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# Calcium Ion-Dependent Myosin from Decapod-Crustacean Muscles

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$Ca^{2+}$ regulation of arthropod actomyosin adenosine triphosphatase is associated with both the thin filaments, as in vertebrates, and with the myosin, as in molluscs. The actomyosin of decapod-crustacean fast muscles was previously considered to be an exception, displaying only a $Ca^{2+}$-regulatory system linked to the thin filaments and not a myosin-linked regulatory system. In the present study, myosin regulation is demonstrated in a variety of decapod muscles when they are tested under more physiological ionic conditions. Myosin regulation is shown by using mixtures of pure rabbit actin with myofibrils, with actomyosin and with purified myosin, and in each case the adenosine triphosphatase is $Ca^{2+}$-dependent. Myosin regulation may also occur in vertebrate striated muscle, but seemingly is lost during purification of the myosin.

Comparative studies have shown that muscles in all animals are regulated by changes in $Ca^{2+}$ concentration. Two distinctly different regulatory systems responsive to $Ca^{2+}$ occur in different animals, one acting by means of the thin filaments and the other via the myosin. The former system was first shown in vertebrate striated muscle (Ebashi, 1963), and the latter in molluscan muscle (Kendrick-Jones *et al.*, 1970). These comparative studies also revealed that most of the higher invertebrates (e.g. arthropods and annelids) possess dual-regulated simultaneously operated systems (Lehman & Szent-Györgyi, 1975).

Decapod crustacea appeared to be aberrant among arthropods, since most decapod muscles examined showed only thin-filament regulation, but the reason for this apparent difference remained unclear (Lehman & Szent-Györgyi, 1975). Moreover, in the lobster, in which both fast and slow muscles were examined, only the fast muscles seemed to have the thin-filament control, whereas the slow muscles showed dual regulation (Lehman & Szent-Györgyi, 1975). Thus the lobster was the only invertebrate species studied thought to possess muscle types with differing regulatory systems. These anomalous results become even more difficult to understand after finding crayfish fast muscles, homologous to the lobster fast muscles, with dual regulation.

In the present study these confusing results have been found to be a consequence of the ionic conditions under which ATPase* measurements were made. It is shown that myosin regulation can only be demonstrated in fast muscles from marine decapods when preparations are assayed at ionic strengths more closely approaching those *in vivo*. In previous studies (Lehman & Szent-Györgyi, 1975) ATPase assays were performed at univalent cation concentrations between 20 and 40mM to maximize the ATPase rate, whereas the free univalent cation concentration in lobster sarcoplasm, for example, is at least 100mM (Hinke, 1959). A possible explanation for the particular susceptibility of decapod myosin to ionic concentration is proposed in the discussion of the results.

* Abbreviations: ATPase, adenosine triphosphatase; Mg-ATPase, $Mg^{2+}$-activated ATPase.

## Materials and Methods

### *Muscles*

The deep medial tail-extensor muscle of the crayfish *Procambarus clarkii* was used, since this fast muscle is homologous in structure and function to its counterpart in lobster (Atwood, 1972; Lehman & Szent-Györgyi, 1975). The tail-flexor muscle of the lobster *Homarus americanus* was used because of its large size, facilitating myosin preparation. Leg muscles taken from within the carapace of the spider crab *Libinia emarginata* and the claw muscles of the rock crab *Cancer irroratus* were also used for myosin preparations. Rabbit myofibrils and myosin were prepared from back muscles.

### *Preparations*

All preparations were carried out between 0° and 4°C. Rabbit actin free of tropomyosin contamination was prepared by the method of Straub (1942), by using the modification of Drabikowski & Gergely (1964).

Myofibrils were prepared by the method of Bullard *et al.* (1973) with some modification. In preparing rabbit myofibrils, back muscle was ground in a meat grinder and then homogenized in a

solution containing 0.3M-sucrose, 0.1M-NaCl, 1mM-$MgCl_2$, 1mM-EGTA, 1mM-$NaN_3$, 10mM-sodium phosphate buffer, pH7.0, with a Sorval Omni Mixer (DuPont Instruments, Newtown, CO, U.S.A.), and then centrifuged at 6000*g* for 10min. Lobster myofibrils were prepared from minced muscle by homogenization in the above solution and then sedimentation at 15000*g* for 10min. After these initial steps, both lobster and rabbit preparations were carried out in the same manner.

