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Novel Thick Filament Protein of Chicken Pectoralis Muscle: the 86 kd Protein

II. Distribution and Localization

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Antibodies specific for the novel 86 kd protein purified from chicken pectoralis myofibrils stained by indirect immunofluorescence the middle third of each half A-band of isolated myofibrils and myotubes. Pectoralis muscle 86 kd protein, like pectoralis C-protein, displayed a fibre-type specific distribution by being restricted to fast twitch fibres and absent in slow tonic and heart muscle fibres. This was demonstrated by immunoblotting experiments with tissue extracts and by immunofluorescence labelling of cryosections. In primary cell cultures prepared from embryonic chicken breast muscle, 86 kd protein, C-protein and myomesin were all detected in post-mitotic myoblasts where fluorescence was found in a cross-striated pattern along strands of nascent myofibrils. Fluorescence due to the 86 kd protein was restricted to myofibrils within myotubes and no significant labelling of the sarcoplasm was evident. Glycerinated fast twitch muscle fibres, after incubation with antibodies to 86 kd protein, revealed in each half of the A-band nine distinctly labelled stripes, spaced about 43 nm apart. Simultaneous incubation of fibres with antibodies against 86 kd protein and C-protein showed a co-localization of the seven C-protein stripes (stripes 5 to 11), with seven stripes of 86 kd protein. The two additional stripes (stripes 3 and 4) labelled by anti-86 kd antibody continued towards the M-band at the same periodicity from the last C-protein stripe (stripe 5). Thus, partial co-localization of two different thick filament proteins is demonstrated and the identity of transverse stripes at positions 3 and 4 attributed in part to the presence of the new 86 kd protein.

1. Introduction

In each half A-band of skeletal muscle myofibrils, a set of 11 transverse stripes that are ascribed to non-myosin proteins are revealed by electron microscopy (Draper & Hodge, 1949; Huxley, 1967; Hanson *et al.*, 1971; Craig, 1977; Sjöström & Squire, 1977). The stripes are spaced by about 43 nm apart. In negatively stained isolated A-segments, Craig (1977) found that they correspond to the exact cross-bridge repeat of 42·9 nm and three times the axial separation of myosin cross-bridges (approximately 14·3 nm) within vertebrate thick filaments. Using negatively stained ultrathin cryosections, Squire *et al.* (1982), however, reached a different conclusion; namely, that the two repeats differ by 5 Å. So far, two thick-filament-associated proteins, C-protein and H-protein, responsible for some of the stripes have been identified and characterized. C-protein has been shown by immunological methods to be responsible for seven or eight of the stripes depending on the fibre types (Offer, 1972; Pepe & Drucker, 1975; Craig & Offer, 1976; Starr & Offer, 1983). It has been shown that in birds and mammals several distinct isoforms of C-protein seem to exist, e.g. fast, slow and heart-muscle C-protein, each corresponding with a specific muscle type (Callaway & Bechtel, 1981; Jeacocke & England, 1980; Reinach *et al.*, 1982; Yamamoto & Moos, 1983). Since no specific function has been attributed to C-protein, it remains to be established whether these different polypeptides really have a similar function. In a study by Dennis *et al.* (1984), the prospective C-protein isoforms were attributed to different sets of stripes or different numbers of stripes within the A-bands of different chicken

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muscle fibre types. On the other hand, H-protein (Starr & Offer, 1971), the function of which is also unknown, is located in the majority of fibres of rabbit psoas muscle at one position on either side of the A-band, that is, at stripe 3 counted from the M-band (Craig, Bennett, Starr & Offer, personal communication; see Craig & Mergerman, 1979; Starr & Offer, 1982), but seems to be absent in slow muscle (Starr & Offer, 1983) as well as in heart (Yamamoto, 1984). In the accompanying paper (Bähler *et al.*, 1985*b*), we have reported on the purification of the novel 86 kd protein from chicken pectoralis muscle and shown that this protein is a myofibrillar component presumably associated with myosin. In this paper, we describe the localization within myofibrils, the distribution within different muscle fibre types, the developmental appearance in primary cell cultures and, finally, the ultrastructural localization of the 86 kd protein within the A-band of the sarcomeres, all in comparison to C-protein.

2. Materials and Methods

(a) *Myogenic cell cultures*

Cells from breast muscle tissue of 11-day-old chicken embryos were obtained as described (Turner *et al.*, 1974; Wallimann *et al.*, 1983). Cells were plated at an apparent density of 6×10^5/4 ml into 60 mm gelatinized tissue culture dishes (Falcon). Normally, the medium was not changed.

(b) *Antibodies*

The characterization of anti-86 kd protein and anti-chicken C-protein antibodies is described in the accompanying paper (Bähler *et al.*, 1985*b*). The antibodies against 86 kd protein used for immunoblotting (Fig. 3), immunofluorescence labelling (Figs 1 and 2), and for immunoelectron microscopy (Figs 5 and 6) were prepared against the 86 kd protein band cut out from polyacrylamide gels and were termed α-d86 antibody (Bähler *et al.*, 1985*b*). Identical results were obtained by using either of three independently prepared antibodies, no matter whether the whole serum or the immunoglobulin G fraction was used. Goat anti-rabbit C-protein antibodies, a kind gift from Mr R. Starr and Dr G. Offer, Meat Research Inst. Langford, Bristol (GB), were affinity-purified according to Olmsted (1981) as described in detail for the production of anti-M-protein and anti-myomesin antibodies (Bähler *et al.*, 1985*a*).

