

# Isoforms of C-protein in Adult Chicken Skeletal Muscle: Detection with Monoclonal Antibodies

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**ABSTRACT** Monoclonal antibodies (McAbs) specific for the C-proteins of chicken pectoralis major and anterior latissimus dorsi (ALD) muscles have been produced and characterized. Antibody specificity was demonstrated by solid phase radioimmunoassay (RIA), immunoblots, and immunofluorescence cytochemistry. Both McAbs MF-1 (or MF-21) and ALD-66 bound to myofibrillar proteins of ~150,000 daltons; the former antibody reacted with pectoralis but not ALD myofibrils, whereas the latter recognized ALD but not pectoralis myofibrils. Chromatographic elution of the antigens from DEAE-Sephadex, and their distribution in the A-band, support the conclusion that both of these antibodies recognize variant isoforms of C-protein. Since both McAbs react with a protein of similar molecular weight in the A-band of all myofibrils of the posterior latissimus dorsi (PLD) muscle, we suggest that either another isoform of C-protein exists in the PLD muscle or both pectoralis and ALD-like isoforms coexist in the A-bands of PLD muscle.

C-protein, first detected as a contaminant in conventional myosin preparations (21), has been shown to be a component of myofibrils in striated muscle (19). It is arranged in 14 transverse stripes within the A-band (2, 18, 20) and is a component of thick myofilaments (3, 17). C-protein binds the myosin rod and light meromyosin (14), subfragment-2 (22), and actin (15). The binding of C-protein to thin myofilaments is calcium-sensitive, being inhibited by EGTA and restored upon raising  $Ca^{++}$  concentrations (16). In addition, C-protein modifies myosin aggregation (10, 11) but no definite role has been demonstrated in thick filament length regulation, myofibrillar assembly, or muscle contraction.

Recently, it has been shown that different isoforms of C-protein exist in striated muscles of various fiber type. In the rabbit, C-protein from the soleus muscle differs in molecular weight and amino acid composition from that in the psoas (1). Polyclonal antibodies against C-protein from fast muscle were reported not to stain slow myofibrils but similar antibodies to C-protein from slow muscle reacted with myofibrils from both fast and slow muscle (1). Rabbit cardiac C-protein is also different from that in both red and white muscle (25), and there is evidence that it can be phosphorylated (9).

In the course of preparing McAbs against myosin from

chicken skeletal muscle (4, 12) we isolated several antibodies which bound to specific C-protein isoforms. In this paper, we have characterized these antibodies, determined the distribution of C-protein isoforms in pectoralis, ALD, and PLD muscles, and shown that determinants specific for ALD and pectoralis C-protein coexist in the A-bands of PLD myofibrils.

## MATERIALS AND METHODS

### *Protein Preparations: Myosin*

ALD and PLD muscles were homogenized in a Waring blender (Waring Products, New Hartford, CT) for 10 s at full speed (4°C) in the presence of 0.1 M KCl, 20 mM potassium phosphate buffer, 0.1 mM ethyleneglycol-bis-( $\beta$ -amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), 2 mM  $MgCl_2$ , 0.1 mM phenylmethyl sulphonyl fluoride (PMSF), pH 6.5, followed by centrifugation at 14,000 g for 10 min. After repeating this procedure once, the pellet was extracted in 4 vol of a modified Guba-Straub solution (0.3 M KCl, 0.1 M  $KH_2PO_4$ , 0.05 M  $K_2HPO_4$ , 5 mM ATP, 5 mM  $MgCl_2$ , 0.1 mM PMSF) for 15 min at 4°C. For pectoralis muscle, the tissue (pectoralis major and minor after removal of the red stripe) was minced in a meat grinder and immediately extracted with Guba-Straub solution (no MgATP or PMSF). Extraction was terminated by centrifugation (14,000 g for 15 min) for ALD and PLD muscles, or by threefold dilution with water followed by gauze filtration for the pectoralis muscle. After this step, all muscles were processed identically. Myosin was precipitated by dilution (1 < 0.05) and collected by centrifugation (14,000 g for 10 min). The pellet was

resuspended in 0.5 M KCl, 5 mM MgCl<sub>2</sub>, 5 mM ATP (from a 100 mM ATP stock made pH 7.0 with NaOH) and cleared by centrifugation at 40,000 rpm for 2 h in a Beckman Ti-45 rotor (Beckman Instruments, Inc., Fullerton, CA). This step pelleted most of the F-actin, and myosin was recovered from the supernatant by centrifugation (14,000 g for 10 min) after low ionic strength precipitation. The myosin was used immediately to prepare C-protein or stored at -20°C in 50% glycerol, 0.3 M KCl. This preparation is termed NCP-myosin (noncolumn purified myosin).