The myofibrillar pellets were resuspended in 0.1M-NaCl, 1mM-$MgCl_2$, 0.2mM-EGTA, 1mM-$NaN_3$, 10mM-sodium phosphate buffer, pH7.1, and sedimented at 6000*g* for 10min, and then redispersed in the latter solution; the sedimentation and redispersion step was repeated twice more.

Lobster actomyosin was extracted from myofibrils as previously described (Lehman & Szent-Györgyi, 1975). A single preparation of lobster actomyosin was additionally precipitated and redissolved as follows. The actomyosin, containing 0.6M-NaCl, was dialysed against 15vol. of 5mM-sodium phosphate buffer (pH7.0) to give a final NaCl concentration of approx. 40mM, and then diluted with 4vol. of 40mM-NaCl/5mM-phosphate buffer (pH7.0). The actomyosin was collected by centrifugation (6000*g* for 10min), resuspended in the above solution, re-collected by centrifugation, and this step was repeated twice more. Sufficient 4M-NaCl was then added to the actomyosin pellet to give a final concentration of 0.6M-NaCl.

Lobster myosin was prepared by a method similar to that of Regenstein (1972). Actomyosin extracts, prepared as described above, containing 1.5mM-ATP and 1mM-$MgCl_2$ were sedimented at 130000*g* for 4h. The contents from the top two-thirds of the centrifuge tube were removed and sedimented for an additional 2h at 130000*g*. This ensured that sedimentation of the remaining actin was not hindered by the high viscosity at the base of the centrifuge tube. After centrifugation, the supernatant containing the myosin was dialysed to a final concentration of 40mM-NaCl against 5mM-sodium phosphate buffer, pH7.0. The precipitated myosin was then collected, washed and redissolved by the same procedure used for the precipitation and resuspension of lobster actomyosin. The redissolved myosin was clarified by sedimentation at 200000*g* for 30min.

Attempts to purify rabbit myosin from actomyosin by the same method used for lobster yielded a preparation heavily contaminated with actin. The actin contamination could be eliminated by lengthening the ultracentrifugation. The centrifugations at 130000*g* and 200000*g* were increased to 16h each. An alternative method also yielding pure myosin is as follows: 100g of ground back muscle was mixed with 200ml of 0.6M-NaCl/5mM-sodium phosphate buffer, pH7.0, and 1mM-MgATP, pH7.0; ATP and NaCl from stock solutions were added to give final concentrations of 0.6M-NaCl and 1mM-ATP in the mixture. Myosin was extracted for 12min and the unextracted muscle removed by sedimentation at 30000*g* for 10min. Subsequent steps were identical with the method used in preparing lobster myosin.

*Analysis*

The actomyosin $Mg^{2+}$-ATPase was measured in a pH-stat at various NaCl concentrations (pH7.5, 25°C), as described by Szent-Györgyi *et al.* (1971). The duration of an assay was from 4 to 15min. Unless otherwise indicated, 0.7mM-ATP and 1mM-$MgCl_2$ were used as substrate. $Ca^{2+}$-sensitivity was measured by comparing the ATPase rates in the presence of 0.1mM-EGTA alone ($ATPase_{EGTA}$), and with 0.1mM-EGTA containing 0.2mM-$CaCl_2$ ($ATPase_{Ca}$), by using the following relationship:

$$Ca^{2+}\text{-sensitivity} = \frac{ATPase_{Ca} - ATPase_{EGTA}}{ATPase_{Ca}} \times 100$$

The competitive actin-binding ATPase assay (Lehman & Szent-Györgyi, 1975) was extensively used in the present investigation. The following is a brief summary of its application: pure rabbit actin is mixed with myofibrils or actomyosin and the mixture assayed in EGTA; if the addition of pure actin activates the $Mg^{2+}$-ATPase to the activity found in the presence of $Ca^{2+}$, actin regulation is demonstrated, since the myosin is free to interact with the actin; if the ATPase activity remains low in EGTA, this demonstrates that the myosin is not available to interact with the actin in the absence of $Ca^{2+}$ and is evidence for myosin regulation.