(c) *Frozen sections*

Small pieces of pectoralis major, anterior latissimus dorsi and heart muscle of chicken were immersed in Tissue Tek II (Lab Tek products, Division of Miles Laboratories, Inc., Naperville, IL), frozen in liquid freon and stored at −20°C. Sections of 7 μm thickness were cut on a cryo-cut microtome (American Optical Model 840C, Scientific Instruments Division, Buffalo, NY) and mounted on glass slides, which had been coated with chrome/alum/gelatine by immersion in a solution of 0·05% $(NH_4)CrO_4$ and 0·5% gelatine. Subsequently, the sections were air-dried.

(d) *Indirect immunofluorescence on myofibrils*

A drop of a myofibril suspension prepared as described (Bähler *et al.*, 1985*a*) was applied on a cover-slip that had been placed in a plastic dish. Myofibrils not adsorbed to the cover-slip were washed off with a solution containing 0·1 M-KCl, 5 mM-EDTA, 1 mM-EGTA, 1 mM-2-mercaptoethanol (pH 7·0). Then 100 μl of antibodies appropriately diluted in the same solution were put on the coverslip for 20 min at room temperature. The primary antibodies were washed off and 100 μl of diluted fluorescein-conjugated second antibody were applied for another 20 min. After washing, the cover-slips were mounted on glass slides with 50% (v/v) glycerol, 0·1 M-glycine/NaOH (pH 9·0).

(e) *Indirect immunofluorescence staining of cell cultures and cryosections*

Cells grown in tissue culture dishes were rinsed 3 times with PBS (phosphate-buffered saline), fixed for 30 min with 3% (w/v) paraformaldehyde in PBS and quenched for 30 min in 0·1 M-glycine in PBS. After washing with PBS, 100 μl of 1 : 100 diluted antibody were placed on the cells for 30 min. After washing with PBS, cells were incubated for another 30 min with a second fluorescein-conjugated antibody (Wallimann *et al.*, 1983). Double immunofluorescence was performed using rabbit anti-chicken 86 kd protein and goat anti-rabbit C-protein antibodies at the dilutions indicated above, followed by fluorescein-conjugated swine anti-rabbit antibody diluted by 1 : 100 and consecutively by rhodamine-conjugated rabbit anti-goat antibody diluted by 1 : 100 (Cappel Laboratories Inc., Cochranville, PA). A drop of mounting solution containing 50% glycerol in 0·1 M-glycine/NaOH (pH 9·0) was placed onto the cells, which then were covered by a cover-slip.

For staining cryosections of different muscles, glass slides with adsorbed cryosections were first immersed in PBS to wash off the Tissue Trek II mounting medium. Then, a drop of diluted antibody (1 : 100) was placed on the sections followed, after washing with PBS, by a 1 : 500 diluted second fluorescein-conjugated antibody. The sections were then processed as described above.

(f) *Electron microscopy*

Chicken pectoralis major fibre bundles were tied onto glass sticks and immersed in rigor solution (0·1 M-KCl, 1 mM-$MgCl_2$, 10 mM-potassium phosphate (pH 7·0), 0·1 mM-EGTA) containing 50% glycerol and kept for 24 h at 4°C. After changing the solution, the fibre bundles were kept at −20°C for at least 1 week, before thawing, washing off the glycerol with rigor solution and teasing them with injection needles into small fibre bundles. These small fibre bundles were immersed overnight at 4°C in rigor solution containing either total immunoglobulin G (IgG) at 15 mg/ml or affinity-purified antibodies against 86 kd protein or against chicken C-protein (1 mg/ml). IgG was prepared from serum by 1-fold dilution with PBS, followed by the addition of saturated $(NH_4)_2SO_4$ containing 0·1 mM-EGTA and 1 mM-$MgCl_2$ at pH 7·0 to give a final concentration of 50% in $(NH_4)_2SO_4$ sufficient to precipitate the IgG. After incubation with antibodies, fibres were washed for at least 2 h with rigor solution and then fixed for 2 h with 2·5% (v/v) glutaraldehyde in rigor solution and postfixed with 1% (w/v) OsO_4 in PBS for another hour. Specimens were rapidly dehydrated with 2,2-dimethoxypropane (Müller &

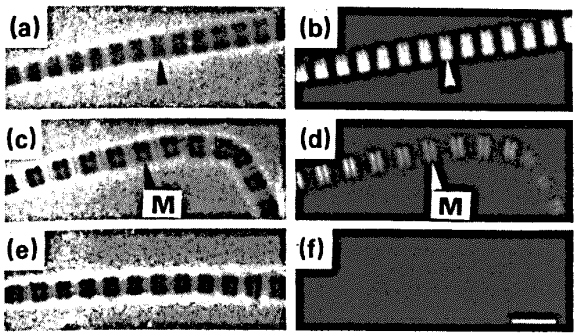

Figure 1. Localization of 86 kd protein and C-protein in isolated chicken pectoralis myofibrils detected by indirect immunofluorescence. Isolated chicken pectoralis myofibrils after incubation with (a) and (b) α-d86 rabbit anti-86 kd protein antibodies or (c) and (d) with rabbit anti chicken C-protein antibodies followed by fluorescein-conjugated goat anti-rabbit IgG. Note that both antibodies are binding in the middle of each half of the A-band ((b) and (d)). Myofibrils after incubation with preimmune-sera revealed no labelling ((e) and (f)). Phase contrast (left) and fluorescence (right) images are paired. M, M-line region. The bar represents 5 μm.

