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Immunofluorescent Subcellular Localization of Some Muscle Proteins: A Comparison Between Tissue Sections and Isolated Myofibrils*

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Summary: The localization of parvalbumin in fish white muscle and of the calcium binding protein, of arginine kinase and of glycogen phosphorylase in crayfish tail muscle have been investigated by immunofluorescence using isolated myofibrils and muscle sections as starting materials.

It is shown that the four proteins appear to be localized on the thin filaments when myofibrils are used as starting material. This result contrasts with previous observations where it appeared that parvalbumin in fish muscle and arginine kinase in crayfish muscle were distributed uniformly within the cell. This discrepancy is discussed in relation to the high solubility of these proteins.

In the light of the present knowledge about striated muscles from these two organisms, it seems that the roles of parvalbumin in fish and of the calcium binding protein in crayfish are probably different.

Introduction

Calcium appears to be involved in numerous biological processes such as, for example, motility in muscular (Gergely, 1976) and non muscular cells (Hitchcock, 1977), secretions in various cells (Dreifuss, 1973) and activation of enzymological processes (Fischer et al., 1970).

In muscle cells the role of calcium has been emphasized for a very long time (Ringer, 1883; Heilbrun and Wiercinski, 1947). This ion regulates muscular contraction either through the calcium binding subunit of troponin in most striated muscles (Gergely, 1976; Ebashi, 1974), or through the light chains of myosin in vertebrate smooth muscles (Hitchcock and Kendrick Jones, 1975) or in some

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invertebrate muscles through both systems (Lehman, 1976; Kendrick Jones et al., 1970). In addition calcium is a regulator of energy producing systems in such cells acting, for instance, through the activation of glycogen phosphorylase (Fischer et al., 1970).

Various calcium binding proteins have been found in muscle cells (Pechère et al., 1977; Benzonana et al., 1974). The precise function of these proteins with regard to their calcium binding capacity is the subject of several studies (Benzonana et al., 1977b; Gerday and Gillis, 1976) and hypotheses have been presented for their possible functions.

During studies relating to the control of muscular contraction in fresh water crayfish, a new calcium binding protein was purified (Benzonana et al., 1974). Cox et al. (1976) have studied its structure and calcium binding properties and this protein appears somewhat different from a parvalbumin, its apparent homologue in vertebrate white muscles.

In previously published work (Benzonana et al., 1977b) the localizations of the crayfish calcium binding protein and of a fish parvalbumin were determined on cryostat sections from the muscles. Nearly at the same time a somewhat different localization of parvalbumin was reported (Häuptle et al., 1976). It thus appeared worthwhile to reexamine our previous work in the light of recent hypotheses concerning calcium binding proteins. Moreover it seemed useful to perform a critical comparison between two techniques of immunofluorescent localization, i.e. on isolated myofibrils and on histological sections. This would, perhaps, explain the discrepancies between our results and those of other workers and might also contribute to a better understanding of the apparently homologous proteins, crayfish calcium binding protein and vertebrate parvalbumin.

Materials and Methods

1. Preparation of Proteins Used as Antigens

Parvalbumin, Arginine Kinase and Crayfish Calcium Binding Protein were prepared according to Pechère et al. (1971) for parvalbumin and Benzonana et al. (1974) for the two other proteins. Some comparative properties of these three proteins have been already described (Benzonana et al., 1977b).

Crayfish Glycogen Phosphorylase was purified from crayfish according to the following technique (modified from a method already briefly described by Benzonana, 1976). Crayfish tail muscles (about 50 g wet weight) were removed immediately after sacrifice of the animals and homogenized (for 2×30 s in a blade-type homogenizer) with 100 ml of the following solution: imidazole-HCl buffer 10 mM pH 7.2, KCl 50 mM, mercaptoethanol 3 mM, ethylene glycol bis (β-aminoethylether)-N,N′ tetraacetate (EGTA) 0.2 mM. The homogenate thus obtained was centrifuged for 15 min at $27{,}000 \times g$. The clear supernatant was filtered through a 16×160 mm column of 5′ AMP Sepharose 4B (Pharmacia, Uppsala, Sweden) at a rate of 25 ml/h. The elution rate was lowered to 12 ml/h and the column was rinsed successively with 20 ml of buffer A, 30 ml of buffer A supplemented with KCl 400 mM, and 30 ml of buffer A supplemented with KCl 1,000 mM. Phosphorylase was eluted with 30 ml of buffer A containing KCl 1,000 mM and AMP 4 mM. The yield was about 85% and the enzyme was in the active (or "a") form (Fischer et al., 1970) despite the presence of EGTA in all the solutions. The most active fractions were pooled and a maximum volume of 12 ml was filtered at a rate of 40 ml/h through a column of Sephadex G 25 (15×400 mm) equilibrated in β glycerophosphate 10 mM pH 7.2, mercaptoethanol 3 mM. KCl and AMP were

completely removed and the enzyme thus obtained was usually pure as judged by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In some cases a small amount of a low molecular weight impurity was observed by SDS-PAGE. Removal of this impurity was achieved by chromatography through a column of DEAE-cellulose (10 × 60 mm) equilibrated with imidazole-HCl buffer 5 mM pH 7.2, EGTA 0.2 mM, mercaptoethanol 1.5 mM. The purified enzyme was eluted after a small peak of impurity by means of a linear KCl gradient (0–200 mM).

2. *Preparation of Antibodies*

Antibodies against Actin, Carp or Perch Parvalbumin, Crayfish Arginine Kinase and Calcium Binding Protein were obtained as previously described (Benzonana et al., 1977b). Specificity of each of the antibodies directed against fish parvalbumin or crayfish calcium binding protein was ascertained by the double immunodiffusion method (Ouchterlony and Nilsson, 1974). A single precipitation arc was observed with each antibody when assayed against a crude soluble muscle extract (1/10 w/v in imidazole-HCl buffer 40 mM pH 7.2, KCl 100 mM) of either fish (for parvalbumin) or crayfish (for the two other proteins).