### C-protein

C-protein in crude form was obtained from the myosin preparations above using DEAE-Sephadex A-50 (19). The pectoralis C-protein was further purified by hydroxylapatite chromatography (23; C. Moos, personal communication). The voided proteins from the DEAE-column were precipitated at 2 M ammonium sulfate and resuspended in hydroxylapatite column buffer (0.3 M NaCl, 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 0.5 mM DTT (Dithiothreitol), 10 mM potassium phosphate buffer, pH 7.0). After dialysis against the same buffer, the sample (5 ml containing 6 mg/ml total protein) was applied to a 30 x 1.5-cm column packed with hydroxylapatite (Calbiochem-Behring Corp., San Diego, CA) and proteins eluted with a linear phosphate gradient from 0.01 to 0.3 M in the same column buffer.

### Radioimmunoassay (RIA)

Protein samples (1 µg in 25 µl of 0.6 M NaCl unless otherwise specified) were applied to wells of polyvinyl chloride flexible microtiter plates, air-dried, and stored at 4°C. For assay, the plates were washed three times in PBS (0.12 M NaCl, 10 mM potassium phosphate buffer, pH 7.4) and once in PBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, Cohn Fraction V). Each well was sequentially reacted with 40 µl of monoclonal antibody supernatant and <sup>125</sup>I-goat anti-mouse IgG (<sup>125</sup>I-GAM) (5 × 10<sup>4</sup> cpm/40 µl) in PBS/BSA for 30 min at room temperature. Extensive washing of the wells with PBS preceded and followed the <sup>125</sup>I-GAM incubation. <sup>125</sup>I bound to the microwells was measured by γ-spectrometry. Background counts were not subtracted. McAb dilutions were prepared in PBS/BSA.

### Preparation of <sup>125</sup>I-GAM

Goat anti-mouse IgG (Cappel Laboratories, Inc. Cochranville, PA) was radioiodinated while bound to a mouse IgG-Sepharose 4B column by the chloramine-T procedure (7). After removal of free <sup>125</sup>I with PBS, the labeled GAM was eluted with 0.2 M glycine-HCl (pH 2.3) containing 1% BSA, neutralized with 0.2 M Tris (tris (hydroxymethyl)aminoethane)-HCl (pH 8.6) and passed through a Sephacryl S-300 column (1.5 x 50 cm) equilibrated with PBS to remove protein aggregates. The <sup>125</sup>I-GAM had a minimum specific activity of 3 × 10<sup>6</sup> cpm/µg.

### Monoclonal Antibodies

Mice were injected three times subcutaneously with 100 µg of NCP-myosin preparations. The first sample injection contained complete Freund's adjuvant; subsequent sample injections were emulsified in incomplete Freund's adjuvant. 3 d after the final injection, splenocytes were fused in polyethylene glycol-1000 (5, 6) with either P3U-1 (pectoralis myosin) or P3NP (ALD myosin) HAT-sensitive myeloma cells obtained from Drs. J. Unkeless and M. Scharff, respectively. After culture for 2 wk in HAT medium, microwells were screened against the immunogen by solid phase RIA and cloned twice in 2% Sea Plaque agarose. Clonal cell lines were expanded in DME supplemented with glucose (4.5 g/l) and 20% fetal calf serum (Gibco, Grand Island Biological Co., Grand Island, NY). All experiments described below were done with unpurified McAbs in undiluted conditioned medium from hybridoma cultures stored at 5°C with 0.01% Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> unless otherwise specified.

### Electrophoresis and Immunoblot Procedures

SDS PAGE was performed using 5 × 10 × 0.1 cm electrophoresis units from Marysol Co., Tokyo, Japan (13). After termination of electrophoresis, peptides were transferred electrophoretically to nitrocellulose paper (40 mM Tris, 240 mM glycine, 20% ethanol) at a constant current of 100 mA for 2 h (24). To improve transfer of myosin heavy chain and C-protein, 0.1% SDS was added to the buffer soaking the backing sponge (cathodal side of the gel). Antigen was detected by autoradiography with x-ray film after incubation of the nitrocellulose paper for 5 min each in McAb followed by <sup>125</sup>I-GAM, using the same blocking and washing protocols described for the solid phase RIA (above).

### Immunofluorescence Microscopy

Glycerinated muscle was prepared essentially as described (8) with 0.1 mM PMSF, 1 mM EGTA in the glycerination medium. Small strips (10 mm × 1 mm)

were equilibrated in 0.1 M NaCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.5 and dispersed in a Dounce homogenizer. While adherent to glass microscope slides, the myofibrils were washed in PBS and then stained indirectly with McAb followed by fluorescein-labeled, goat anti-mouse IgG (FITC-GAM). Each antibody incubation was done for 15 min at room temperature followed by thorough washing in PBS. The FITC-GAM was purchased (Cappel Laboratories, Inc.) and then affinity purified on a mouse IgG-Sepharose 4B column by a procedure similar to that used for <sup>125</sup>I-GAM (above). Specimens were mounted in PBS, sealed with nail polish, and examined and photographed with a Zeiss epifluorescent photomicroscope. Muscle for frozen sections was immersed in liquid N<sub>2</sub>-cooled isopentane, cut at 10 µm, and sections were mounted on chrome alum (0.1%-gelatin (1%) subbed slides. After indirect immunofluorescent staining, as described above, sections were fixed in 4% formaldehyde, 0.05 M phosphate buffer (pH 7.4) and mounted in 90% glycerol-10% 0.2 M Tris-HCl, pH 8.6.