Jacks, 1975) and were then, after 10 to 15 min incubation with water-free acetone, embedded in Epon. Longitudinal sections 100 to 200 nm thick were stained with 2% (w/v) aqueous uranyl acetate and lead citrate (Reynolds, 1963) and viewed at 100 kV in a Siemens 102 or a Jeol 100C electron microscope.

(g) *Gel electrophoresis and immunoblotting*

Gel electrophoresis and immunoblotting were performed as described in the preceding paper (Bähler *et al.*, 1985*b*).

3. Results

(a) *Localization in myofibrils of 86 kd protein by indirect immunofluorescence*

Localization of 86 kd protein within isolated pectoralis myofibrils that had been washed several times was, independent of sarcomere length, confined to the middle third of each half A-band and the fluorescence pattern (Fig. 1(b)) was similar to that obtained by antibodies against C-protein (Fig. 1(d): and see Pepe & Drucker, 1975). The two fluorescent bands obtained with antibodies against 86 kd protein labelled a distance of about 1·2 μm of the A-bands and were spaced in the M-region about 0·2 μm apart. Controls with the corresponding

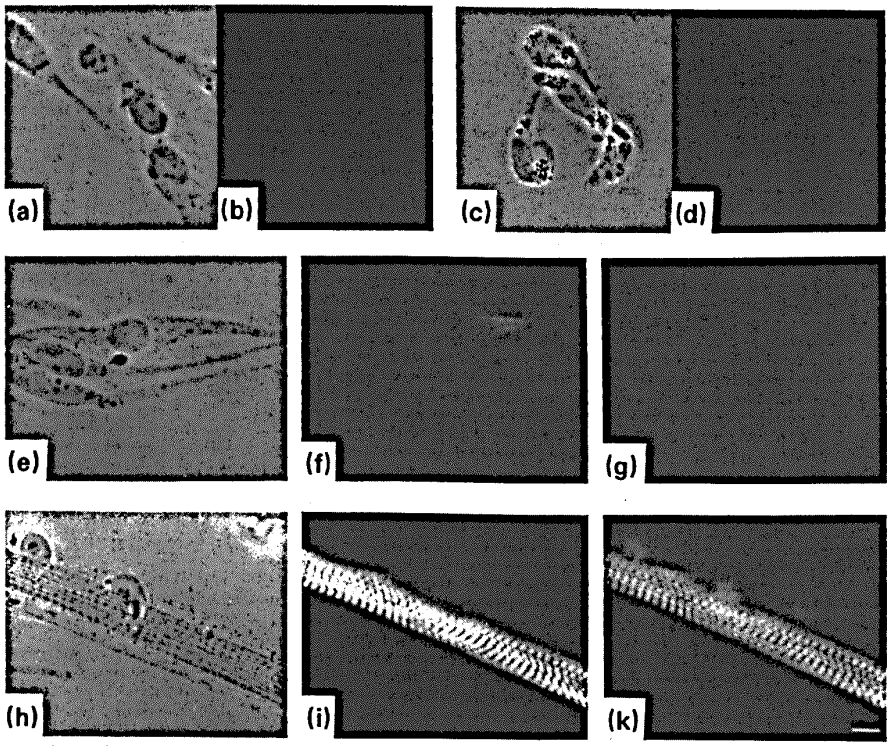

Figure 2. Detection of 86 kd protein and C-protein in primary cell culture from chicken breast muscle by indirect immunofluorescence. (a) to (d) Cells 13 h, (e) to (g) 39 h and (h) to (k) 4 days after plating. Cells 13 h after plating separately incubated with (a) and (b) anti-86 kd protein antibodies or (c) and (d) anti chicken-C-protein antibodies followed by fluorescein-conjugated second antibodies. Indirect double-immunofluorescence staining was performed with cells cultured for (e) to (g) 39 h and (h) to (k) 4 days. Cells incubated with (f) and (i) rabbit anti-chicken 86 kd protein antibodies and (g) and (k) goat anti-rabbit C-protein antibodies followed by rhodamine and FITC-conjugated second antibodies, respectively. (e) and (h) Phase contrast and fluorescence (right) photographs are paired. The bar represents 10 μm.

preimmune sera showed no fluorescence staining (Fig. 1(f)). This clearly demonstrates that 86 kd protein, like C-protein, is a component of the myofibrillar A-band associated with the thick filaments.