Antibodies Against Crayfish Phosphorylase. Rabbits (2.5–3 kg) were first injected subcutaneously (intracutaneously whenever possible) in the neck with about 5 mg of pure protein in 1 ml of Freund's *incomplete* adjuvant. After 2, 4 and 6 weeks an additional 1 mg of protein in 0.5–1 ml of NaCl 150 mM emulsified with an equivalent volume of Freund's incomplete adjuvant was injected. Starting one week after the last injection, 20–30 ml of blood were collected weekly from an ear vein. Immunoglobulins were not isolated in pure form but only partially purified and concentrated by ammonium sulfate precipitation as described by Harboe and Ingild (1973). The specificity of the fraction thus obtained was checked by the double diffusion method (Ouchterlony and Nilsson, 1974).

3. *Preparation and Staining of Myofibrils for Immunofluorescence*

Since the staining was made in order to demonstrate the localization of *soluble* proteins (Benzonana et al., 1977b), it was necessary to prepare the myofibrils as quickly as possible. Muscles were removed from either fish (perch or carp) or crayfish immediately after sacrifice of the animals. Fragments of muscle (1–2 g) were cut with scissors into small pieces around 2–3 mm wide and 10 mm long and gently hand homogenized until complete dilaceration in a Potter homogenizer fitted with a tight teflon pestle in imidazole-HCl buffer 40 mM pH 7.2, KCl 100 mM (10 ml of buffer per gram of fresh tissue). The homogenate was centrifuged for 3 min at 1,000 × *g* in a swinging bucket rotor. The supernatant was discarded, the pellet gently rehomogenized in the Potter with the same amount of buffer as removed and then forced through a Type NY 100 HD nylon gauze (Zürcher Beuteltuchfabrik AG, Rüschlikon. Switzerland). The filtrate was centrifuged as before and the pellet containing the myofibrils was kept after a control under a phase contrast microscope. It was used the same day for immunofluorescence studies. For staining, a suitable amount of the pellet was resuspended in 200 µl of imidazole-HCl buffer 40 mM pH 7.2, KCl 100 mM (buffer B). About 15 µl of human antiactin autoantibodies (Gabbiani et al., 1973; Lidman et al., 1976; Benzonana et al., 1977b) were added and the mixture left for 30 min at room temperature and occasionally stirred. It was then washed (and centrifuged) with 3 × 3 ml of buffer B. The suspension in a volume of about 200 µl was mixed with 10µl of fluorescein labelled γ globulin fraction of goat serum against human γ globulin (Miles Laboratories, Lausanne, Switzerland) for 30 min and washed as before. Staining for parvalbumin, or calcium binding protein, or arginine kinase, or glycogen phosphorylase was performed as before, the fluorescent marker being a rhodamine (or fluorescein) labelled γ globulin fraction of sheep serum against rabbit γ globulin. The rhodamine was coupled to γ globulin in our laboratory. Control experiments were made as follows: myofibrils were incubated with serum from non immunized rabbit or purified non specific γ globulins at a protein concentration equivalent to the one used for normal experiments. In all cases it was impossible to localize any fluorescence on the micrographs after 2.5 min exposure time.

The preparation and staining of sections for immunofluorescence was performed as previously described (Benzonana et al., 1977b). To avoid any possible diffusion of a small molecular weight protein (like parvalbumin), the following slight modification of the original technique was introduced. Immediately after sacrifice of a fish (or crayfish), a muscle fiber was carefully removed from

the tail, slightly stretched between 2 forceps and maintained in this state during inclusion into Ames O.C.P. compound embedding medium. Freezing was performed with a stream of carbon dioxide. The preparation was thus completely frozen within less than 5 min after the death of the animal. After sectionning and fixation, the sections were stained as previously described. Controls were done with rabbit non specific γ globulins at a dilution similar to the one used for specific staining.

4. Electron Microscopy

Myofibrils freshly prepared as above were fixed for 5 h with 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 20° C. They were washed 3 times with cacodylate at 4° C, postfixed with 2% osmium tetroxide for 2 h in collidine buffer 0.2 M pH 7.4 at 4° C. Preparations were stained "en bloc" for 2 h at 4° C with 2% uranyl acetate in sodium maleate buffer 0.05 M pH 5.2 and washed 3 times with the same buffer, then dehydrated through graded alcohols and propilene oxide before embedding in Epon 812. Sections were cut with a diamond knife on a LKB III ultrotome, stained with uranyl acetate and lead citrate and examined on a Philips EM 300 microscope.

5. Sodium Dodecyl Sulfate Disc Gel Electrophoresis

It was performed according to the method of Weber and Osborn (1961).

Results

Specificity of Antisera

Figure 1 shows the results observed with the double diffusion tests. In all cases a single precipitation arc is visible. The case of the crayfish calcium binding protein has been discussed in another publication (Cox et al., 1976) and is not presented here. The peculiar aspect of the parvalbumin test is mostly due to the large difference between diffusion rates of the parvalbumin and the immunoglobulin; this induces precipitation arcs which are never at mid distance between the center and the peripheral holes.

Fish Parvalbumins

Figure 2 shows an example of a section of a fish muscle stained for actin (a) and for parvalbumin (b). In contrast to the actin fluorescence where clear striations are visible and as previously reported (Benzonana et al., 1977b), striations are never seen when staining is done for parvalbumins. Micrographs (c) and (d) are controls. A fish muscle section stained with purified unspecific γ globulins from rabbit is seen under phase contrast (c) and under ultra violet illumination after staining with fluorescein conjugated γ globulins of sheep serum against rabbit γ globulins. No fluorescence is present on the micrograph (d).