In practice, actomyosin or solubilized myofibrils are pre-mixed with pure rabbit actin before ATPase assays are performed to ensure that the myosin is able to react with the actin. Myofibrils (10–15mg/ml) are solubilized by adding sufficient 4M-NaCl to give a final concentration of 0.6M and by adding 100mM-ATP (pH7.0) to a final concentration of 1mM. Solubilized myofibrils or actomyosin (8–13mg/ml) are mixed together with various amounts of rabbit F-actin (6–8mg/ml), with the aid of a Pasteur pipette. The mixtures are maintained at 0–4°C and can be used immediately for ATPase analysis. Generally, in assays where the ATPase activity exceeds 0.5$\mu$mol of ATP split/min per mg of protein, 1mg of either myofibrils, actomyosin or myosin is introduced into a total of 10ml of ATPase assay solution. For ATPase activities less than 0.5unit, 2mg of protein is used. The NaCl content of the samples is taken into account to compute the univalent ion concentration in the assay solution.

Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin standards, which in turn were standardized by nitrogen determination (Strauch, 1965).

## Results

### *Crayfish actomyosin*

The $Mg^{2+}$-ATPase of crayfish deep extensor-muscle actomyosin is activated by the addition of $Ca^{2+}$. This $Ca^{2+}$-dependency remains when pure rabbit actin is introduced into the ATPase assay, even when the actin is in an excess (Table 1). The competitive actin-binding assay thus indicates the presence of a myosin-linked $Ca^{2+}$-regulatory system.

### *Lobster preparations*

*Rationale.* The $Mg^{2+}$-ATPase activity of crayfish muscle actomyosin is less than that of actomyosin from homologous fast-muscles in the lobster (Lehman & Szent-Györgyi, 1975). Thus a possible connexion between the magnitude of the ATPase rate and the detection of myosin regulation was considered. It was thought that myosin-linked regulation might be observed in lobster fast muscles if its ATPase rate was decreased. The $Mg^{2+}$-ATPase activity of muscle decreases with increasing ionic strength, and the effect of ionic strength on various lobster preparations was tested.

*Competitive actin-binding assays.* Competitive actin-binding assays show that lobster actomyosin, in the presence of rabbit actin, shows a complete lack of $Ca^{2+}$-sensitivity when the NaCl concentration is lower than 50 mM (Table 2, also cf. Lehman & Szent-Györgyi, 1975); similarly, pure rabbit actin diminishes the $Ca^{2+}$-sensitivity of lobster myofibrils at low NaCl

Table 1. *ATPase activity of crayfish actomyosin*
These data are averages from experiments on three different preparations of actomyosin. The values in parentheses are the ranges of values observed. In each assay 1 mg of actomyosin was tested in 1 mM-$MgCl_2$, 0.7 mM-ATP, 27 mM-NaCl, pH 7.5, at 25°C; see the Materials and Methods section.

| Ratio rabbit actin/ actomyosin (mg/mg) | $Mg^{2+}$-ATPase ($\mu$mol of ATP split/min per mg of actomyosin) | | Calcium-sensitivity |
|---|---|---|---|
| | +EGTA | +Calcium | |
| 0.0* | 0.012 | 0.52 | 98 (96–98) |
| 0.33 | 0.11 | 0.73 | 85 (82–89) |
| 1.0 | 0.17 | 0.72 | 76 (74–79) |

* No rabbit actin present in assay.

Table 2. *ATPase activity of lobster actomyosin*
In a second lobster actomyosin preparation $Ca^{2+}$-sensitivities ranging between 71 and 76% were observed at elevated [NaCl] (actin/actomyosin, 0.33 mg/mg). A third preparation of lobster actomyosin which was additionally precipitated and redissolved (see the Materials and Method section) and assayed at 84 mM-NaCl gave a $Ca^{2+}$-sensitivity of 74% and an ATPase rate of 0.73 $\mu$mol of ATP split/min per mg of actomyosin in the presence of $Ca^{2+}$ and 0.33 mg of rabbit actin/mg of actomyosin.