(b) *Appearance of 86 kd protein during differentiation of cultured myogenic cells*

By indirect immunofluorescence, no staining was revealed for 86 kd protein or for C-protein in proliferating presumptive myoblasts at 13 hours in culture (Fig. 2(a) to (d)). However, after 39 hours in culture, nascent myotubes showed some distinct fibrillar fluorescence, mostly resolved in a cross-striated pattern when stained for either of the two proteins (Fig. 2(e) to (g)). Whenever C-protein was detected, 86 kd protein was present in the same cell; that is, double immunofluorescence experiments did not show the presence of one antigen without the other (Fig. 2(f) and (g), (i) and (k)). Occasionally, labelling was detected in mononucleated, presumably post-mitotic myoblast cells (Fig. 2(e) and (f)). Myotubes at three to four days in culture are packed with myofibrils and, as shown also for isolated myofibrils from adult chicken (Fig. 1(b)), labelling of the middle third of each half of the A-band by anti-86 kd protein as well as anti-C-protein antibodies was easily resolved (Fig. 2(h) to (k)). Controls performed with the corresponding preimmune sera showed no labelling (not shown). No sarcoplasmic 86 kd protein or C-protein was detected by these means. In addition, 86 kd protein and C-protein seemed to be expressed and incorporated into the myofibrillar apparatus at about the same time as the M_r 185,000 M-band protein myomesin (Grove *et al.*, 1984*a*,*b*), for no difference in the time of detection of all three antigens was resolved by double immunofluorescence (not shown here).

(c) *Fibre-type specificity of 86 kd protein*

Since the localization of C-protein is fibre-type-specific (Callaway & Bechtel, 1981; Starr & Offer, 1982; Reinach *et al.*, 1982), we investigated the distribution of 86 kd protein in different muscle fibres. Chicken pectoralis major (fast-twitch) myofibrils, from which 86 kd protein was purified, showed a strong fluorescent band on immunoblots labelled with anti-86 kd protein antibody (Fig. 3(b)). By contrast, anterior latissimus dorsi, mainly a slow-tonic muscle, showed only a weak fluorescent band of identical molecular weight, and heart myofibrils did not show any reaction at all with anti-86 kd protein antibodies. Identical results were obtained with anti-fast-twitch C-protein antibodies (Fig. 3(c)). Immunohistochemistry with antibodies against pectoralis 86 kd protein or against pectoralis C-protein on serial frozen sections of different muscle types show that all fibres of the white portion of pectoralis major were labelled uniformly by both antibodies (Fig. 4(b) and (c)). By

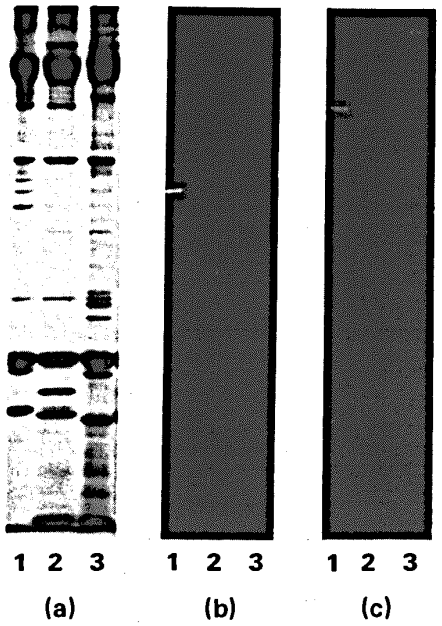

Figure 3. Fibre-type specificity of 86 kd protein and C-protein revealed on immunoblots. Lane 1, pectoralis major; lane 2, anterior latissimus dorsi; and lane 3, heart myofibrils from chicken were run in triplicates on 8% polyacrylamide gels in the presence of sodium dodecyl sulphate. (a) The first gel shown after staining with Coomassie blue for protein. The other two gels transferred to nitrocellulose and (b) reacted with anti-86 kd protein antibody (α-d86); or (c) anti-C-protein antibodies; both followed by fluorescein-conjugated second antibody.

contrast, in anterior latissimus dorsi, only a very small population of fibres representing the few fast fibres in this muscle were stained by both antibodies (Fig. 4(e) and (f)). In heart fibres, no labelling was detected (Fig. 4(h) and (i)). Thus, the antibodies against fast-twitch 86 kd protein and fast-twitch C-protein react in a fibre-type specific way exclusively with fast-twitch fibres.

(d) *Ultrastructural localization of 86 kd protein by immunoelectronmicroscopy*

For the ultrastructural localization in fast-twitch muscle of 86 kd protein, antibodies were used that were prepared either by separately cutting out from polyacrylamide gels the 86 kd protein band (α-d86) or the 79 kd degradation band (α-d79 antibody) and injecting the emulsified gel pieces into rabbits or, as an alternative method, by injecting native purified 86 kd protein (α-n86 antibody: see the preceding paper, Bähler *et al.*, 1985*b*). For immunolabelling, the IgG fraction of the three different antisera were used directly. In addition, affinity-purified antibody prepared against native 86 kd protein was used. All these antibodies have been shown to be specific for 86 kd protein and not to cross-react with any other myofibrillar component, e.g. C-protein or rabbit H-protein (Bähler *et al.*, 1985*b*). All these antibodies labelled in longitudinal sections of chicken