### RESULTS

Approximately 30 hybridoma clones were obtained from each of the cell fusions after immunization of mice with pectoralis or ALD NCP-myosin preparations (4, 12). In this report we describe the properties of antibodies MF-1 and 21 (pectoralis NCP-myosin immunization) and ALD-66 (ALD NCP-myosin immunization). Although we have evidence that clones MF-1 and MF-21 secrete different antibodies, both exhibit similar specificities and are treated collectively in this manuscript.

Using solid phase RIA, we tested the binding of MF-1 and ALD-66 to NCP-myosin preparations from pectoralis, ALD, and PLD muscles, both as a function of antibody and antigen concentrations (Fig. 1). Each of the antibodies bound to their respective immunogens, but MF-1 did not bind to NCP-myosin from ALD muscle under any of our assay conditions. However,

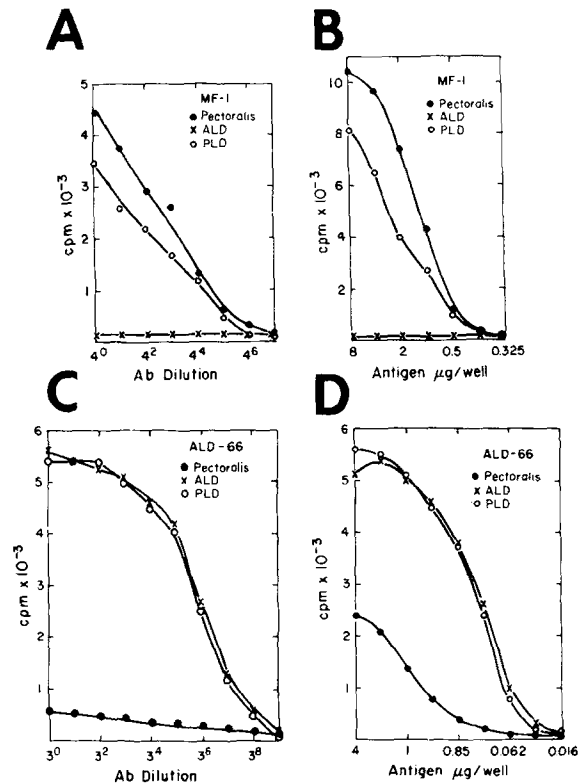


FIGURE 1 Monoclonal antibodies MF-1 (A, B) and ALD-66 (C, D) were reacted with NCP-myosin from pectoralis ALD and PLD muscles. In A and C, a fixed amount of antigen (1 µg/well) was reacted with a serial dilution of the antibodies. In B and D, a fixed concentration of the McAbs (0.25 and 0.125 dilution of culture supernatants MF-1 and ALD-66, respectively) was reacted with a serial dilution of the antigen.

ALD-66 did exhibit low but significant binding activity to NCP-myosin from pectoralis muscle at high antigen concentrations. Both MF-1 and ALD-66 bound to PLD NCP-myosin.

To identify the component in NCP-myosin which was binding the antibodies, the myosin was further purified in a DEAE-Sephadex column. The antigens recognized by both MF-1 and ALD-66 were restricted to the void volumes of the respective columns (Fig. 2). Both antibodies did not bind to the purified myosins from pectoralis, ALD, or PLD muscles. Control anti-

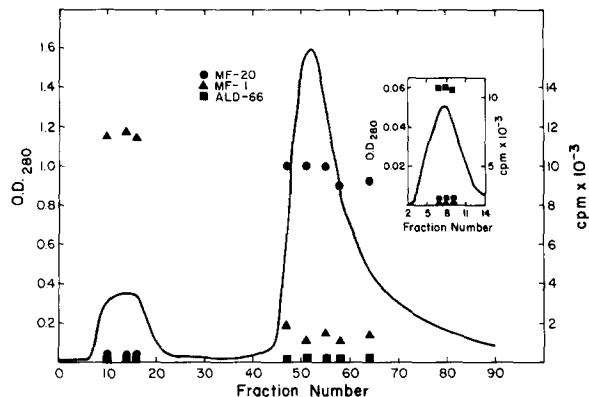


FIGURE 2 DEAE-Sephadex chromatography of NCP-myosin from pectoralis muscle and the antigen reactivity in 1  $\mu$ g of selected column fractions measured by solid phase RIA. McAb MF-20 which binds myosin heavy chain was compared with MF-1 and ALD-66. The insert shows the voided peak of a similar fractionation of NCP-myosin from ALD muscle. MF-20 bound only to protein in the myosin peak while MF-1 bound to antigen(s) in the void volume. As expected, ALD-66 did not react with either peak of the pectoralis preparation but bound to the voided peak of the ALD preparation. MF-20 and MF-1 did not bind to this fraction.