Phase contrast and parvalbumin fluorescence of a preparation of fish myofibrils are shown on Figure 3a, b. In contrast to Figure 2b, the parvalbumin fluorescence (Fig. 3b) reveals striations on a few large myofibrils; these striations correspond to the clear bands (actin or thin filaments) seen on phase contrast

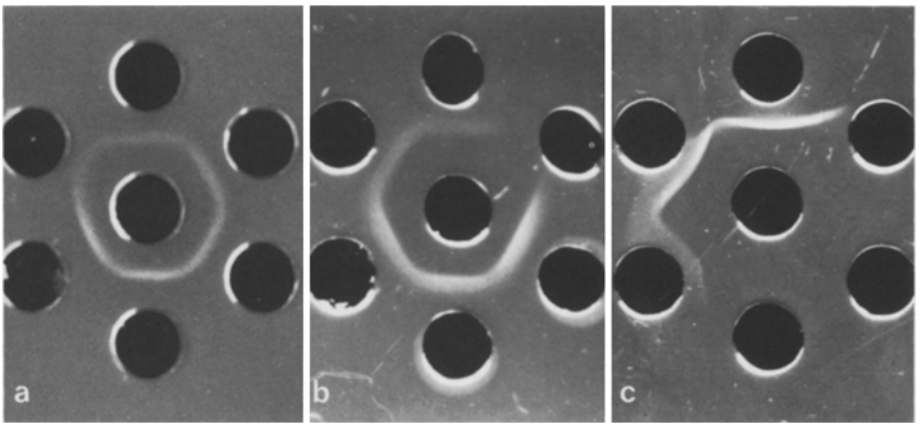

Fig. 1 a–c. Specificity of antisera by double immunodiffusion. **a** Arginine kinase. **b** Glycogen phosphorylase. **c** Perch parvalbumin. Center wells: crude crayfish muscle extract (in **a** and **b**), crude perch muscle extract (in **c**). External wells: antibodies at variable concentrations

(see Fig. 3c, d). Many myofibrils seen in phase contrast on Figure 3a are not fluorescent (Fig. 3b). Moreover the fluorescence of the small S shaped lateral myofibril in Figure 3b is not clear but diffuse.

Actin in Crayfish Myofibrils

The typical appearance of a section from a crayfish muscle seen in phase contrast is shown in Figure 4. The muscle shows the usual alternating clear and dark bands. In the case of invertebrate muscle, however, due to the peculiar properties of contractility (see below) it is not easy to ascertain whether the I band is dark or light, as the dark and light bands are equally wide, actin fluorescence does not allow a clear cut identification (Fig. 4b). In many places in Figure 4a, between the large dark bands, very thin lines can be seen and in Figure 4b there are thin lines in the dark bands (arrowed). This suggests that the I bands in phase contrast are probably dark. The myofibril preparation enables this to be confirmed, because better optical resolution is possible and landmarks can be readily identified in phase contrast and fluorescence micrographs. In Figure 5a, c, crayfish myofibril preparations can be seen at the same scale and after the same treatment as the fish myofibrils of the Figure 3. The appearance of the two types of myofibrils is strikingly different. Instead of the large regular dark bands corresponding to myosin seen on the fish preparation, in the crayfish deeply dark bands alternate with thin gray ones on clear background. In some cases the darkest bands protrude from the borders of the myofibrils (double arrows Fig. 5a and 5c), an appearance caused by the phenomenon of *supercontraction*, a physiological state that is commonly seen in some invertebrate muscles (Hoyle et al., 1965; Uehara et al., 1976). When muscles are not stretched during fixation, this is often observed. In our crayfish myofibril preparations, supercontraction was the rule, and the correspondence between myofi-