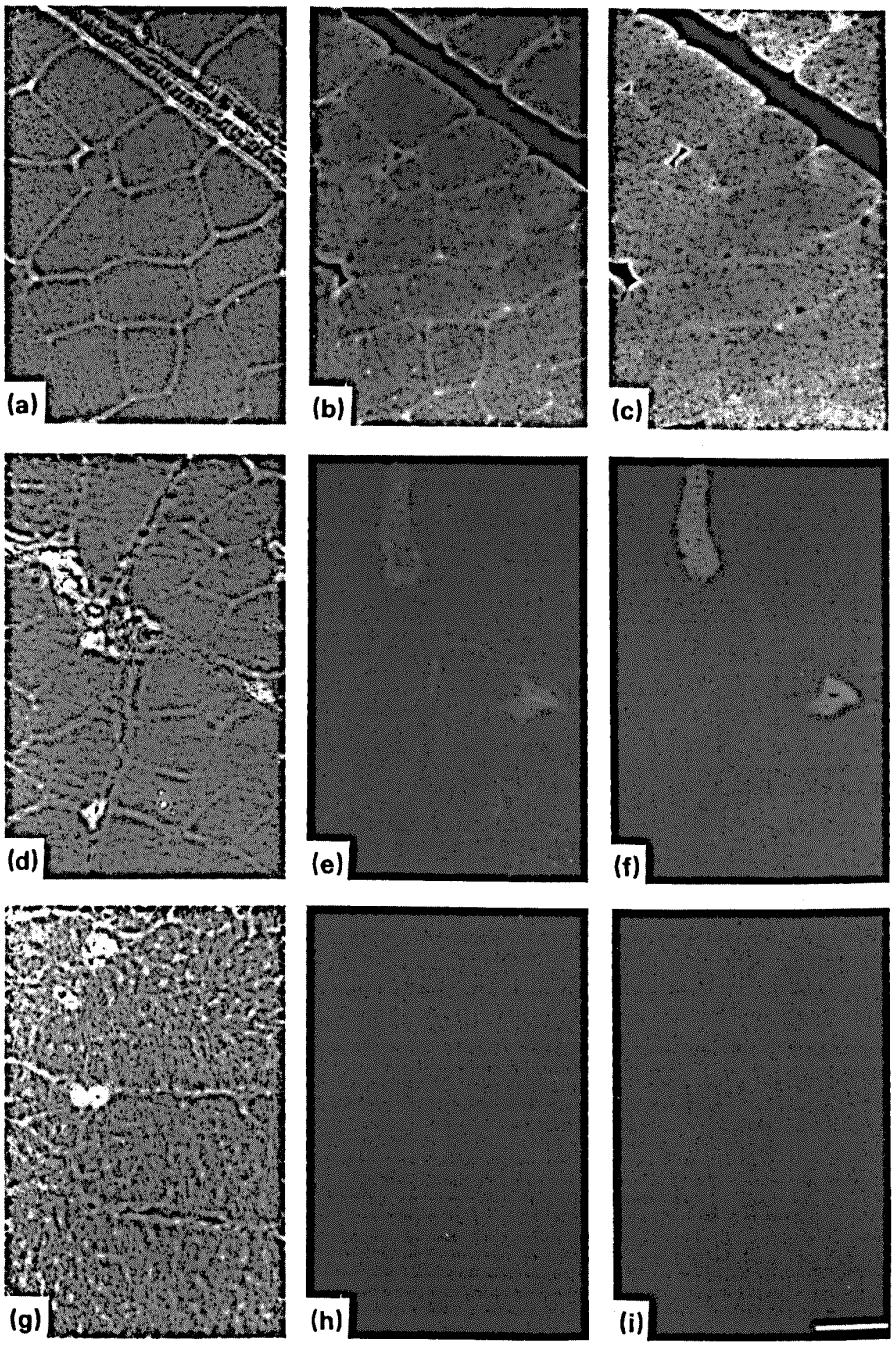

Figure 4. Fibre-type specificity of 86 kd protein and C-protein revealed in cryosections. Frozen serial sections (7 μm) of (a) to (c) pectoralis major, (d) to (f) anterior latissimus dorsi and (g) to (i) heart muscle incubated with anti-86 kd protein antibodies (α-d86: middle row; (b), (e) and (h)); anti chicken-C-protein antibodies (right row; (c), (f) and (i)); followed by a second fluorescein-conjugated antibody. Phase contrast (left) and epifluorescence pictures (middle and right). The bar represents 10 μm.

pectoralis muscle fibres nine stripes in each half of the A-band (Figs 5(b) and 6(b)). Assuming a length of 1·6 μm for the A-band, the stripes were spaced about 43 nm apart. Sometimes, the two stripes nearest to the M-band, at position 3 and 4 (according to the nomenclature used by Craig, 1977), were labelled somewhat more strongly (Fig. 5(b)). The location and periodicity of the labelling strongly suggested that seven or eight stripes stained by anti-86 kd protein antibody coincided with the localization of C-protein. Therefore, for comparison, pectoralis fibres were incubated with anti C-protein antibodies. Strong labelling of seven stripes spaced again by about