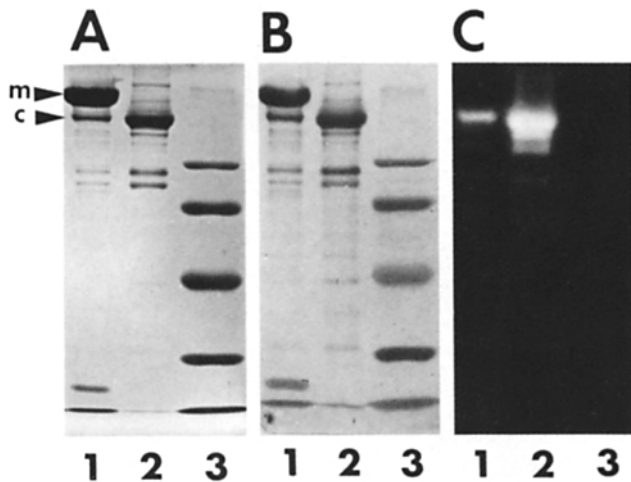


FIGURE 3 NCP-pectoralis myosin (1), proteins eluted in the void volume of the DEAE-Sephadex chromatography of pectoralis NCP-myosin (2), and molecular weight markers (3) were run in three identical 7.5% SDS polyacrylamide gels. The first gel was stained with Coomassie Brilliant Blue (A). The second was transferred to nitrocellulose paper which was stained (24) with amido black (B), and the third (C) after transfer to the nitrocellulose, was reacted with MF-21 followed by  $^{125}$ I-labeled GAM IgG. C is an autoradiogram printed in reverse contrast. All proteins present in the sample (A), including myosin heavy chain, were transferred to nitrocellulose (B), but only a 150 kD band reacted with MF-21 in both NCP myosin and in the void volume of the DEAE-Sephadex column. No reaction was observed with MHC or with the molecular weight markers. Arrowheads indicate the myosin heavy chain (m) and C-protein (c).

bodies specific for the myosin heavy chain did bind to the myosin peak but not to proteins in the void volume. Antibody specificities remained the same: antigenic material in the void volume of the pectoralis myosin column would bind MF-1 and MF-21 but not ALD-66 (Fig. 2) while the opposite specificity was observed with the ALD preparation (Fig. 2 insert). When pectoralis NCP-myosin and proteins in the void volume of a myosin DEAE-Sephadex column were analyzed by immunoblots with MF-21, we observed that of the many peptides present, only one band of 150,000 daltons (150 kD) would bind the antibody (Fig. 3). When the void volume proteins from the DEAE-Sephadex columns were further fractionated with hydroxylapatite, the antigen bound by MF-1 (or MF-21) was present in the main peak containing the 150 kD peptide (Fig. 4). Some binding of both MF-1 and ALD-66 could be observed in a later eluting peak that contained some 150-kD material

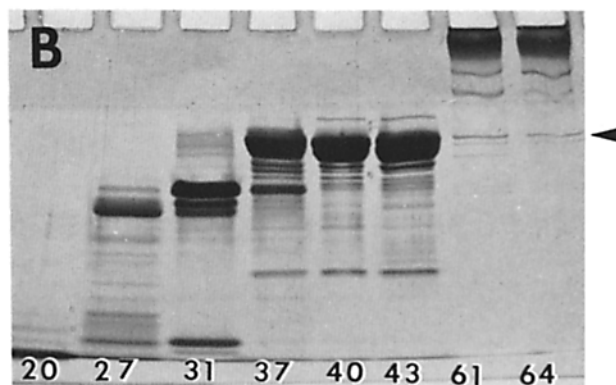
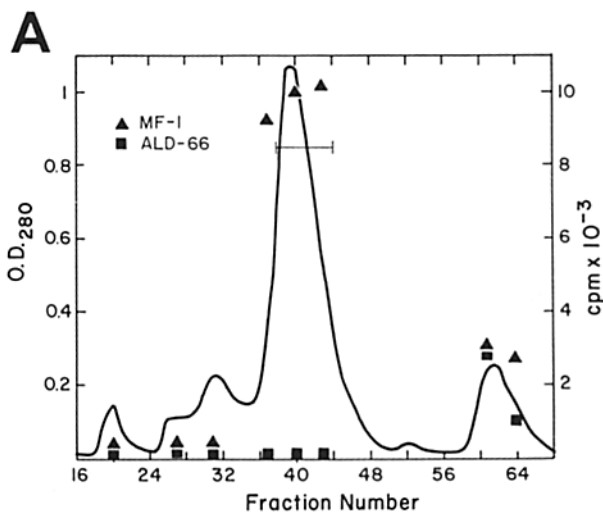


