heavy chain modification. The proteolysis kinetics reported in fig.4b was obtained using a myosin species in which 50% LC2 was removed. The salient observation is that the susceptibility of the N-M site becomes ionic strength-independent. In the monomer, the cleavage rate is then very close to that of the filament. This experiment provides some insight into the role played by the C-terminal portion of LC2 in structure-mediated LC2 function. As expected, nucleotides exert a strong protective effect with the rates being also very close at high and low ionic strengths.

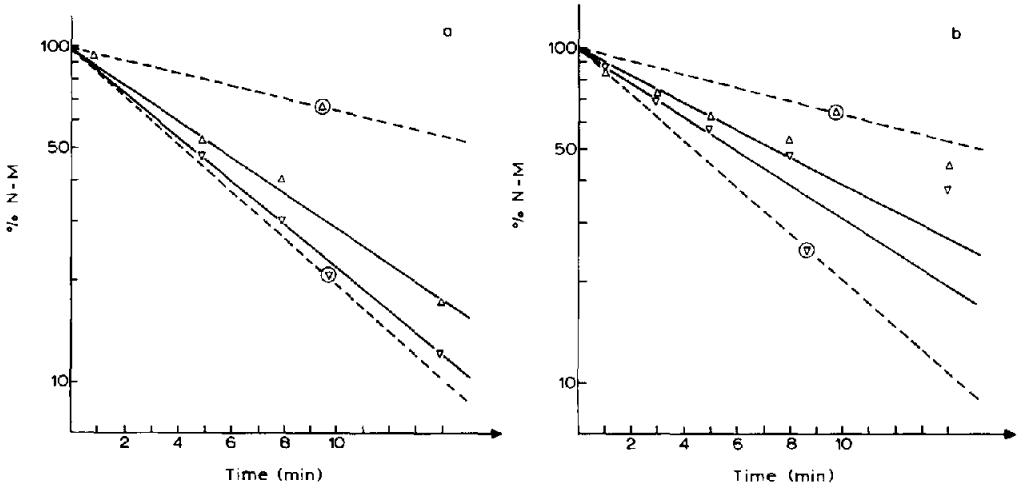

Fig.5. Compared proteolysis rates of HMM (a) and S1 (b). Reaction carried out in 0.02 M imidazole-HCl (pH 6.8) in the presence of 1 mM $CaCl_2$ and 5 mM $MgCl_2$ with (△) 0.6 M NaCl; (▽) 0.06 M NaCl. Temperature, 24°C; papain concentation, 0.03 mg/ml. Broken lines are the rate curves for myosin under the same conditions (and equivalent concentrations) identified with the corresponding encircled symbols (experimental points not shown).

In a fourth type of experiment (fig.5) the N-M susceptibility of myosin fragments, HMM and S1, was shown to be also much less dependent on ionic strength. In this experiment the LC2 ratio was LC1:LC2:'LC3' = 1.13:0.93:1.94. The abnormal relative amounts of LC2 and LC3 indicate that the LC2 → LC2'' conversion is produced by chymotryptic proteolysis. Site concentrations were adjusted to be equal (12 mg/ml in myosin equivalent) in the three species compared in fig.5. The ionic strength effect is much smaller and hardly significant in HMM and S1 species which cannot form the organized structure (filament) found in myosin. Another characteristic of these reactions is that the rates for these two species are intermediary between those of filament and monomeric myosin.

4. DISCUSSION

The present results indicate quite clearly that a heavy chain site (25–50 kDa junction), susceptible to papain, is efficiently protected by nucleotides, thus extending to intact myosin previous results obtained with S1 [12–14]. A second observation is that this site is much more susceptible when myosin is in the form of filaments. Other experiments, reported elsewhere, and the present kinetic studies of HMM and S1 at low and high ionic strengths, provide evidence that this effect is truly structural rather than being due to ionic strength per se. Thirdly, a correlation between the 25–50 kDa junction susceptibility and the status of LC2 draws attention to a possible relationship between the ATPase active site and some interaction of the LC2 N-terminal segment, since when this segment is absent as in the modified species myosin-[(T)-LC2'] the filament specific behaviour (enhanced susceptibility) is lost. Thus, it seems clearly established that the 25–50 kDa maximum susceptibility requires both the filament structure and the integrity of LC2 and, in particular, the trypsin-cleavable N-terminal segment. The results in this study can therefore be explained by the following scheme: in filament formed with intact myosin LC2 N-terminus interaction with the shaft results in the displacement of a specific zone in the LC2 C-terminal segment unmasking the N-M cleavage site; in the monomer (high ionic strength) the LC2 N-terminus finds no interacting zone, the 'relaxed' C-terminal segment protects the N-M site which is therefore slowly cleaved; when the LC2 N-terminus is severed (in myosin-[(T)-LC2']) the interaction is no longer possible in the filament and cleavage is also slow; when the whole chain is removed (LC2-deleted myosin) the masking element is lacking and the cleavage is fast. LC2 appears to establish some communication between the ATPase active site and some as yet undefined but probably specific interaction zone. Because the LC2 extremity has been located [7] close to the neck of myosin, it is not unreasonable to view this interaction zone as being in the filament shaft. In the light of recent findings that LC2 interacts with the C-terminal region of S1 [8], it must be assumed that the ATPase site transconformation is conveyed through this segment, believed to be also involved in actin binding [21,22]. This suggests that the attribution of specialized functions to particular 'domains' delimited along the linear amino acid sequence is an oversimplified picture; the three tryptic peptides in S1 were previously shown to be maintained in a stable functional tertiary structure [23], evidence that these three domains present a considerable degree of entanglement.