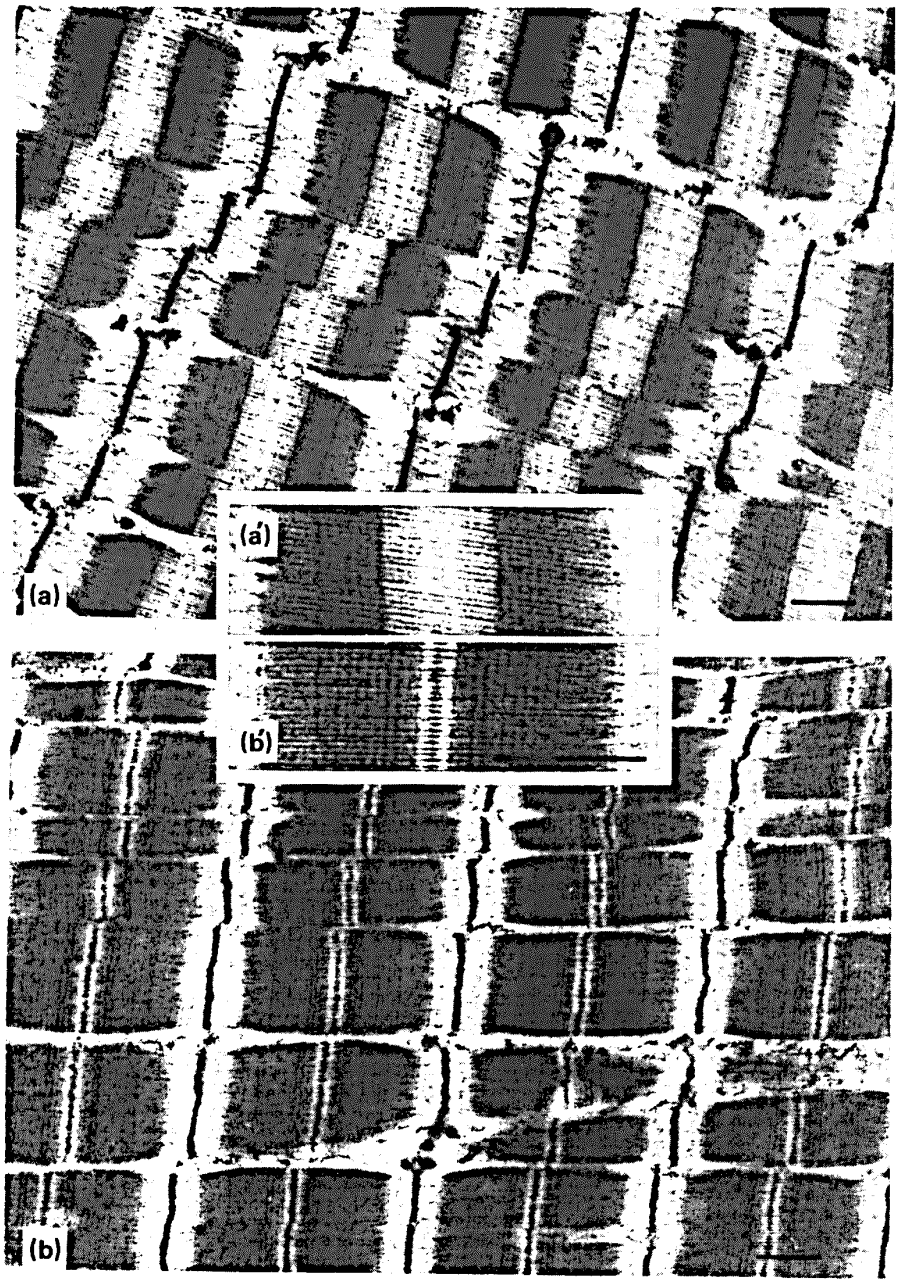

Figure 5. Ultrastructural localization of 86 kd protein. Immunolabelling of chicken pectoralis major fibres (a) with affinity-purified anti chicken-C-protein antibodies; and (b) with anti-86 kd protein antibodies (α-d86), see also Fig. 6(b). Insert: Higher magnification view of aligned labelling patterns obtained with antibodies against (a') chicken C-protein; and (b') 86 kd protein. The bars represent 0·5 μm.

43 nm was observed (Fig. 5(a)). Measurements of the distance between the centre of the A-band and the outermost band (stripe 11) labelled by antibodies against 86 kd protein or C-protein gave in both cases a value of 0·52 μm, supporting the co-localization of C-protein with seven positions of the 86 kd protein. In addition, after simultaneous incubation with antibodies against C-protein and 86 kd protein, a labelling of nine stripes being identical (Fig. 6(a) and (c)) to the labelling with anti-86 kd protein antibodies alone (Figs 5(b) and 6(b)) could be observed. In fibres that had been double-labelled, the stripes were spaced again by about 43 nm and no significant broadening of stripes was observed, indicating that the seven C-protein stripes fully overlap with seven of the nine 86 kd protein stripes (Fig. 6(a) and (c)). The two additional stripes seen with anti-86 kd protein antibody (stripes 3 and 4) followed at the same spacing interval the last C-protein stripe (stripe 5)

