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REGULATORY PROTEINS OF CRAYFISH TAIL MUSCLE*

GILBERT BENZONANA, LAURE KOHLER and ERIC A. STEIN

Department of Biochemistry, University of Geneva (Switzerland)

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SUMMARY

Native tropomyosin and fragmented sarcoplasmic reticulum from crayfish tail muscle have been obtained in a pure state. After isoelectric precipitation, the former system yields two biologically active components, troponin and tropomyosin. Troponin binds calcium and can dissociate into three subunits of molecular weight 54 000, 29 000 and 16 000. The tropomyosin monomer has a molecular weight of 40 000. Desensitized actomyosin becomes calcium sensitive in the presence of native tropomyosin or tropomyosin plus troponin. The properties of crayfish fragmented sarcoplasmic reticulum (ATPase activity, calcium binding and uptake, inhibition of actomyosin superprecipitation) are characteristic of a calcium pump.Comparison with the regulatory system of rabbit skeletal muscle reveals many similarities between crayfish and mammalians; indeed crayfish fragmented sarcoplasmic reticulum or native tropomyosin can influence the calcium sensitivity of rabbit actomyosin.

INTRODUCTION

The mechanism of actin–myosin interaction in vertebrate skeletal muscle has been the subject of numerous investigations (see ref. 2 for a review of the literature). Much less has been achieved in the instance of lower animals, although the structure of invertebrate muscle is well known [3]. The aim of this work is to determine whether or not crustaceans have a more primitive regulatory system for muscular contraction than the one already characterized for rabbit. More specifically, is the actin–myosin interaction in crayfish tail muscle mediated by a calcium-dependent troponin–tropomyosin system, and, if so, how are calcium ions pumped and stored? The crayfish was chosen as a model because it is an ancient animal that evolved probably some 400 million years before mammalians.

*A preliminary report on this work was presented at the 6th annual meeting of the Union of Swiss Societies for Experimental Biology at Lausanne, May 11–12th, 1974 [1].

Abbreviations: EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetate; ATPase, ATPphosphohydrolase (EC 3.6.1.3); TNT, TNI, TNC, components already characterized in rabbit troponin, respectively, tropomyosin-binding, inhibitory, calcium-binding.

METHODS

A. Preparations of proteins

Rabbit myofibrils

These were prepared either from fresh or frozen [4] white skeletal muscle, essentially according to Perry *et al.* [5, 6]. Crude myofibrils were washed and centrifuged seven times. Myofibrils were stored at 0 °C in an ice bath in 10 mM KCl, 1 mM dithiothreitol, 10 mM Tris–HCl buffer pH 7.4. This medium induces the solubilization of native tropomyosin from myofibrils and was used only for the preparation of desensitized actomyosin: When natural actomyosin had to be prepared, myofibrils were stored in 0.1 M KCl, 1 mM dithiothreitol, 39 mM sodium borate buffer pH 7.1.

Rabbit natural actomyosin

This was prepared from myofibrils according to Schaub and Perry [7], except that no more than 30 min were allowed for the precipitation of actomyosin (Schaub, personal communication). Natural actomyosin was stored at 0–4 °C in 10 mM Tris–HCl buffer pH 7.4 for one week at most, as the protein becomes desensitized in this low ionic strength medium.

Rabbit desensitized actomyosin

The suspension of natural actomyosin was centrifuged at 12 000 × *g* for 10 min, the precipitate was resuspended and carefully homogenized in 4 vol. of 2 mM Tris–HCl buffer pH 7.6 and again centrifuged as above. At least seven washings and centrifugations were required for complete desensitization of natural actomyosin when freshly prepared, whereas three washings were sufficient after 2–7 days storage in 10 mM Tris–HCl buffer pH 7.4. In the instance of frozen muscle, actomyosin swelled so fast at low ionic strength that it could not be centrifuged and washed more than four times; complete desensitization occurred spontaneously upon 24 h storage at 0–4 °C in 2 mM Tris–HCl buffer pH 7.6.