| [NaCl] (mM) | Ratio rabbit actin/actomyosin (mg/mg) | $Mg^{2+}$-ATPase ($\mu$mol of ATP split/min per mg of actomyosin) | | Calcium-sensitivity |
|---|---|---|---|---|
| | | +EGTA | +Calcium | |
| 26 | 0.0 | 0.037 | 0.9 | 96 |
| 26 | 0.33 | 0.9 | 0.9 | 0 |
| 46 | 0.33 | 0.9 | 0.9 | 0 |
| 66 | 0.33 | 0.57 | 0.8 | 29 |
| 86 | 0.33 | 0.24 | 0.74 | 68 |
| 106 | 0.33 | 0.13 | 0.38 | 66 |
| 126 | 0.33 | 0.034 | 0.19 | 82 |

Table 3. *ATPase activity of lobster myofibrils*
Two additional preparations of lobster myofibrils were assayed in the presence of rabbit actin at high NaCl concentration. One preparation gave 81% $Ca^{2+}$-sensitivity [at 78 mM-NaCl, ratio of 0.33 (mg/mg) actin/myofibrils], and the other 95% $Ca^{2+}$-sensitivity [at 86 mM-NaCl, ratio of 0.33 (mg/mg) actin/myofibrils].

| [NaCl] (mM) | Ratio rabbit actin/myofibrils (mg/mg) | $Mg^{2+}$-ATPase ($\mu$mol of ATP split/min per mg of myofibrils) | | Calcium-sensitivity |
|---|---|---|---|---|
| | | +EGTA | +Calcium | |
| 27 | 0.33 | 1.47 | 1.9 | 23 |
| 47 | 0.33 | 0.60 | 2.0 | 70 |
| 67 | 0.33 | 0.095 | 1.14 | 92 |
| 87 | 0.33 | 0.036 | 0.70 | 95 |
| 67 | 1.0 | 0.098 | 0.90 | 89 |
| 67 | 2.0 | 0.095 | 0.81 | 88 |

Table 4. *ATPase activity of lobster myosin*

Results are averages from two preparations of lobster myosin; values in parentheses represent individual values. In a third experiment, myosin was prepared using two 16 h ultracentrifugations instead of the 6–7 h ultracentrifugation. The ATPase activities in this preparation were about one-fifth that shown above, but retained about 55% $Ca^{2+}$-sensitivity when assayed at 70 mM-NaCl.

| [NaCl] (mM) | Ratio rabbit actin/myosin (mg/mg) | $Mg^{2+}$-ATPase (μmol of ATP split/min per mg of myosin) +EGTA | $Mg^{2+}$-ATPase (μmol of ATP split/min per mg of myosin) +Calcium | Calcium-sensitivity |
|---|---|---|---|---|
| 20 | 0.0 | 0.013 | 0.012 | — |
| 20 | 0.33 | 1.13 | 1.13 | 0 (0, 0) |
| 43 | 0.33 | 1.06 | 1.11 | 4 |
| 63 | 0.33 | 0.72 | 0.79 | 9 (7, 12) |
| 84 | 0.33 | 0.23 | 0.57 | 60 (55, 65) |
| 96 | 0.33 | 0.18 | 0.40 | 55 (50, 60) |

Table 5. *Calcium-sensitivities of other myosins*

These myosins were prepared by the procedure used for lobster myosin, and were mixed with rabbit actin in a ratio of 3:1 (mg/mg). The experiment was performed once for each myosin.

| Source of myosin | [NaCl] (mM) | Calcium-sensitivity |
|---|---|---|
| Spider crab | 26 | 0 |
| | 86 | 57 |
| Rock crab | 30 | 20 |
| | 95 | 53 |

concentrations (Table 3). Both lobster myofibrils and actomyosin, however, show a $Ca^{2+}$-dependent ATPase in the presence of rabbit actin when the NaCl concentration is raised (Tables 2 and 3), even if the actin is present in a large excess (Table 3). The same response is also observed if KCl is substituted for NaCl.