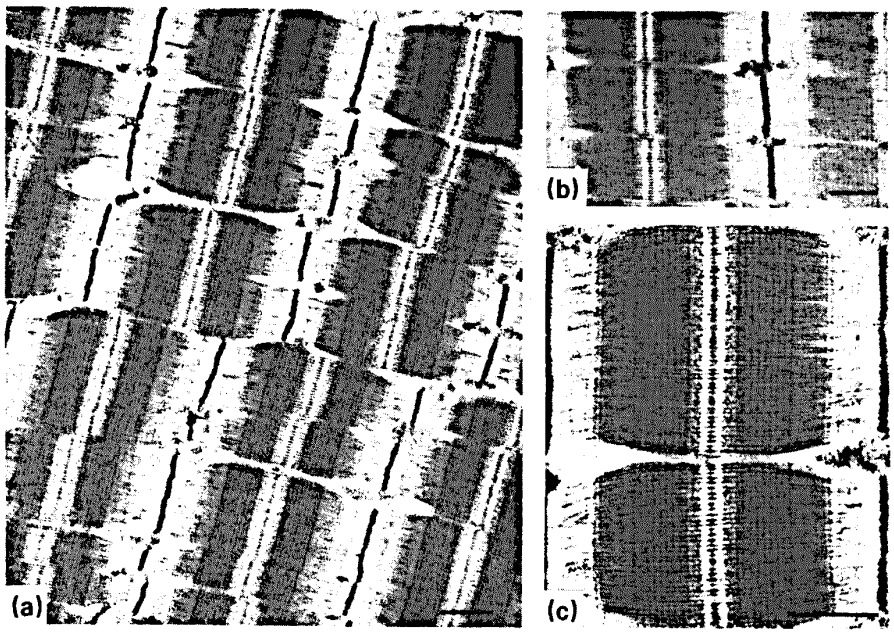

Figure 6. Ultrastructural double immunolabelling with anti-86 kd and anti-C-protein antibodies. (a) Chicken pectoralis major fibres incubated with a mixture of antibodies against both 86 kd protein (α-d86) and chicken C-protein. (b) For comparison, an immunolabelling obtained by anti-86 kd protein antibodies alone. Note the similar intensity of labelling of all 9 stripes as opposed to Fig. 5(b). (c) Higher magnification of view shown in (a). The bars represent 0·5 μm.

in the direction towards the M-band. The results shown here were obtained with anti-86 kd protein antibody prepared against the 86 kd protein band that was cut out from polyacrylamide gel (α-d86 antibody). However, identical staining patterns, number of stained positions and spacing of antibody stripes within the sarcomeres were obtained with the antibodies against native 86 kd protein (α-n86) and against the cut-out M_r 79,000 degradation product of 86 kd protein (α-d79). The same was true with affinity-purified anti-86 kd protein antibody (not shown here: for characterization of antibodies, see Bähler *et al.*, 1985*b*). In addition, anti-86 kd protein antibody affinity-purified by elution from nitrocellulose blots was tested by immunofluorescence on myofibrils and gave the same staining pattern. No change in the staining pattern was observed with respect to number, position or spacing of stripes from the outside of the fibre bundle towards the centre, thus excluding the possibility that anti-86 kd protein antibodies were contaminated with anti-C-protein antibodies. Controls performed by incubation of fibres with preimmune IgG instead of immune IgG were shown to produce no labelling.

4. Discussion

We report here the ultrastructural localization of yet another thick-filament-associated minor muscle protein, the 86 kd protein, which is different from any known thick filament protein. Evidence is presented that the individual transverse stripes of 43 nm periodicity within the A-band of chicken pectoralis sarcomeres are composed of more than one protein species, e.g. it was shown that stripes 5 to 11 in chicken pectoralis muscle (according to the nomenclature of Craig, 1977), so far attributed only to C-protein, contain also the 86 kd protein. Double immunoelectronmicroscopy with anti-86 kd protein and anti-C-protein antibodies revealed that the outer seven of the nine labelling positions obtained with anti-86 kd protein antibodies coincided with the seven transverse stripes also labelled by anti-C-protein antibodies alone. The two additional stripes labelled by anti-86 kd-protein antibodies, stripes 3 and 4, directly followed in the direction towards the M-band at the same periodicity as the last C-protein stripe. The fact that stronger labelling of the two innermost stripes was sometimes observed with anti-86 kd protein antibodies (Fig. 5(b)), although it was not a consistently observed feature (see Fig. 6(b)), may indicate that either a larger amount of 86 kd protein is present at stripes 3 and 4 compared to stripes 5 to 11 or that in the latter stripes, 86 kd protein is less accessible to antibodies, possibly due to masking of antigenic sites as a result of interaction of 86 kd protein with C-protein in this region or simply due to the thin filament overlap.