Another interesting piece of information derived from the proteolytic probe technique is the interrelationship between LC1 and LC2 light chain extremities, since the presence of the LC2 N-terminal segment results in a slight but significant protection of the papain-susceptible site in LC1. Clear evidence for the LC1-LC2 interaction is also provided by LC2 dissociation studies [24] and ^{1}H-NMR studies of the LC1 and LC2 *N*-trimethylalanine resonances which are broad in intact myosin filament and become sharp as in the monomer when either the LC1 or LC2 N-terminal segment is selectively removed [11].

Since the modulation of site susceptibility as tested here establishes a communication function for LC2, this leads to several possible physiologically significant hypotheses which are not mutually exclusive. The LC2 N-terminus interaction could result in a mechanical effect pulling myosin heads away from the thin filament in the detached-state step of the cross-bridge cycle (return spring hypothesis). It is also possible that the LC2 N-terminal interaction modulates substrate or product affinity or influences the rate of some step in the ATP-hydrolysis process. Alternatively, the significance of this interaction could

be in the relax-active switch mechanism in relation with phosphorylation at Ser-15. It has been observed recently that the appearance of tarantula thick filament changes with phosphorylation of LC2 [25]. The substantial ordering of heads observed in frog sartorius filaments [26] is also consistent with the strong low-angle diffraction pattern obtained earlier [27] from relaxed whole frog muscle. Specific steric relation of the myosin head to the thick filament is also suggested when the strongly periodic cross-bridge arrangement seen in relaxed insect flight muscle is reversibly lost under conditions that induce rigor in intact muscle fiber [28]. Thus, the present experiments appear as the biochemical counterpart to structural X-ray and electron microscopy investigations and may contribute the submolecular view. Efforts are being pursued to fit the presently postulated process into the detailed mechanisms available (e.g. [29–31]).

REFERENCES

[1] Morgan, M., Perry, S.V. and Ottaway, J. (1976) Biochem. J. 157, 687–697.
[2] Pemrick, S.M. (1980) J. Biol. Chem. 255, 8836–8841.
[3] Moore, R.L. and Stull, J.T. (1984) Am. J. Physiol. 247, C462–C471.
[4] Persechini, A., Stull, J.T. and Cooke, R. (1985) J. Biol. Chem. 260, 213–219.
[5] Moss, R.L., Giulian, G.G. and Greaser, M.L. (1982) J. Biol. Chem. 257, 8588–8591.
[6] Cardinaud, R. and Kakol, I. (1985) Biochim. Biophys. Acta 832, 80–88.
[7] Waller, G.S. and Lowey, S. (1985) J. Biol. Chem. 260, 14368–14373.
[8] Mitchell, E.J., Ross, J. and Kendrick-Jones, J. (1986) Eur. J. Biochem. 161, 25–35.
[9] Henry, G.D., Winstanley, M.A., Dalgarno, D.C., Scott, G.M.M., Levine, B.A. and Trayer, I.P. (1985) Biochim. Biophys. Acta 830, 233–243.
[10] Roux-Fromy, M. and Cardinaud, R. (1984) FEBS Lett. 172, 198–204.
[11] Cardinaud, R. and Roux-Fromy, M. (1985) J. Muscle Res. Cell Motil. 6, 67–68.
[12] Muhlrad, A. and Hozumi, T. (1982) Proc. Natl. Acad. Sci. USA 79, 958–962.
[13] Mocz, G., Szilagyi, L., Chen Lu, R., Fabian, F., Balint, M. and Gergely, J. (1984) Eur. J. Biochem. 145, 221–229.
[14] Applegate, D. and Reisler, E. (1984) Biochemistry 23, 4779–4784.
[15] Korner, M., Thiem, N.V., Cardinaud, R. and Lacombe, G. (1983) Biochemistry 22, 5843–5847.
[16] Mahmood, R. and Yount, R.G. (1984) J. Biol. Chem. 259, 12956–12959.
[17] Cardinaud, R. (1980) Biochimie 62, 135–145.
[18] Godfrey, J.A. and Harrington, W.F. (1970) Biochemistry 9, 886–893.
[19] Cardinaud, R. and Drifford, M. (1982) J. Muscle Res. Cell Motil. 3, 313–332.
[20] Cardinaud, R. (1982) Eur. J. Biochem. 122, 527–533.
[21] Katoh, T. and Morita, F. (1984) J. Biochem. (Tokyo) 96, 1223–1230.
[22] Mornet, D., Bertrand, R., Pantel, P., Audemard, E. and Kassab, R. (1981) Biochemistry 20, 2110–2120.
[23] Cardinaud, R., Dassin, E. and Pelletier, F. (1973) Biochem. Biophys. Res. Commun. 52, 1057–1063.
[24] Pastra-Landis, S.C. and Lowey, S.J. Biol. Chem. 261, 14811–14816.
[25] Crowther, R.A., Padron, R. and Craig, R. (1985) J. Mol. Biol. 184, 429–439.
[26] Stewart, M. and Kensler, R.W. (1986) J. Mol. Biol. 192, 831–851.
[27] Huxley, H.E. and Brown, W. (1967) J. Mol. Biol. 30, 383–434.
[28] Clarke, M.L., Hofmann, W. and Wray, J.S. (1986) J. Mol. Biol. 191, 581–585.
[29] Huxley, H.E. (1984) in: Contractile Mechanisms in Muscle (Pollack, G.H. and Sugi, H. eds, pp. 161–175, Plenum, New York.
[30] Huxley, H.E. and Kress, M. (1985) J. Muscle Res. Cell Motil. 6, 153–161.
[31] Kress, M., Huxley, H.E., Faruqi, A.R. and Hendrix, J. (1986) J. Mol. Biol. 188, 325–342.
[32] Weeds, A.G. and Taylor, R.S. (1975) Nature 257, 54–56.