Rabbit native tropomyosin

(*a*) *From myofibrils.* 1 g of proteins from fresh myofibrils (as measured by means of the biuret technique) were homogenized at 0–4 °C in 100 ml of 0.1 M KCl, 40 mM Tris–HCl buffer pH 7.0 with a Sorvall Omnimixer at full speed for 30 s. The homogenate was centrifuged for 10 min at 7700 × *g*, the supernatant discarded, the precipitate rehomogenized as above and the process repeated four times. The final precipitate was suspended in 30 mM mercaptoethanol, 10 mM Tris–HCl buffer pH 7.4 (20–30 ml per g of protein) and dialyzed for 30 h against 10 vol. of the same buffer changed twice. The content of the dialysis bag was centrifuged at 36 000 × *g* for 75 min and the supernatant retained. The precipitate was resuspended in half the original volume of buffer, stirred for 4–12 h at 0 °C and again centrifuged. Occasionally, a third cycle was performed to increase the yield. The supernatants (native tropomyosin) were combined and used for the preparation of pure troponin and tropomyosin either at once or after freeze-drying.

(*b*) *From the low ionic strength supernatants of myofibrils or actomyosin*

Native tropomyosin can be prepared as above using the supernatant from storage of myofibrils, or the washings resulting from desensitization of actomyosin. When the solutions were too diluted, vacuum dialysis was used for concentration.

Rabbit tropomyosin and troponin

Pure tropomyosin and troponin were prepared from native tropomyosin by

the method of Schaub and Perry [7] or by that of Greaser and Gergely [8].

Rabbit fragmented sarcoplasmic reticulum

Fragmented sarcoplasmic reticulum was prepared by the method of MacLennan [9] up to the step "R_1 washed". This preparation lost all activity within 24 h at 0 °C or −20 °C, irrespective of the buffer used for storage. However, addition of 1 mM dithiothreitol to the buffer [10] allowed the preparation to be kept active for at least one week at 0 °C and more than one month at −20 °C.

Crayfish tail muscle proteins

Crayfish (*Astacus* (*pontastacus*) *leptodactylus leptodactylus*) were obtained from a local fish farm. The tails were removed from the live animal and the muscle separated from the exoskeleton and the digestive tract. Myofibrils, natural and desensitized actomyosins, native and pure tropomyosins, and troponin were prepared by the methods used for rabbit muscle proteins. However, precipitation of crayfish tropomyosin by ammonium sulphate occurred between 30 and 55 % saturation instead of 40–70 % for the homologous rabbit protein (11). Sarcoplasmic reticulum was purified according to the method of MacLennan for rabbit fragmented sarcoplasmic reticulum [9], modified as follows: (a) the first centrifugation was performed at $2000 \times g$ for 15 min; (b) the extraction medium was 88 mM KCl, 1 mM dithiothreitol, 5 mM imidazole–HCl buffer pH 7.4.

B. Enzyme assays

ATPase activity of actomyosin. ATPase activities from rabbit or crayfish were determined by titration of inorganic phosphorus according to Fiske and SubbaRow [12]; 0.2–0.4 mg/ml of protein were incubated at 25 °C in 2 ml of 2.5 mM $MgCl_2$ and 25 mM Tris–HCl buffer pH 7.2. No extra calcium was needed to obtain optimum ATPase activity. For ATPase assay in the absence of Ca^{2+}, 3 mM EGTA was added to the incubation medium. The reaction was initiated by the addition of 5 μmoles ATP, and stopped 5 min later with 1 ml of 20 % trichloroacetic acid. After 5 min centrifugation at $5000 \times g$, 1 ml aliquots were withdrawn for phosphorus determination.

ATPase activity of fragmented sarcoplasmic reticulum. This was determined according to the method of MacLennan [9].

Protein concentrations. These were determined either by the method of Lowry [13] or by the biuret technique scaled down to 0.2–1.0 mg protein, using bovine serum albumin as standard.

Superprecipitation. This was followed at 660 nm, 23 °C with a double beam spectrophotometer (Varian 635), as described by Ebashi [14].

Free calcium. This was determined by atomic absorption spectrophotometry as described previously [15].

Fixation of ^{45}Ca by proteins. This was measured as reported for the determination of calcium binding by parvalbumins [15].

Calcium pump of sarcoplasmic reticulum. This was assayed as specified in the text, tables or figures.

Disc gel electrophoresis. Disc gel electrophoresis in the presence of 0.1 % sodium dodecylsulphate, was run according to Weber and Osborn [16]. Application of the samples on top of the gels was performed in the presence of urea [8]. The gels were stained with 0.25 % G 250 coomassie brilliant blue in 45 % methanol and 9 %

acetic acid for at least 4 h. Destaining was performed in 7.5 % acetic acid with a custom-made electrophoretic apparatus, the gels being transversally to the current. With 200 V (100 mA per tube), destaining was achieved within 40 min. Thereafter the gels were stored in water in the dark.