In the white portion of chicken pectoralis major muscle used in this study, only seven (5 to 11) and not eight stripes were labelled by our polyclonal anti-pectoralis major C-protein antibody, which is in agreement with earlier studies on chicken muscle (Pepe & Drucker, 1975). Stripes 3 and 4 were not seen to contain C-protein, although the presence of C-protein within stripe 3 but not stripe 4 has been

reported in chicken muscle (Dennis *et al.*, 1984). Stripe 3 is also the position where H-protein has been localized in rabbit (Craig & Megerman, 1979), whereas stripe 4 is the optional position reported by Starr & Offer (1982, 1983) where an eighth C-protein stripe is located in many rabbit psoas fibres. Thus, the question arises as to whether the 86 kd protein is bound to the thick filament at nine specific locations by direct interaction with myosin or whether additional proteins, like C-protein and H-protein, which 86 kd protein may be binding to, are needed to facilitate binding of 86 kd protein to the thick filament. Direct binding of 86 kd protein to myosin is supported by the fact that 86 kd protein is a persistent contaminant in preparations of chicken myosin and that it is retarded on myosin affinity columns. However, more detailed studies on the interaction of this protein with myosin and its subfragments as well as with the myosin-associated proteins are needed to answer this question.

By gel permeation experiments with purified 86 kd protein, an average apparent M_r of 370,000 was estimated for the native protein, suggesting either the existence of a polymeric form of the polypeptide with a subunit M_r of 86,000 or a highly asymmetric shape of the molecule (Bähler *et al.*, 1985*b*), and yet the nine stripes stained by our polyclonal antibodies showed a rather narrow width of approximately 12 to 15 nm, suggesting that 86 kd protein, like C-protein, has a very limited axial extent of approximately 10 nm. Both of these requirements may be met, however, by the possibility that the polymeric or extended 86 kd protein, instead of lying along may be wrapped around the thick filaments, or stick out from the inside of the thick filaments at periodic positions to give rise to a nine-stripe labelling pattern.

By densitometric determination of the 86 kd protein band relative to actin (Offer *et al.*, 1973) of washed pectoralis myofibrils, after separation of the myofibrillar proteins by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and subsequent staining with Fast Green, an approximate value of two 86 kd protein molecules at each of the 18 positions in a complete thick filament was calculated according to Offer *et al.* (1973), assuming that all 18 stripes in an A-band were to contain an equal number of 86 kd protein molecules. This corresponds approximately to a 1 : 1 molar ratio of 86 kd protein to C-protein in pectoralis myofibrils (see Bähler *et al.*, 1985*b*). This is reflected by simply looking at gels of pectoralis myofibrils (Fig. 3(a), lane 1), where the intensity of the 86 kd protein band is approximately half of the C-protein band, the latter, however, exhibiting an apparent M_r value of almost twice that of 86 kd protein. However, in order to obtain a more accurate estimation on the number of 86 kd molecules per stripe, again, direct interaction studies between 86 kd protein and myosin including myosin subfragments are needed. Also, the effects of 86 kd protein on myosin self-assembly have to be investigated in a qualitative manner. C-protein, besides its possible structural role in the assembly of thick filaments, might have an enzymatic or regulatory function as indicated by the facts (1) that it can influence the actin-activated Mg-ATPase (Moos & Feng, 1980), (2) that it can bind to thin filaments in a calcium-regulated way (Moos, 1981) and (3) that cardiac C-protein can be phosphorylated (Jeacocke & England, 1980; Hartzell & Titus, 1982). Therefore, it would be an attractive working hypothesis to postulate for the 86 kd protein a complementary enzymatic activity, e.g. a kinase or phosphatase activity, so that both proteins together with myosin may form a structural and functional multienzyme complex. So far, however, we have been unable to attribute any such functions to the 86 kd protein.

As far as a possible role of 86 kd protein in thick filament formation and myofibril assembly is concerned, our double-immunofluorescence studies, although of limited sensitivity, show that 86 kd protein is incorporated at very early stages of muscle development into nascent myofibrils together with C-protein and myomesin. Thus, a role of these proteins in the assembly process of the contractile apparatus has to be considered. Here, it is worth mentioning that cardiac C-protein is expressed also in cultured myogenic cells (Bähler *et al.*, 1985*c*).

Neither 86 kd protein nor C-protein were found in gizzard, brain, liver or kidney, only in striated muscle. The polyclonal antibody against the 86 kd protein seems very useful for fibre typing as marker for fast-twitch muscle fibres, since no cross-reacting antigen was detectable by these antibodies in slow fibres or heart muscle.

The labelling by rabbit anti-chicken 86 kd protein antibody of nine periodic positions within each half A-band of chicken myofibrils seems to be extremely reminiscent of the results obtained on the location of rabbit X-protein in rabbit muscle (Bennet, Starr & Offer, personal communication). However, compared to chicken, rabbit X-protein is of significantly higher apparent molecular weight (M_r 152,000, Starr & Offer, 1982, 1983) and, unlike 86 kd protein, is not restricted to fast white fibres. In addition, the amino acid composition of rabbit X-protein and H-protein, and chicken 86 kd protein differ markedly (Bähler *et al.*, 1985*b*). From this evidence, it is unlikely that X-protein and 86 kd protein or H-protein and 86 kd protein are very similar proteins. However, we cannot exclude the possibility that these proteins may be related structurally and functionally and that the differences described are in fact differences between species. It remains to be established whether there exist also fibre-type-specific isoforms of the 86 kd protein, as reported for C-protein (Callaway & Bechtel, 1981; Reinach *et al.*, 1982; Yamamoto & Moos, 1983) or whether the 86 kd protein is limited to fast-twitch muscles (as indicated by our results) where its presence may be correlated specifically with the structural and functional needs of fast-twitch muscle.

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