*Lobster myosin and rabbit actin.* As with lobster myofibrils and actomyosin, the actin-activated $Mg^{2+}$-ATPase of purified lobster myosin is $Ca^{2+}$-independent at low NaCl concentration, but becomes $Ca^{2+}$-dependent at higher ionic strengths (Table 4). The calcium-dependency at higher ionic strength could be a non-specific bivalent-ion effect; however, this is unlikely, since at 84 mM-NaCl/0.7 mM-ATP the same $Ca^{2+}$-sensitivity and specific activity are observed when 3 mM-$MgCl_2$ (a 30-fold excess over [$CaCl_2$]), instead of the standard 1 mM-$MgCl_2$, is used.

Muscles from rock crabs and spider crabs are also among the decapod muscles classified by Lehman & Szent-Györgyi (1975) as solely thin-filament regulated. In the present study, the same relationship between univalent ion concentration and expression of myosin regulation is found. The actin-activated ATPase of these myosins are also $Ca^{2+}$-independent at low ionic strength, and $Ca^{2+}$-sensitivity appears only after the univalent-ion concentration is raised (Table 5).

*Rabbit preparations*

*Competitive actin-binding assays.* Competitive actin-binding assays, showing vertebrate skeletal muscles to be solely thin-filament regulated, were also performed by Lehman & Szent-Györgyi (1975) at 20–40 mM-NaCl, but may, as observed in decapod crustaceans, show evidence of myosin regulation at higher NaCl concentration. Competitive actin-binding assays carried out in the present study show that pure actin abolishes the $Ca^{2+}$-sensitivity of rabbit myofibrils only at lower ionic strength, but has no effect at higher NaCl concentration (Table 6). Again, neither an excess of $Mg^{2+}$ nor an excess of actin decreases the $Ca^{2+}$-sensitive response at the higher NaCl concentrations (Table 6). The same response is observed when KCl is substituted for NaCl.

As outlined in the Materials and Methods section, myofibrils are solubilized and premixed with pure rabbit actin in the myofibrillar competitive actin-binding assay. The same results are obtained when undissolved intact rabbit myofibrils are combined with pure actin, namely a marked diminution of $Ca^{2+}$-sensitivity at low NaCl concentration and a $Ca^{2+}$-sensitive response at higher NaCl.

*Rabbit myosin and rabbit actin.* In marked contrast with results with lobster myosin, rabbit myosin purified by similar methods does not show a $Ca^{2+}$-dependent ATPase activity at any of the NaCl concentrations tested when recombined with pure actin (Table 7).

## Discussion

It was previously shown that decapod crustaceans, like vertebrates, possess a thin-filament-linked $Ca^{2+}$-regulatory system, operating by means of troponin and tropomyosin (Regenstein & Szent-Györgyi, 1975). Peculiarly, only lobster slow muscles showed evidence of dual regulation, whereas other decapod muscles showed solely a thin-filament-linked system (Lehman & Szent-Györgyi, 1975). In the present

Table 6. *ATPase activity of rabbit myofibrils*

Competitive actin-binding assays were performed on three different preparations of rabbit myofibrils; the Table shows the results obtained after varying [$MgCl_2$], [ATP], [NaCl] and the ratio of actin/myofibrils. Variation in the $Ca^{2+}$-sensitivity in the different preparations is indicated by the values in parentheses.

| Assay solution composition (mM) | | | Ratio rabbit actin/myofibrils | $Mg^{2+}$-ATPase (μmol of ATP split/min per mg of myofibrils) | | Calcium-sensitivity |
|---|---|---|---|---|---|---|
| [$MgCl_2$] | [ATP] | [NaCl] | (mg/mg) | +EGTA | +Calcium | |
| 1.0 | 0.7 | 34 | 0.0 | 0.10 | 0.23 | 57 (52–59) |
| 1.0 | 0.7 | 88 | 0.0 | 0.044 | 0.21 | 79 |
| 1.0 | 0.7 | 94 | 0.0 | 0.041 | 0.20 | 80 (71–93) |
| 1.0 | 0.7 | 26 | 0.33 | 0.33 | 0.38 | 13 |
| 1.0 | 0.7 | 34 | 0.33 | 0.36 | 0.38 | 5 (0–10) |
| 1.0 | 0.7 | 54 | 0.33 | 0.27 | 0.38 | 30 |
| 1.0 | 0.7 | 74 | 0.33 | 0.18 | 0.38 | 53 |
| 1.0 | 0.7 | 88 | 0.33 | 0.073 | 0.30 | 76 |
| 1.0 | 0.7 | 94 | 0.33 | 0.068 | 0.28 | 76 (68–81) |
| 1.0 | 0.7 | 99 | 0.33 | 0.068 | 0.27 | 75 (68–78) |
| 1.0 | 0.7 | 99 | 1.0 | 0.071 | 0.21 | 66 (62–70) |
| 3.0 | 0.7 | 94 | 0.33 | 0.053 | 0.19 | 72 |
| 3.0 | 3.0 | 94 | 0.33 | 0.037 | 0.22 | 83 |