Electron microscopy. Pellets from rabbit or crayfish fragmented sarcoplasmic reticulum were first fixed for at least 24 h in Karnovsky's medium [17] at 0–4 °C, and then postfixed in 1 % osmium tetroxide, 0.1 M phosphate buffer pH 7.2 for 2 h at 0–4 °C, dehydrated in the usual way with alcohol and propylene oxide and embedded in Epon 812 or Durcopan. Ultrathin sections, made with a diamond knife, were post-stained with lead citrate.

RESULTS

Tropomyosin and troponin from crayfish tail muscle

The affinity of troponin for tropomyosin appears stronger in crayfish than in rabbit muscle, therefore the complete separation of these two proteins is difficult to achieve. Moreover crayfish troponin is quite unstable, as compared to rabbit troponin. Fig. 1 shows the densitometric scannings of disc gels pertaining to crayfish and rab-

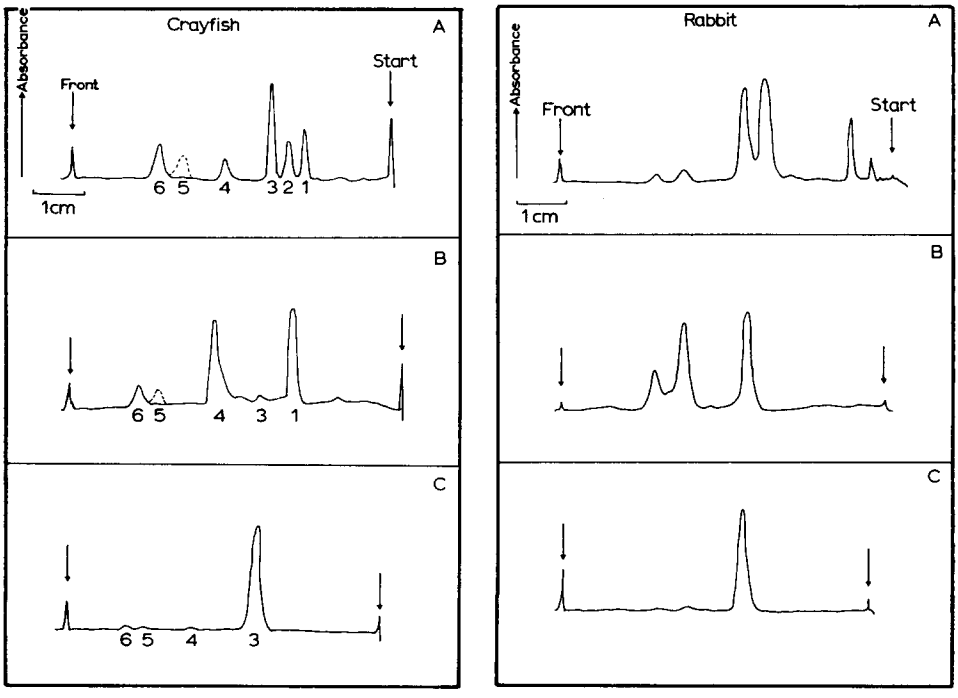

Fig. 1. Densitometric scannings of disc gel electrophoreses of crayfish and rabbit proteins in the presence of sodium dodecylsulphate. (A) Native tropomyosin; (B) troponin; (C) tropomyosin. The concentration of acrylamide was 10 %. 10 μl of 8 M urea were added to each sample (10 μg) on top of the gels. The scannings were obtained at 550 nm by means of a Zeiss ZK4. The scale was adjusted to match the highest peak in each recording. The dotted lines show the position and relative height of the component found in some crayfish preparations (component 5).

bit tropomyosin and troponin. Whereas the rabbit troponin shows the three characteristic peaks of the TNT, TNI and TNC fractions and appears to be fairly pure, crayfish troponin, which also yields three peaks (1, 4, 6) is still contaminated by traces of tropomyosin (peak 3) as well as by a high molecular weight component. Occasionally, an additional peak can be detected on troponin scannings (peak 5). Rabbit and crayfish tropomyosins both present one major component (peak 3) with traces of troponin contamination.