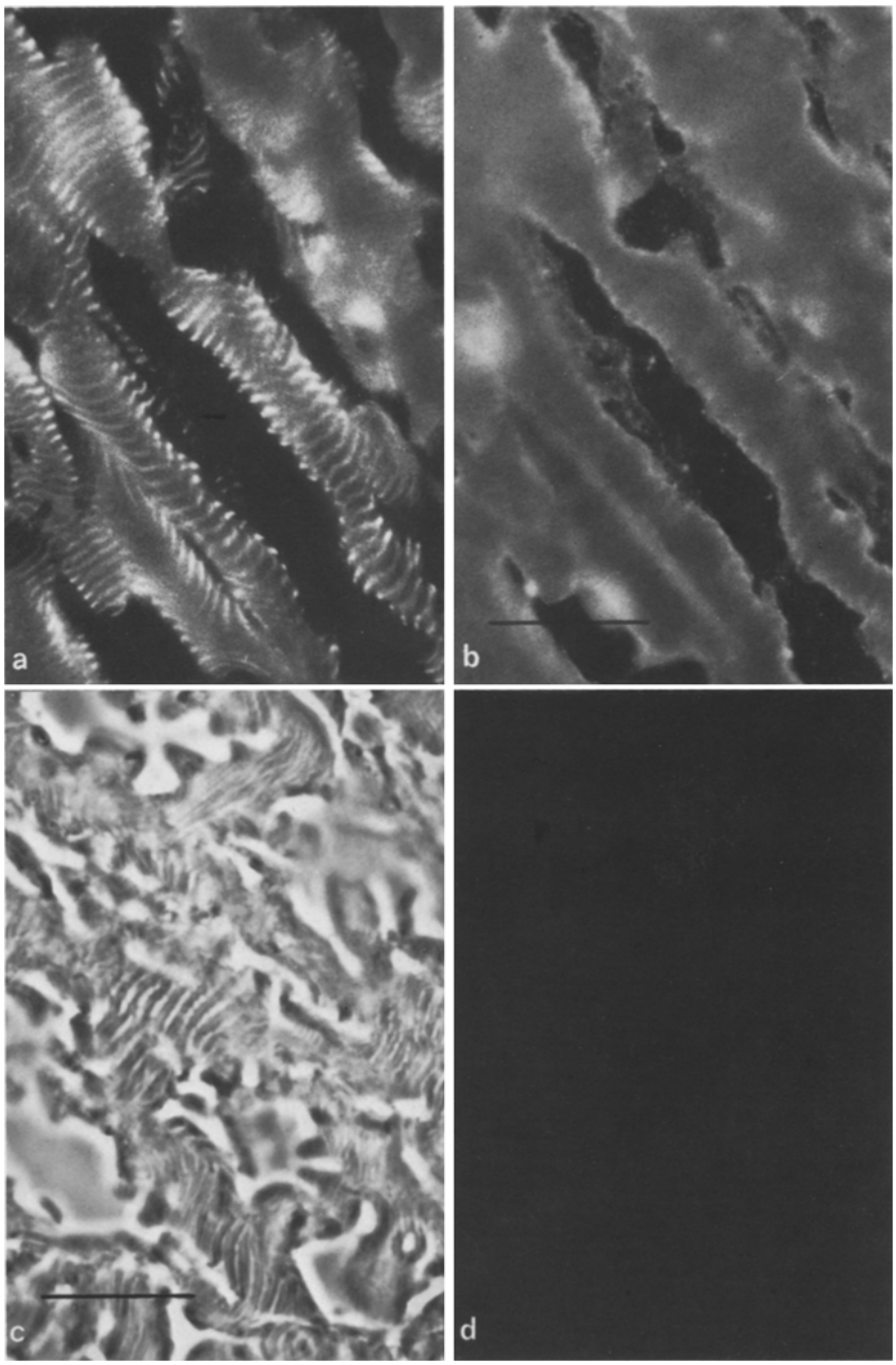

Fig. 2a–d. Perch muscle sections. **a** Actin fluorescence. **b** Parvalbumin fluorescence of the same preparation. **c** Phase contrast appearance of a section stained with non specific rabbit γ globulins then with fluorescein conjugated γ globulins of sheep serum against rabbit γ globulins. **d** Same section as in **c** under ultra violet illumination for fluorescein fluorescence. The bars correspond to 20 μm

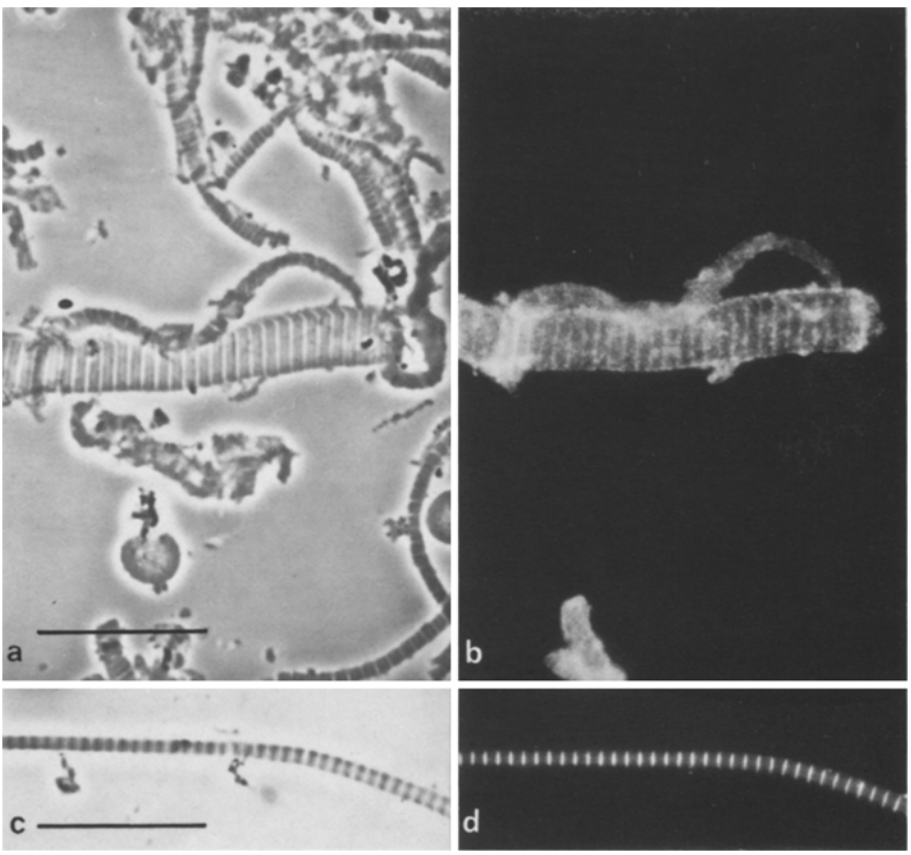

Fig. 3a–d. Perch isolated myofibrils. **a** Phase contrast illumination. **b** The same preparation under ultra violet illumination for parvalbumin fluorescence. **c** Phase contrast illumination. **d** Actin fluorescence. Note the correspondence between the clear zones in **c** (actin zone) and the fluorescent bands in **d**. The bars correspond to 20 μm

brils seen under phase contrast (Fig. 5a, c) and under ultra violet illumination for actin fluorescence (Fig. 5b, d) can be seen. A comparison between these figures indicates that the darkest bands under phase contrast, are fluorescent and selectively labelled for actin.

This extremely surprising aspect is explained by the electron micrographs shown in Figure 6a, b. A supercontracted crayfish myofibril is shown at low magnification in (6a). The thin gray lines seen under light microscopy in the middle of the sarcomeres (see Figs. 5a, 7a) are caused, not by thin filaments but essentially by the overlapping of *thick* filaments. The Z lines protrude form the borders of the myofibrils probably because of the volume increase due to the presence of thick filaments in the middle of these Z lines (Fig. 6b). Most of the thin filaments have disappeared from the myofibrils. In the high magnification micrograph (Fig. 6b) small fragments of thin filaments are seen around and/or in the Z line mostly in disorder. Away from the Z line most