Table 7. *ATPase activity of rabbit myosin*

Results are average values for two preparations of myosin; each was prepared by the different methods presented in the Materials and Methods section.

| [NaCl] (mM) | Ratio rabbit actin/myosin (mg/mg) | $Mg^{2+}$-ATPase (μmol of ATP split/min per mg of myosin) | | Calcium-sensitivity |
|---|---|---|---|---|
| | | +EGTA | +Calcium | |
| 23 | 0.0 | 0.021 | 0.026 | 19 |
| 23 | 0.33 | 0.47 | 0.47 | 0 |
| 83 | 0.33 | 0.37 | 0.37 | 0 |
| 103 | 0.33 | 0.38 | 0.38 | 0 |
| 123 | 0.33 | 0.27 | 0.27 | 0 |
| 163 | 0.33 | 0.15 | 0.15 | 0 |

study, it is concluded that the decapod-crustacean fast muscles are in fact dual-regulated and their myosins can confer a $Ca^{2+}$-dependence on an actomyosin in ATPase. This is only observed, however, at ionic concentrations greater than those used in previous studies (Lehman & Szent-Györgyi, 1975). Thus the seeming absence of myosin-linked regulation in decapods is related to ATPase-assay conditions and not to specific properties of these muscles, as was originally thought (Lehman & Szent-Györgyi, 1975). It appears therefore that myosin regulation is probably universal among invertebrate muscles.

One feature distinguishing decapod fast muscles from other arthropod muscles is their high ATPase activity, being 4–5 times that found in most arthropod muscles. This may be a result of a high affinity of the decapod myosin for actin, and, in turn, may be related to the lack of $Ca^{2+}$-sensitivity observed at low ionic strengths. It is known that increasing ionic strength decreases the apparent binding constant of myosin for actin, thus decreasing ATPase activity (Eisenberg & Moos, 1970). It is therefore possible that at very low ionic concentrations the binding of decapod fast-muscle myosins to pure actin is so high that binding occurs indiscriminately in the presence and absence of $Ca^{2+}$. Only when the ionic strength is increased nearer to the physiological range is $Ca^{2+}$-sensitivity observed, since here the binding of the myosin to actin is lower and can be modulated by $Ca^{2+}$. The results of this study draw attention to the complexities that may occur as a consequence of the different affinities of different myosins for actin. Likewise, it is also possible that actins from different muscles may vary in their binding for a particular myosin; this consideration, however, is improbable owing to the almost invariant amino acid sequence of actins from diverse sources (Elzinga & Collins, 1972).

Results from the present investigations on rabbit striated muscles, however, are more difficult to interpret. As with lobster myofibrils, the competitive actin-binding assay on rabbit myofibrils apparently

shows the occurrence of myosin regulation only at relatively high ionic strength; yet rabbit myosin, isolated and then recombined with pure actin, is $Ca^{2+}$-independent at all ionic strengths tested. The simplest explanation is that myosin regulation is lost during purification. Nevertheless, it is considered that this evaluation is of such importance that one must await direct demonstration of a myosin-linked system by using purified proteins. It is noteworthy that myosin regulation has been demonstrated unambiguously in vertebrate smooth muscles (Bremel, 1974; Mrwa & Rüegg, 1975; Sobieszek & Small, 1976). Smooth muscles are slow muscles, with low ATPase activities, and the relative ease encountered in detecting myosin regulation in slow muscles of both decapods and vertebrates may well have a similar basis.

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