Molecular weight determination of the various components of troponin and tropomyosin was made by disc gel electrophoresis in the presence of dodecylsulphate with various protein markers (Fig. 2). Tropomyosin appears to have a molecular

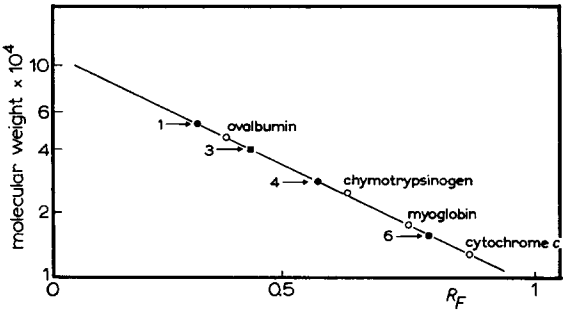

Fig. 2. Plot of the relative mobilities of crayfish troponin and tropomyosin and reference proteins as a function of molecular weight determined by disc gel electrophoresis. Conditions as in Fig.1. Troponin was not separated into its three components prior to the experiment. Markers were ovalbumin (M_r = 45 000), chymotrypsinogen (M_r = 25 000), myoglobin (M_r = 17 800) and cytochrome *c* (M_r = 12 400). ●—●, troponin (components 1, 4 and 6, as characterized in Fig. 1). ■, tropomyosin (component 3).

weight of 40 000 and the three components of troponin of 54 000, 29 000 and 16 000 amounting to 100 000 for the whole complex, assuming that as in rabbit the three components are present in a molar ratio of 1 : 1 : 1 [18]. These values are more scattered than for rabbit troponin where the TNT, TNI and TNC components have molecular weights of 37 000, 24 000 and 20 000, respectively [18].

Crayfish tropomyosin and troponin were studied by observing their effects on the ATPase activity of crayfish desensitized actomyosin [19]. Table I shows these effects in parallel with those of homologous proteins from rabbit muscle. The addition of a mixture of tropomyosin plus troponin or native tropomyosin to desensitized crayfish or rabbit actomyosin has the following effects on the ATPase activity: (a) increase in the presence of calcium; (b) decrease in the presence of EGTA: approximately 60 % of the initial activity is lost. Actomyosin ATPase activity remains insensitive to calcium in the presence of pure tropomyosin. The slight decrease in activity observed when EGTA is added to the actomyosin–troponin system may be ascribed to the traces of contaminating tropomyosin evidenced in Fig. 1 (peak 3).

Addition of native crayfish tropomyosin to an ATPase assay medium containing rabbit desensitized actomyosin restores some calcium sensitivity: the specific ATPase activity of rabbit actomyosin in the presence of native crayfish tropomyosin decreases by 22 % upon addition of 3 mM EGTA.

TABLE I

EFFECT OF REGULATORY PROTEINS ON THE ATPASE ACTIVITY OF CRAYFISH AND RABBIT ACTOMYOSINS

To save material, lower concentrations of regulatory proteins were used in the instance of crayfish, 0.06 mg/ml compared to 0.3 mg/ml for rabbit. This did not affect the results. See Methods for the composition of the assay system. Results expressed as μmoles P_i per min/mg protein.

	Crayfish		Rabbit	
	No additive	3 mM EGTA added	No additive	3 mM EGTA added
Desensitized actomyosin	0.26	0.25	0.28	0.23
+ tropomyosin	0.31	0.31	0.26	0.23
+ troponin	0.28	0.21	0.23	0.20
+ tropomyosin and troponin	0.40	0.15	0.33	0.15
+ native tropomyosin	0.39	0.17	0.33	0.14

Purified crayfish tropomyosin does not bind calcium: before removal of spurious caclium by dialysis, no more than 0.3 gatoms Ca^{2+} per mole of protein (on the basis of a molecular weight of 40 000, see below) are retained. Under the same conditions, troponin retains approximately 1 μg of Ca^{2+} per mg of protein, corresponding to 2.5 gatoms of Ca^{2+} per mole of protein on the basis of a molecular weight of 100 000. After extensive dialysis in the presence of 1 mM EGTA [15] at a troponin concentration of 11 mg/ml and after a second dialysis to remove EGTA, up to 90 % of the initial Ca^{2+} was removed. Troponin binds calcium with a dissociation constant, K_D (ref. 15) of 0.2–0.5 μM; similar values were found for rabbit troponin [20] or fish parvalbumins [15].