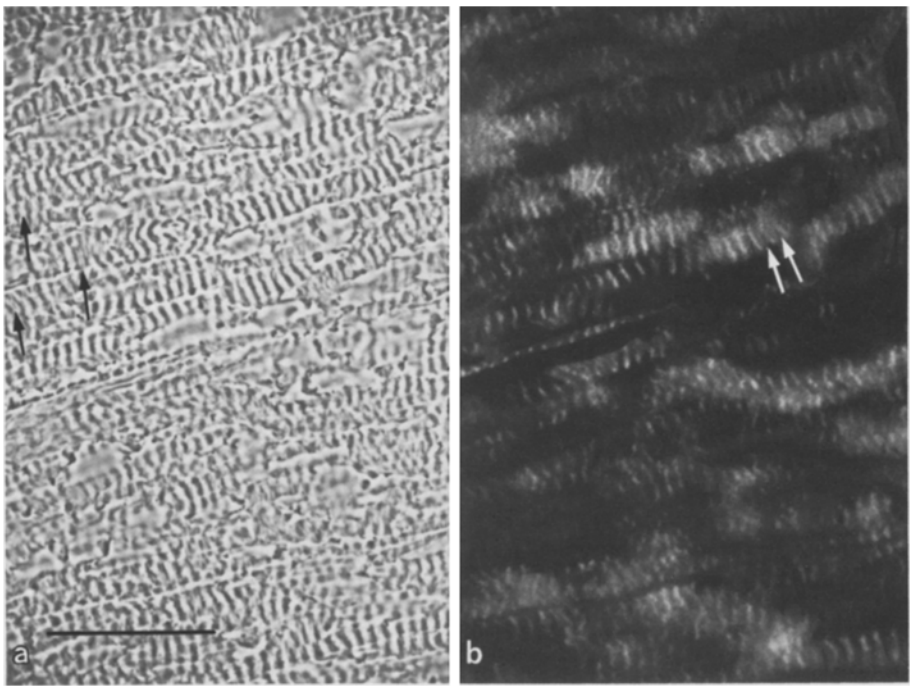

Fig. 4a and b. Crayfish tail muscle section. **a** Phase contrast illumination. Note the thin grey bands (arrows) in the middle of the clear zones. **b** The same preparation under ultra violet illumination for actin fluorescence. Thin light lines appear in some places in the middle of dark zones (arrows). The bar corresponds to 50 μm

of the thin filaments have probably been pushed away due to the rupture and overlapping of thick filaments. This explains the absence of fluorescence in the bulk of supercontracted myofibrils under light microscopy. The Z lines where some thin filaments are remaining are thus the main parts of the myofibrils which appear fluorescent.

The unusual appearance of crayfish myofibrils is not caused by the preparation technique for in crayfish muscle fragments and in fish myofibrils treated in the same way as crayfish myofibrils, all thin filaments are normally present (unpublished observations). Thus crayfish isolated myofibrils reveal unusual properties of crayfish muscle.

Myogenic Proteins in Crayfish Myofibrils

The correspondence between actin and 3 highly soluble proteins, namely arginine kinase, calcium binding protein, and glycogen phosphorylase was studied. It has already been shown that the calcium binding protein is localized similarly to actin in sections of crayfish muscle (Benzonana et al., 1977) while arginine kinase under the same conditions is distributed throughout the cells. In myofi-

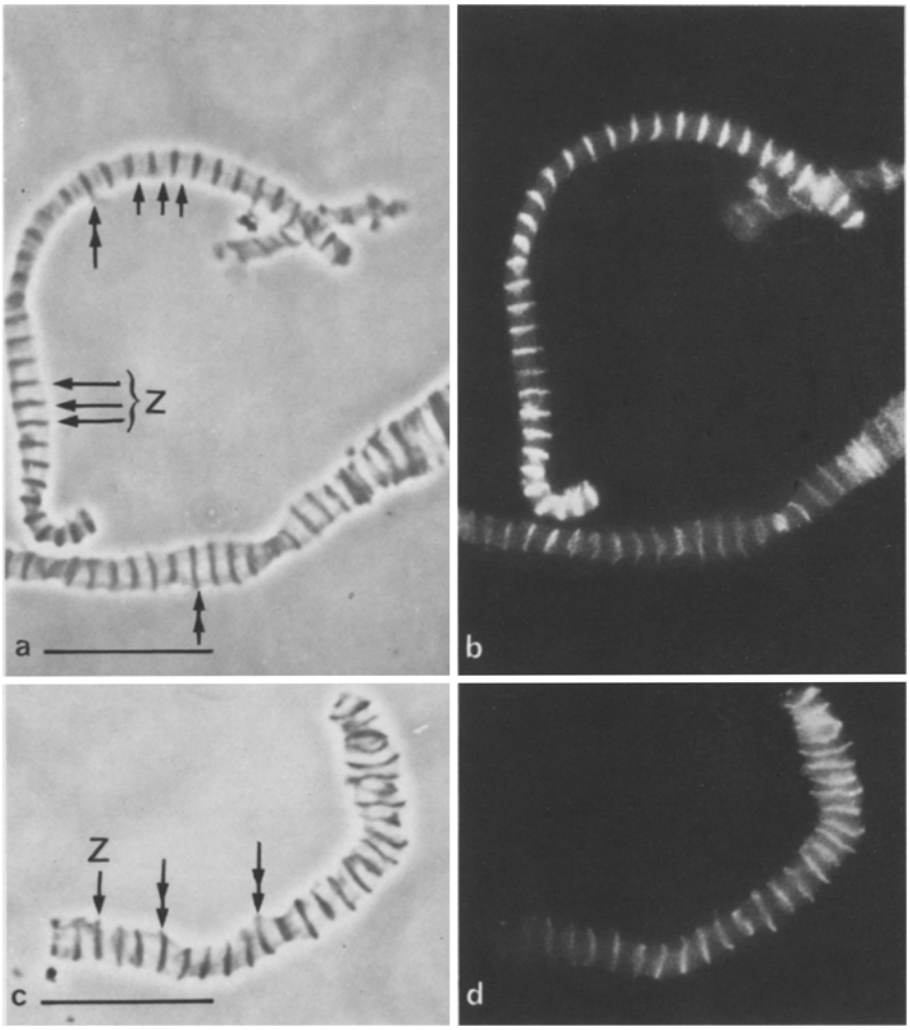

Fig. 5a–d. Crayfish isolated myofibrils. **a** and **c** Phase contrast illumination. **b** and **d** Actin fluorescence. in **a** and **c** dark bands correspond to Z lines (*Z*) in the supercontracted state. The A zone is sometimes divided by a light grey band due to overlapping of thick filaments (arrows). No H zone nor M line is visible. Double arrows indicate protruding Z lines induced by penetration of thick filaments (see Fig. 6). The bars correspond to 20 μm

brils shown in Figure 7a–f actin (now shown to correspond to dark bands in phase contrast illumination) and all three proteins are localized in similar places.