FIGURE 4 A: Hydroxylapatite fractionation of the crude C-protein fraction obtained from DEAE-Sephadex fractionation of pectoralis myosin and the binding activity of 1  $\mu$ g of selected fractions to MF-1 and ALD-66 by solid phase RIA. The horizontal bar indicates the pooled fractions used for the experiment shown in Fig. 5. B: A 7.5% SDS polyacrylamide gel of the peptides present in each fraction used for the solid phase RIA. 7  $\mu$ g of protein were applied per gel lane to permit detection of low molecular weight contaminating peptides. The arrowheads indicate the 150-kD C-protein band. The numbers below each lane correspond to the column fractions in A.

plus some high molecular weight components which fail to enter the separating polyacrylamide gel (Fig. 4).

Analysis of McAb binding to the hydroxylapatite-purified 150-kd peptide as a function of antigen and antibody concentration demonstrated much higher specific binding activity than observed with crude NCP-myosin (Fig. 5). The low levels of ALD-66 binding to NCP-myosin at high antigen concentrations were not observed with the purified 150-kd peptide (Fig. 5). It remains to be seen whether the small amount of ALD-66 binding material present in pectoralis NCP-myosin can be accounted for by an antigen in the later eluting peak of the hydroxylapatite column or by an antigen lost during C-protein purification on this column.

Having purified the peptides containing the antigenic determinants for these McAbs, we considered it necessary to prove that there were no other proteins in the pectoralis, ALD, or PLD muscles which could bind these antibodies. Whole pieces of fresh muscles, solubilized in SDS/ $\beta$ -mercaptoethanol sample buffer, were displayed by SDS PAGE and reacted with MF-1, MF-21, and ALD-66 by the immunoblot procedure. Under these conditions MF-1, MF-21, and ALD-66 only bound to a 150-kd peptide, and this binding exhibited the same muscle specificity previously demonstrated with the respective NCP-myosins (Fig. 6).

To localize the respective antigens within the contractile apparatus, we performed indirect immunofluorescent staining with glycerinated myofibrils from pectoralis and PLD muscles (Fig. 7). Staining with both McAbs was restricted to the A-bands in a pattern characteristic of C-protein (2, 20). Fluorescence was absent from the lateral and central zones of each A-band and the longitudinal distance from the outer border (i.e., side closest to the Z-band) of one fluorescent band to the outer border of the next was  $\sim 1 \mu\text{m}$ . All of the myofibrils from the PLD muscle stained positively with both MF-21 and ALD-66, and the staining patterns of both McAbs appeared identical. ALD-66 was unreactive with pectoralis myofibrils but these same myofibrils stained strongly with MF-21 (Fig. 7).

Since MF-1 (or 21) bound specifically to pectoralis but not ALD C-protein whereas ALD-66 had basically the opposite characteristics, we decided to examine possible fiber type C-

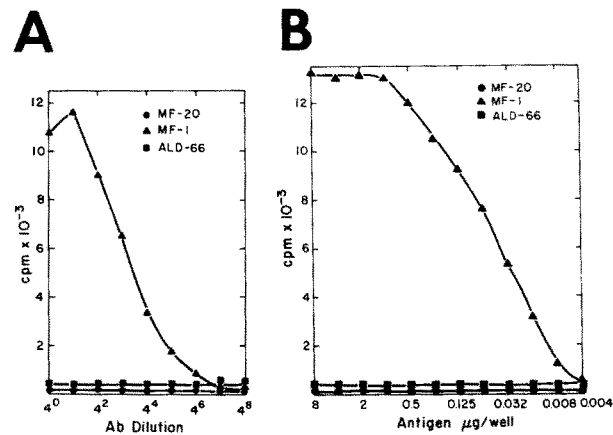


FIGURE 5 Solid phase radioimmunoassay of the antigenic reactivity of purified pectoralis C-protein from the hydroxylapatite column with MF-20, MF-1 and ALD-66. In *A*, a fixed amount of antigen (1  $\mu\text{g}$ ) was reacted with serial dilutions of the McAb supernatant. In *B*, undiluted supernatants were reacted with serial dilution of the antigen. Only McAb MF-1 bound to pectoralis C-protein.