Sarcoplasmic reticulum from crayfish tail muscle

A. Morphology. The electron microscope examination of crayfish tail muscle reveals some interesting differences compared to rabbit muscle: (a) the myofibrils are much larger in the invertebrate; (b) the relative positioning of actin and myosin filaments is significantly different; (c) no clear H band appears in the middle of the crayfish A band. Otherwise both muscles are rather similar (to be published). Subcellular formations reminiscent of rabbit sarcoplasmic reticulum are apparent between the crayfish myofibrils.

The isolation procedure of MacLennan [9] was applied to crayfish up to the step "R_1 washed". Electron micrographs reveal the vesicle picture typical of fragmented membranes. Taking into account the difference in magnification, Fig. 3 shows that the crayfish vesicles are two or three times smaller than those of rabbit fragmented sarcoplasmic reticulum. As crayfish muscle is not nearly so tough as rabbit skeletal muscle, homogenization probably brings about a more complete fragmentation of the cellular structure, explaining the smaller size of the crayfish vesicles. The black spots seen on Fig. 3B are probably artifacts due to lead precipitation. The membraneless grey fuzzy formations are recognizable on electron micrographs of muscle preparations from several organisms, even at a high state of purity. They probably correspond to membranes that escaped the microtome knife because of flattening during centrifugation.

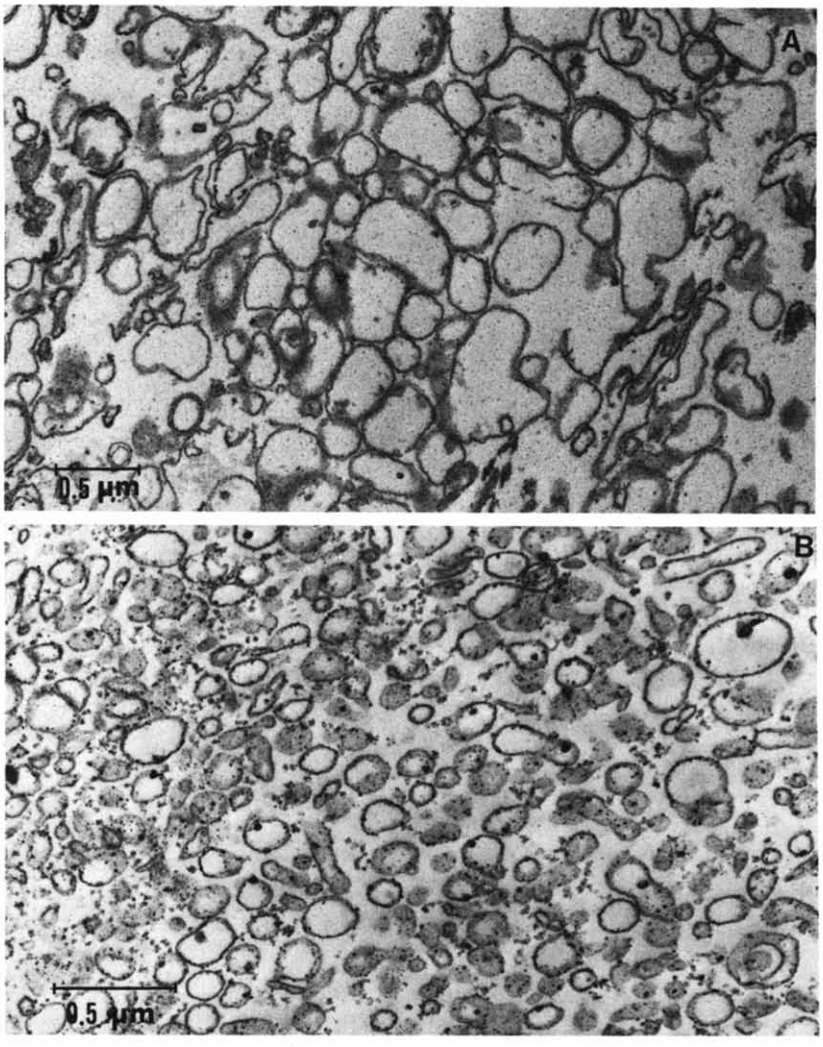

Fig. 3. Electron micrographs of rabbit (A) and crayfish (B) fragmented sarcoplasmic reticulum at the same stage of purity. Samples were prepared and stained as described in Methods. (A) Philips EM 300, ×22 000, (B) AEI-6 B, ×32000.