The localization of the calcium binding protein (Fig. 7d) in myofibrils is in accordance with the results already obtained on sections of muscle. As for glycogen phosphorylase its localization in sections of rabbit muscle has already been shown to be within the actin band together with many other glycolytic enzymes (Pette, 1975). We find the same localization in sections of crayfish

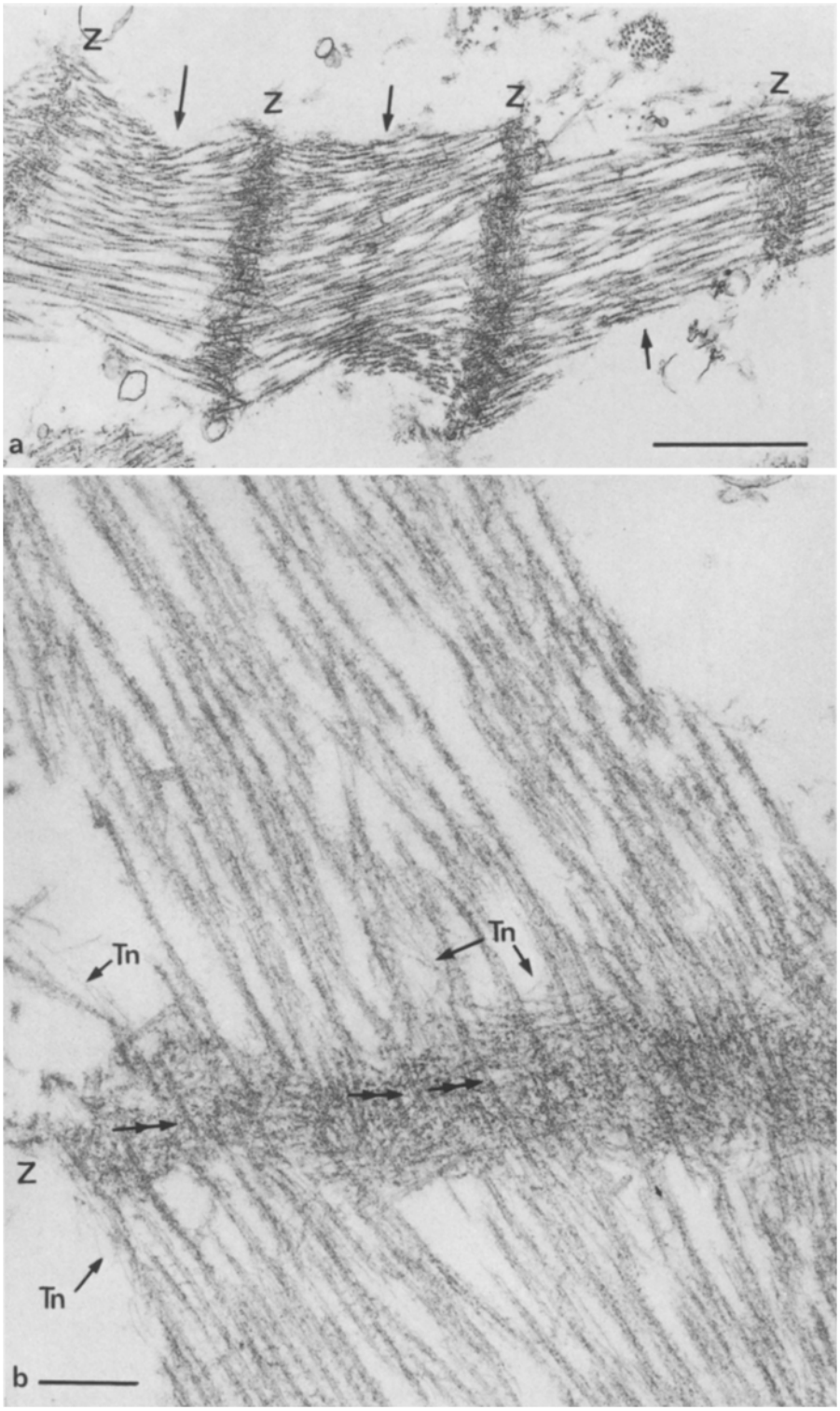

Fig. 6a and b. Crayfish isolated myofibril under electron microscopy.. **a** General appearance of a myofibril at low magnification. Between two Z lines (*Z*) the light grey bands (arrows) are caused by overlapping of *thick* filaments. Note the complete disappearance of thin filaments. **b** Detail from **a** at high magnification. Thick filaments penetrate into the Z line (double arrows). Thin filaments (*Tn*) are visible only near the Z band. The hollow structure of thick filaments and the myosin heads are clearly visible. The bars correspond to 1 μm (in **a**) and 0.2 μm (in **b**)