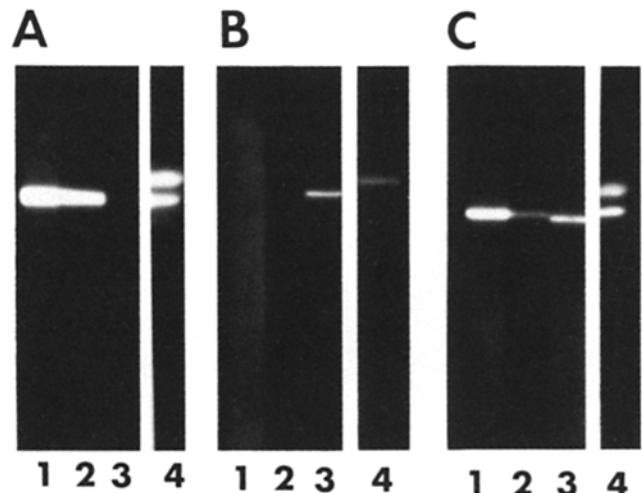


FIGURE 6 Pieces of fresh pectoralis (*A*), ALD (*B*), and PLD (*C*) muscle from adult chickens were solubilized in SDS- $\beta$ -mercaptoethanol sample buffer, separated by SDS PAGE (7.5% polyacrylamide), and transferred to nitrocellulose paper. Separate lanes of the extract of each muscle were reacted with MF-1 (1), MF-21 (2), and ALD-66 (3). A mixture of MF-20 and MF-21 (4) was used to locate the positions of the MHC and C-protein. In the pectoralis extract, only one band of  $\sim 150$  kd reacted with MF-1 and MF-21, but there was no reaction with ALD-66. The opposite was observed with the ALD extract. All three antibodies reacted with a 150-kd band in the PLD extract.

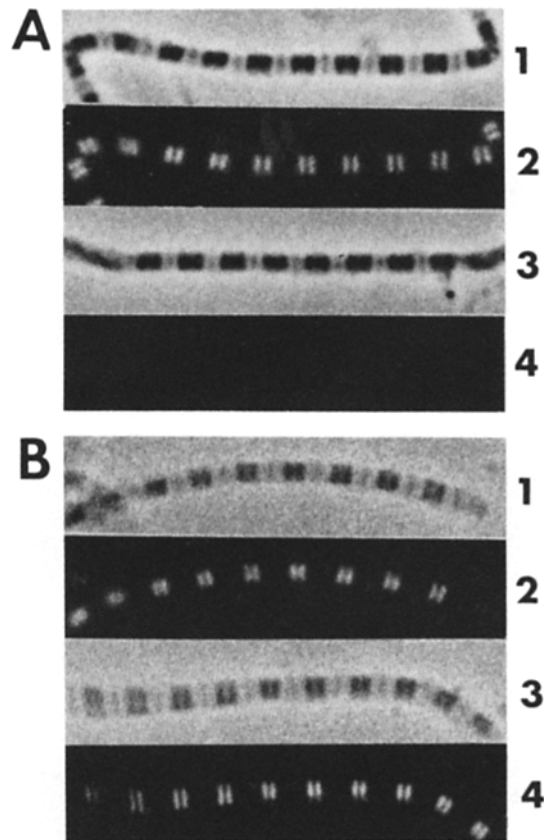


FIGURE 7 Glycerinated myofibrils from pectoralis (*A*) and PLD (*B*) muscles were stained by indirect immunofluorescence with McAbs MF-21 (1, 2) and ALD-66 (3, 4). The photographs are paired with phase contrast and fluorescent images. Both antibodies reacted with PLD myofibrils; only MF-21 stained the A-bands of pectoralis myofibrils.  $\times 2,000$ .

protein heterogeneity in the PLD muscle. All myofibers in frozen sections of the pectoralis major stained positively with MF-1, but a small percentage of muscle cells also reacted with ALD-66 (Fig. 8 *A*). All myofibers observed in the ALD muscle stained intensely with ALD-66 but were negative with MF-1 or MF-21 (Fig. 8 *B*). The percentage of ALD-66-positive myofibers in the pectoralis muscle varied from chicken to chicken and between different areas of the same muscle but was always <10% of the total fiber population. It was interesting to note

that these fibers also stained with MF-1. Presumably, these myofibers were the source of ALD-66 antigen present in pectoralis NCP-myosin and in the late-eluting peak of the hydroxylapatite column. In the PLD muscle, all myofibers stained at comparable intensity with MF-1 or ALD-66 (Fig. 8 *C*), although with the latter some variation in intensity of staining was also noted. The behavior of the PLD fibers resembles the small percentage of fibers in the pectoralis muscle that stain with both McAbs.