B. Preparation and properties of crayfish fragmented sarcoplasmic reticulum. The amount of protein and of ATPase activity at the last steps of the purification are given in Table II. The yield of a fragmented sarcoplasmic reticulum purification (3.5 % of the whole muscle protein) can hardly be expressed in ATPase units because (a) the ATPase activity of fragmented sarcoplasmic reticulum cannot be distinguished from that of the other ATPases present at the beginning of the purification and (b) ATPase activities increase markedly with ageing, or freezing and thawing. Thus the specific activity of fragmented sarcoplasmic reticulum can vary from 1 to 7 units at the same stage of purity ("R_1 washed"). Similar activity increases have been obtained for rabbit fragmented sarcoplasmic reticulum.

TABLE II

PROTEIN AND ATPase ACTIVITY AT THE END OF CRAYFISH FRAGMENTED SARCOPLASMIC RETICULUM PURIFICATION

Purification stage	Total protein (mg)	Total activity (μmoles P_i/min)	Specific activity* (μmoles P_i/min/mg protein)
"R_1"	200	264	1.32
Supernatant 7500 $\times g$	59.6	133.6	2.32
"R_1 washed"	42	116.3	2.77

* As measured 10 h after sacrifice.

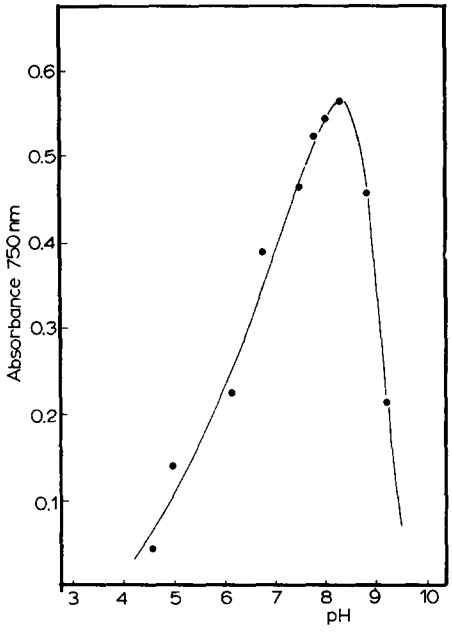

Fig. 4. ATPase activity of crayfish fragmented sarcoplasmic reticulum as a function of pH. The pH of the assay system was adjusted by means of 1 M HCl or NaOH and remained stable throughout the assays.

The pH activity curve is given on Fig. 4. The optimum pH is 8.5 as compared to 7.2–7.5 for rabbit [9]. The former value differs significantly from the physiological pH of crayfish: both haemolymph and freshly homogenized muscle have a pH of 7.4.

The usual assay conditions for the ATPase activity of rabbit fragmented sarcoplasmic reticulum (37 °C, 5 min) are also optimum in the case of crayfish, a fresh water animal accustomed to relatively cold temperatures, as can be seen in Fig. 5. The reaction rate is constant for at least 6 min up to 37 °C. At 50 °C, the fragmented sarcoplasmic reticulum is completely inactivated within the 3 min required to bring the enzyme to temperature equilibrium prior to assay.

As in rabbit [14], crayfish fragmented sarcoplasmic reticulum ATPase is apparently associated with a calcium pump, the activity of which is given in Fig. 6