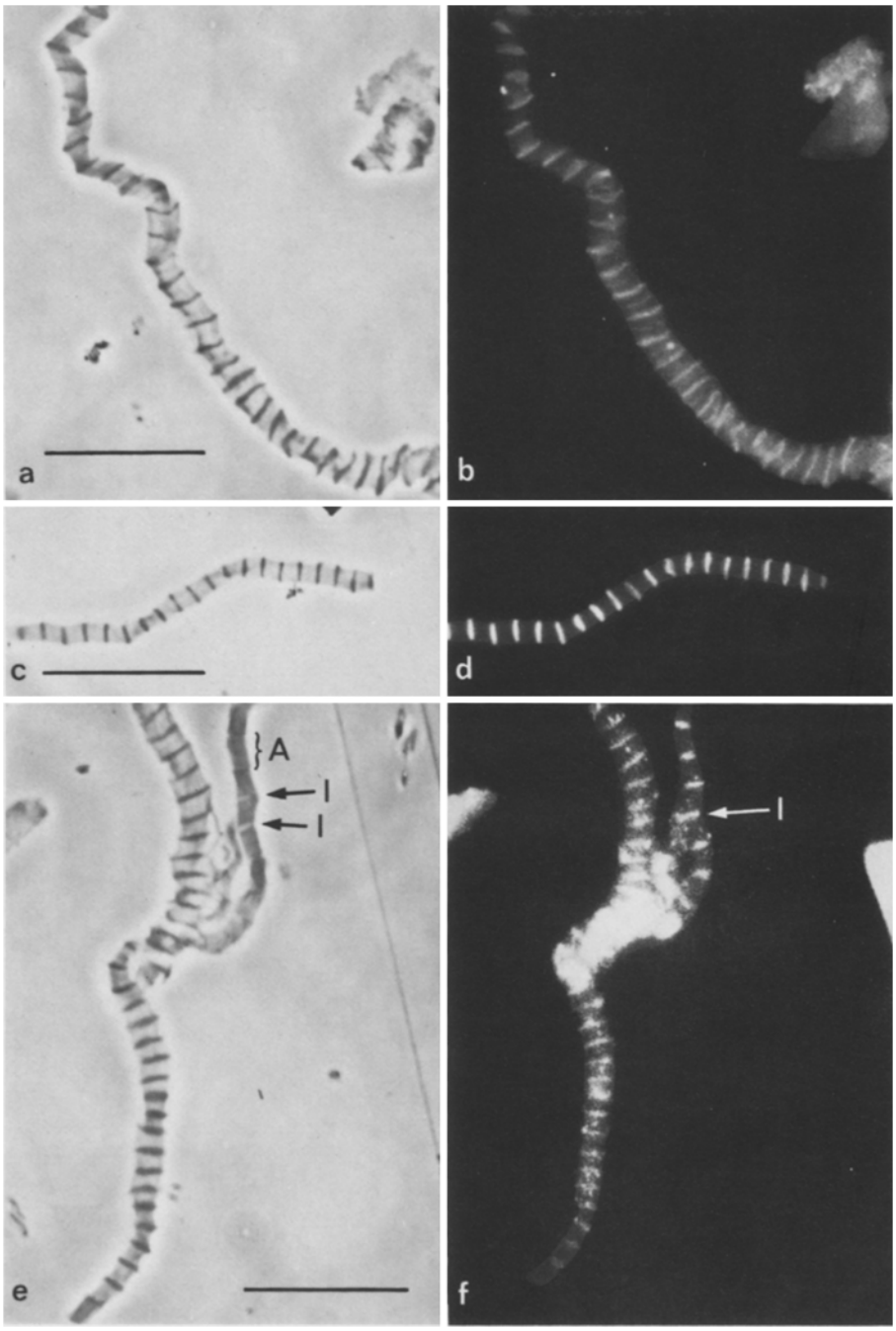

Fig. 7a–f. Crayfish myofibrils. **a**, **c** and **e** Phase contrast illumination. **b** Arginine kinase fluorescence. **d** Calcium binding protein fluorescence. **f** Glycogen phosphorylase fluorescence. Note in **e** a small partially relaxed myofibril with clear I bands (I) and grey A zones (A). In **f** the corresponding I bands are strongly fluorescent. The bars correspond to 20 μm

muscle (not shown here) and in isolated crayfish myofibrils (Fig. 7f) where again, the glycogen phosphorylase is present only on remaining thin filaments (Z lines here).

Crayfish arginine kinase, which has been shown to be present in whole muscle sections (Benzonana et al., 1977) without any preferential localization on the actin filaments, is again clearly localized on thin filaments from isolated myofibrils (Fig. 7b).

Discussion

The following points will be discussed:

a) The apparently different localizations of parvalbumin in fish white muscle depending on the preparation: sections or myofibrils.

b) The unusual appearance of crayfish myofibrils as compared with the vertebrate ones and again the differences observed in the localization of crayfish soluble proteins depending on the preparation (as in a).

c) The possible functions of the crayfish calcium binding protein.

Fish Parvalbumins

Previous work done on muscle sections (Benzonana et al., 1977b) suggested that in fish white muscle parvalbumin was not restricted to certain places, but appeared throughout the cells. This is confirmed in the present report which shows that apart form an apparently higher concentration of parvalbumin on the perimembranous area of the muscle cells this protein is present everywhere (Fig. 2b). The apparently higher concentration of protein on the perimembranous area is probably an artefact. It may be caused by a slight diffusion of reagents between the preparation and the glass support, the excess of reagents being incompletely removed during washings. The same phenomenon can be observed with actin (Fig. 2a) and there is no reason to think that this appearance corresponds to any difference in the local concentration of this protein. Hence the localization of parvalbumin that is observed in the myofibril preparation is, at first sight, surprising. The localization of parvalbumin (or a parvalbumin like protein) in the I band of myofibrils (in chicken) has been reported by Häuptle et al. (1976). Parvalbumin indeed appears on our pictures to be preferentially localized on the I bands, however this observation must be interpreted with caution. The striations corresponding to parvalbumin fluorescence are never as distinct as those of actin fluorescence; for the latter, the clear parallel straight bands (Fig. 3d) correspond to the clear bands appearing in the phase contrast (Fig. 3c). For parvalbumin (Fig. 3b), numerous light spots are visible within the light bands and between them. Moreover, in many places where myosin is supposed to be present, the borders of the myofibrils are fluorescent. Another important point is the small number of myofibrils which become fluorescent for parvalbumin as compared with the total number present in the preparations. The example shown in Figure 3a, b, is representative in that

most of the myofibrils are only slightly fluorescent and this fluorescence, when present, is diffuse, as in the case of the S shaped myofibril of Figure 3b. This may be explained by the very high solubility of parvalbumins. It has been shown in our laboratory (P. Lehky, unpublished experiments) that after centrifugation of a fish muscle homogenate at low or medium ionic strength, high amounts of this calcium binding protein appear in the supernatant. The pellet contains only traces of the protein as was demonstrated by SDS-PAGE.