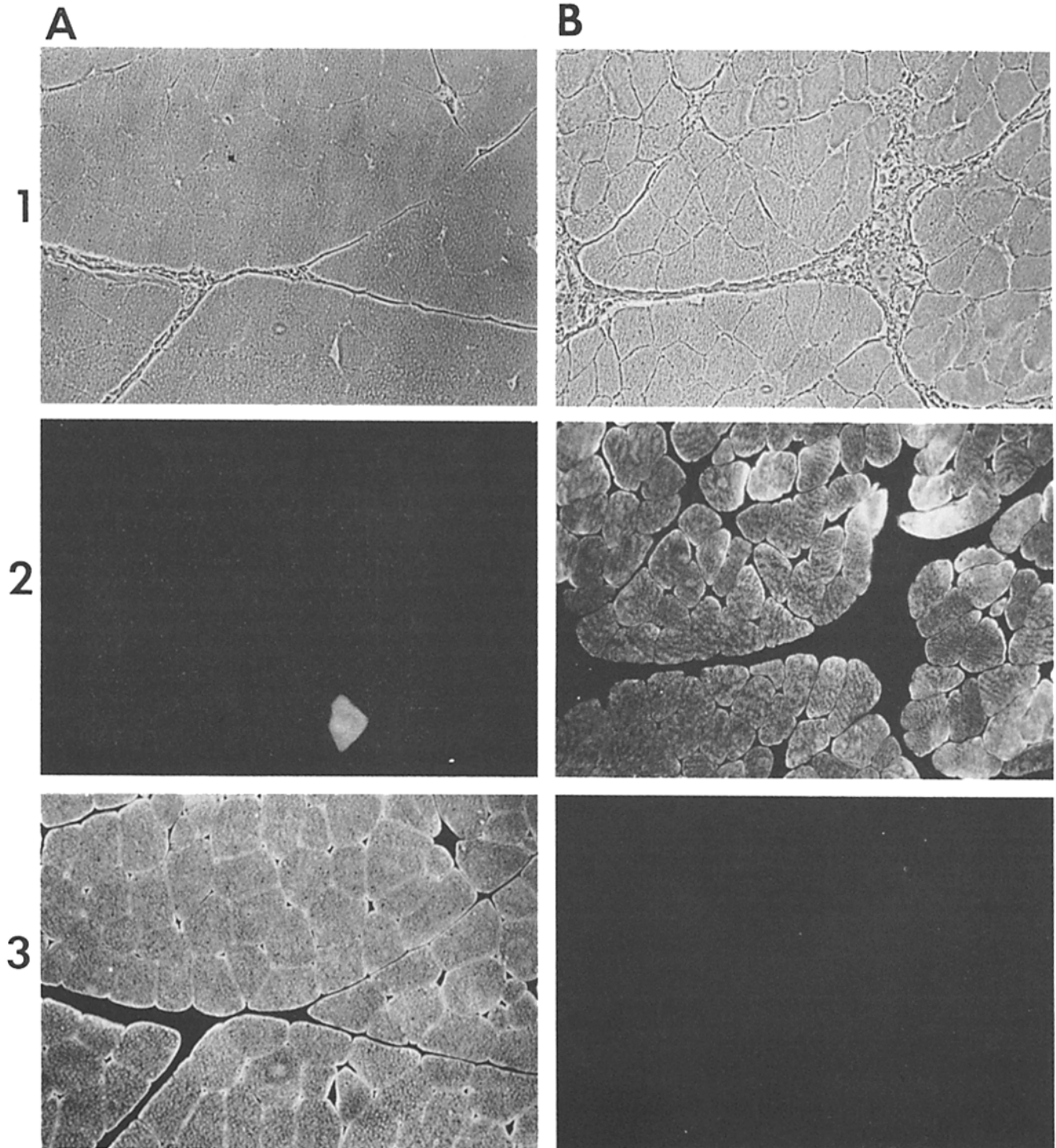


FIGURE 8 Frozen serial sections of pectoralis (*A*), ALD (*B*), and PLD (*C*) muscles were reacted with McAbs ALD-66 and MF-1. Photomicrographs of serial sections were taken with phase optics (1), indirect immunofluorescence using ALD-66 (2), and MF-1 (3).  $\times 140$ .

## DISCUSSION

Two classes of monoclonal antibodies have been generated after immunization of mice with crude myosin preparations from either pectoralis or ALD muscles. One binds to C-protein from pectoralis but not ALD muscle, whereas the other exhibits the reverse binding properties. Both groups bind to C-protein from the PLD muscle.

Evidence that these McAbs react specifically with C-protein is based on three criteria: (a) With immunoblots of NCP-

myosin, the McAbs bind to a protein of 150 kd but not the 200-kd myosin heavy chain. The molecular weight of C-protein (19, 21) is near this value, although some variation has been observed with C-proteins from different muscles (25). (b) The 150-kd antigen can be separated from myosin using DEAE-Sephadex chromatography but coprecipitates with myosin at low ionic strength, a behavior characteristic of C-protein (19). (c) The antigen is localized in the A-band of myofibrils with a distribution identical to that previously described for C-protein (2, 16, 20).

The present work with chicken muscle confirms previous reports that C-protein antigenicity may vary in different rabbit muscles and it supports the concept that this protein occurs in several isoformic variants (1, 25). The fact that these McAbs exhibit the same specificities in assays where the antigen is denatured (RIA and immunoblots) or in its "native" conformation (glycerinated myofibrils) suggests that protein conformation plays a minor role in these specificities. It is likely that differences in primary structure underlie the antigenic variations between C-proteins of ALD and pectoralis muscles, but it remains to be established whether these differences are in amino acid sequence or result from post-translational modifications of identical gene products.