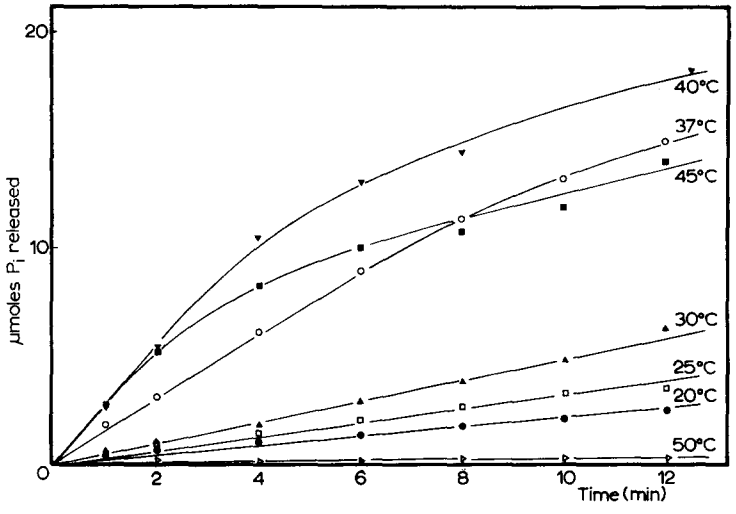

Fig. 5. ATPase activity of crayfish fragmented sarcoplasmic reticulum as a function of temperature. All components of the assay system were first brought to temperature equilibrium except for fragmented sarcoplasmic reticulum, which was added to the reaction mixture 3 min before the beginning of the kinetics, started by addition of ATP. From a total volume of 10 ml, 1 ml aliquots were withdrawn at times indicated and titrated for inorganic phosphorus.

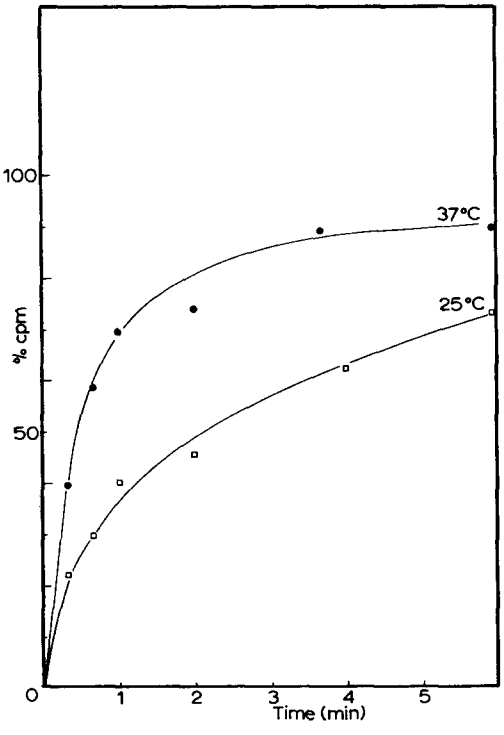

Fig. 6. Calcium uptake by fresh crayfish fragmented sarcoplasmic reticulum at 25 °C (□—□) and 37 °C (●—●). Assay conditions were those described by MacLennan and Wong [18]. 150 000 cpm of ^{45}Ca were used per ml of mixture. From a total volume of 2 ml, 0.2 ml aliquots were withdrawn at times indicated and filtered for ^{45}Ca assay.

as the time course of calcium uptake in sarcoplasmic membranes. For unknown reasons, calcium uptake in rabbit fragmented sarcoplasmic reticulum is usually measured at 25 °C [21], whereas the ATPase activity is followed at 37 °C [9]. For crayfish, calcium uptake was measured both at 25 and 37 °C. It can be seen that, like ATPase activity, calcium uptake is highly temperature dependent. Calcium uptake as a function of time is not linear during the first 6 min contrary to the ATPase activity. At 37 °C, in 1 min about 70 % of the total cpm introduced in the assay medium are incorporated, while in the following 4 min only 20 % more are fixed. An uptake above 90 % could not be achieved although various assay conditions were used (temperature changes, use of calcium–EGTA buffer [21, 22] and of filters with smaller pores); 100 % of the cpm would correspond to an uptake of 0.5 μmole of calcium for about 150 μg of protein or 3.3 μmoles of calcium per mg of protein, a value close to that reported for rabbit fragmented sarcoplasmic reticulum [24, 25]. As usual in such preparations, the oxalate "calcium trap" allows a 25-fold increase in calcium incorporation. In the absence of ATP, Ca^{2+} incorporation is reduced to 0.2–0.5 % of the total cpm, irrespective of the presence of oxalate. The calcium uptake or binding is the same whether the cpm are measured directly on the filter after four washings with 0.5 ml of 0.14 M NaCl (for a filtration volume of 0.2 ml) or calculated from the filtrate obtained without washings (difference between the total cpm introduced and those recovered in the filtrate). This means that, in uptake as in binding Ca^{2+} is firmly bound to insoluble structures.