Thus one hypothesis is that during the preparation of myofibrils, the diffusion of parvalbumin is so fast that most of it is removed from small myofibrils. Nevertheless, some of it is retained in a few large fragments, with a preferential localization for the actin zone. On the other hand incubation of myofibrils with antiparvalbumin antibodies following the addition of parvalbumin did not increase the fluorescence intensity of the myofibrils: only a few ill-defined, highly fluorescent lumps that correspond probably to antigen-antibody precipitates were visible. This means that the "affinity" of parvalbumin for structural muscle proteins in general is very much lower than the affinity of some glycogenolytic enzymes for actin (Pette, 1975). These enzymes appeared to be relatively easily readsorbed on thin filaments in conditions comparable to those used here.

In conclusion, the distribution of parvalbumin in fish white muscle is probably rather uniform as previously demonstrated (Benzonana et al., 1977b), though there may be a slightly higher concentration along the I bands.

It has been proposed that parvalbumins participate in the activation-relaxation cycle of vertebrate skeletal muscle (Gerday and Gillis, 1976; Pechère et al., 1977). The hypothesis is that during relaxation, calcium leaving the myofibrils is bound temporarily to a parvalbumin before being taken up by the sarcoplasmic reticulum. This would allow the myofibrils to release their calcium at a faster rate if the sarcoplasmic reticulum pump was the only effective calcium trap. If this hypothesis is true, the migration or diffusion of calcium from the myofibrils to the sarcoplasmic reticulum would require the continuous presence of this calcium binding protein between these two subcellular elements of the cell.

Myogenic Proteins and Actin in Crayfish

The ultrastructure of invertebrate muscle is somewhat different from that of vertebrate muscle. In the case of crayfish (and of crustacea in general), the sarcomeres are longer than in vertebrates and their length varies between different muscles or even in the same muscle (Atwood, 1972). In the relaxed state, the I band is generally narrower and more diffuse than in vertebrates (Hoyle et al., 1965). If the muscle is slightly contracted or if the fixation is not done under appropriate conditions, the I bands can disappear completely (Brandt et al., 1965; Benzonana and Gabbiani, unpublished), the H bands being no longer visible (Hoyle et al., 1965).

In the present work we used a fast method of preparation of isolated crayfish myofibrils but they were in a supercontracted state, i.e. without visible I bands.

Instead of clear H zones in the middle on the A bands, there were grayish thin lines which correspond to overlapping thick filaments in the case of isolated myofibrils. Treatment of crayfish myofibrils in the relaxing medium of Etlinger et al. (1976) did not lead to any apparent change in structure. This can be easily explained by the partial destruction of the structure of isolated myofibrils after superconcontraction. Most thin filaments being absent (see Fig. 6) the contraction-relaxation process cannot occur any more and the myofibrils remain in their supercontracted state. In all cases this peculiar state of the myofibrils does not appear to interfere with the localization of various proteins.

However, it must be emphasized that actin is frequently not as clearly localized in crayfish myofibrils as it is in vertebrate myofibrils (this is true only for myofibrils, not for sections of muscle in which well defined fluorescent bands are *always* clearly visible as in vertebrate muscles). Again the electron micrographs of Figure 6 explain this disappearance by the absence of most thin filaments (and thus actin). We have also observed that actin in a monomeric form has an anomalous high solubility in crayfish muscle homogenates (unpublished experiments). Whether this results from the destruction of thin filaments during supercontraction, or from another process remains to be established.

The fluorescent striations corresponding to actin on myofibrils in the supercontracted state are clearly coincident with the *dark* bands: the Z lines seem entirely fluorescent and no dark line in their middle was ever visible. This again indicates that the structure of crayfish muscles is different in some way from the structure of vertebrate muscles.

The three myogenic proteins all appear exactly at the same place as actin. This is not surprising for the calcium binding protein (Benzonana et al., 1977b) and for glycogen phosphorylase (Dölken et al., 1975) but it is in contradiction with our previous findings on arginine kinase. A possible explanation for this descrepancy is the high solubility of arginine kinase and, perhaps, a relatively high affinity of the enzyme for thin filaments. Only a small fraction of all the enzymes present in muscle cell is seen on myofibrils; in fact this is true for all three myogenic proteins examined. Indeed the precise localization of various muscle proteins by immunofluorescence is easier on myofibrils than on muscle sections, but the present examples show that it can lead to erroneous interpretation if the solubility of the protein to be tested as well as the peculiar state of isolated myofibrils is not taken into account.

Does the Crayfish Calcium Binding Protein Have a Role Similar to That of Parvalbumin?

The localization of the two proteins is different. Moreover, myofibrils from invertebrate and vertebrate are different, supercontraction being typical of invertebrate muscle. More important is the difference in the role of calcium in the generation and propagation of action potentials. In crustacea, the action potential is due primarily to a flux of calcium ions across the membrane. In vertebrates, sodium ions play this role. It has been suggested that in crustacea the level of free sarcoplasmic calcium is higher in slow than fast muscle fibers

(Atwood, 1972). This is in accordance with the observation that the crayfish calcium binding protein is more abundant in tail than in claws (Cox et al., 1976). In the latter, slow muscles are predominant and the calcium binding capacity is reduced, allowing perhaps more free calcium to be present. The level of the calcium binding protein may thus be in relation with the pattern of muscle innervation of crustacea, which is known to depend on the type of muscle (Atwood, 1972).

In view of the differences observed between:

a) the ultrastructure of crayfish and vertebrate muscle,

b) the respective distribution of the calcium binding protein in crayfish and parvalbumin in vertebrate, and

c) the molecular properties of the calcium binding protein and those of parvalbumin, it appears likely that the two types of proteins do not have the same function in their respective muscles.

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