We conclude that in pectoralis muscle the vast majority of cells possess a C-protein which binds only MF-1 or MF-21 but not ALD-66. This protein can be isolated free of any ALD-66 binding activity. The low levels of pectoralis NCP-myosin reactivity with ALD-66 can be traced to a small population of cells that have the same fluorescence staining properties as PLD myofibers. Whether or not these cells are equivalent to the PLD myofibers cannot be established with these experiments since the minute amounts of this antigen could not be detected with immunoblots or isolated by column chromatography.

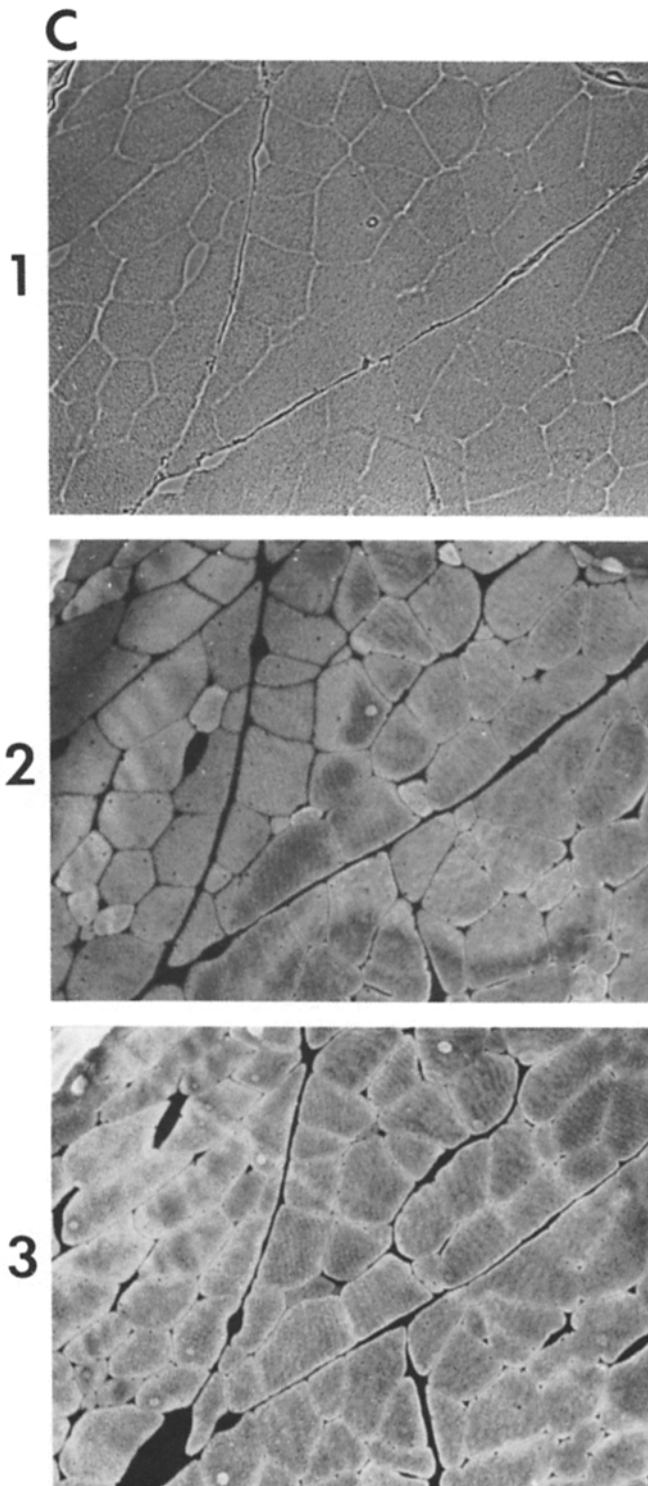
In the ALD muscle, we could not detect any myofibers that would react with pectoralis specific McAbs. In contrast to the antiserum to C-protein from rabbit soleus muscle (1), ALD-66 does not bind to fast (pectoralis) C-protein. We emphasize, however, that our data only prove antigenic differences between ALD and pectoralis C-proteins but cannot exclude the possibility that either or both of these C-proteins are heterogeneous.

Of particular interest is the observation that the antigenic determinants recognized by MF-1 and ALD-66 are both present in virtually all myofibers and myofibrils of the PLD muscle that we examined. Assuming that both ALD-66 and MF-21 epitopes reside on the same C-protein molecules in PLD muscle, one must postulate at least three isoforms in skeletal muscle on the basis of antibody binding to ALD, PLD, and pectoralis muscles. If ALD-66 and MF-21 bind different C-proteins in PLD myofibrils, then one must consider intrasarcomeric C-protein heterogeneity.

Preliminary observations in our laboratory suggest the presence of embryonic C-protein isoforms since MF-21 does not bind to pectoralis muscle from 12-d-old chick embryos (12), yet C-protein is probably present in myofibrils of this tissue. It is apparent that C-proteins must comprise a large family of peptides of unknown function.

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