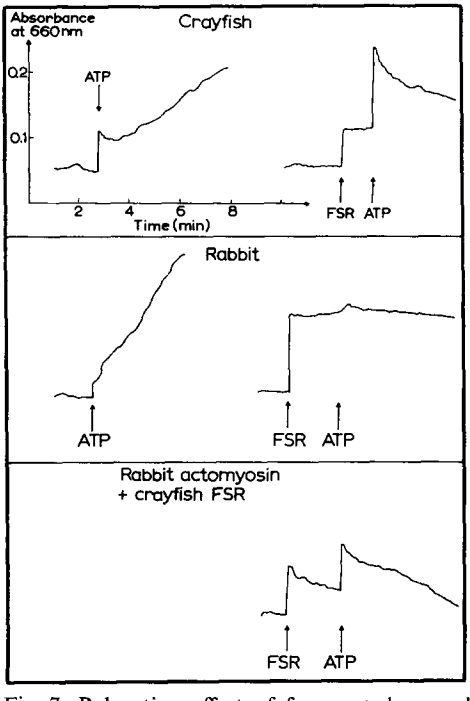

Fig. 7. Relaxation effect of fragmented sarcoplasmic reticulum (FSR) on the superprecipitation of actomyosin. The assay conditions were those of Ebashi [14] except that 2.5 mM oxalate was added to all assays. The scale is the same for all experiments; the concentration of fragmented sarcoplasmic reticulum was 300 μg/ml.

The function of sarcoplasmic reticulum as a calcium pump able to remove free calcium ions is evidenced by the inhibition of actomyosin superprecipitation in the presence of fragmented sarcoplasmic reticulum, as shown in Fig. 7. Natural actomyosin from rabbit and crayfish superprecipitates upon addition of ATP (left part of Fig. 7). However, the addition of crayfish fragmented sarcoplasmic reticulum to either actomyosins inhibits superprecipitation (right half of Fig. 7), provided that oxalate is added to the medium. Oxalate, by itself, does not influence superprecipitation. As the "endogenous" calcium (7.3 μg Ca^{2+} per mg fragmented sarcoplasmic reticulum) was not removed prior to the experiment, it is conceivable that the vesicles were already saturated with calcium, thus explaining the need for a calcium trap.

DISCUSSION

Despite the 400 million years of evolution that separate mammalians from crustaceans, their muscular apparatus appears remarkably similar. From the morphological point of view, both types of fibers present the usual striations across the myofibrils, but in crustaceans the sarcomers are usually much longer and the fibers often appear to branch and anatomose [3, 26]. On the molecular level, the similarity is just as striking: in crayfish as in rabbit, a tropomyosin, a troponin complex able to bind calcium and a sarcoplasmic reticulum functioning as a calcium pump have been identified.

The difference in size between the lighter and the heavier component of troponin is somewhat greater in crayfish (1 : 3) than in rabbit (1 : 2) but the molecular weights are well within the same order of magnitude, for troponin as for tropomyosin. In the striated muscle of the lobster too [27] the lighter and the heavier components of the regulatory proteins recently identified differ in size in the same ratio (1 : 3) as they do in crayfish. The fourth component that appears in some crayfish troponin preparations (Fig. 1, peak 5) does not participate in any of the various functions of troponin tested here; indeed troponin behaves identically whether or not this additional component is present. It may correspond to the inactive TNC component already detected in some fish [28]; peak 6 would then correspond to the typical TNC fraction.

The fragmented sarcoplasmic reticulum purification procedure adopted in this work was that of MacLennan [9] rather than that of Baskin [29]. Although the latter procedure was established for a crustacean (lobster), in the instance of crayfish it yielded fragmented sarcoplasmic reticulum preparations devoid of ATPase activity. In agreement with the results of McFarland and Inesi [30], Meissner and Fleischer [22] or Deamer [31], the specific activity of rabbit fragmented sarcoplasmic reticulum isolated in this laboratory was always lower than that reported by MacLennan [9]. In our hands, crayfish fragmented sarcoplasmic reticulum had an ATPase activity corresponding to about 50 % of that of rabbit, taking into account the influence of ageing or freezing and thawing.

The action of native tropomyosin as well as fragmented sarcoplasmic reticulum of crayfish on the calcium sensitivity of rabbit actomyosin indicates that both contractile systems are related and that the regulation of muscular contraction at this level has not evolved significantly in the last several hundred million years, unless both systems underwent parallel or convergent evolution.

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