M BAND PROTEIN

Two Components Isolated from Chicken Breast Muscle

BARBRA L. EATON and FRANK A. PEPE

From the Biochemistry Graduate Group and the Department of Anatomy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT

M band protein can be specifically extracted from fresh chicken breast muscle myofibrils suspended in 5 mM Tris-HCl pH 8.0. During discontinuous polyacrylamide gel electrophoresis the isolated protein separates into three bands which can be identified as two separate components (A, B) and a complex of the two. When partially purified fractions of the separated components are combined, an increase in the intensity of the band containing the complex can be shown. The polypeptide chain weights of the two components are 100,000 (A) and 40,000 (B) daltons as estimated by sodium dodecyl sulfate- (SDS-) polyacrylamide gel electrophoresis. Antibody prepared against total M band protein stains only the M band of the myofibril and is completely absorbed by M band protein. M band protein also absorbs the M band staining specifically from antibody which stains both I and M bands. Immunodiffusion data indicate that anti-M band is a mixture of two specific antibodies, one against each component.

The M band of skeletal muscle is located in the center of the anisotropic (A) band which contains the thick filaments. Using high resolution electron microscopy, Pepe (1) has shown that in longitudinal section the M band in fish and chicken skeletal muscle is composed of five distinct lines perpendicular to the long axis of the muscle. In cross section, the M band appears as bridges connecting each thick filament to its six nearest neighboring thick filaments. Antibody staining studies by Pepe (2) have demonstrated that the M band is composed of a protein different from myosin, actin, or tropomyosin, which is weakly bound to the center of the thick filament. The M band staining ability of antibodies prepared against other muscle proteins (3-5) must be due to small amounts of M band protein contaminating these preparations.

Several investigators have reported extraction of the M band (6-9), but only Samosudova (6) and Kundrat and Pepe (9) removed the M band specifically. This specific extraction of the M band was based on the low ionic strength extractions reported by Perry and Corsi (10, 11). The preparation of M band protein in this study uses the Kundrat and Pepe (9) extraction modified for fresh muscle, followed by recovery of the extracted protein by isoelectric precipitation. The identity of the protein as M band protein is shown by the following: (a) its ability to induce the production of antibody which stains the M band specifically, (b) its ability to absorb the M band staining from that antibody, and (c) its ability to absorb only the M band staining material from antibody which stains both the I and M bands. It is shown that the isolated protein contains two components which can complex with each other as demonstrated by discontinuous polyacrylamide gel electrophoresis. The molecular weights of the two components are estimated using sodium dodecyl sulfate- (SDS-) polyacrylamide gel electrophoresis. The immunodiffusion patterns of various fractions enriched in
one or the other component are analyzed. An attempt is also made to relate the M band protein obtained by this isolation procedure to those reported by other investigators (12-15). A preliminary report of these studies was given at the 1971 Biophysical Society Annual Meeting (16).

MATERIALS AND METHODS

Isolation Procedure

White Leghorn roosters were anesthetized with Nembutal and exsanguinated by cutting the jugular veins. The pectoralis major and minor were excised, trimmed to remove fat and tendon, and ground in a chilled meat grinder. The ground muscle was washed five times in 3 volumes of cold wash solution containing 0.1 M KCl and 5 mM ethylenediaminetetraacetate (EDTA) at pH 7.0. The suspension was drained through a double thickness of cotton gauze after each 15 min wash. The washed muscle was then resuspended in 2 volumes of wash solution and homogenized in the cold in a blender for 30 sec by cooling in ice. Each batch of 250–300 ml was homogenized three times. The blender was rinsed with 100–200 ml of wash solution. The resulting myofibril suspension was centrifuged at 2°-4°C in a model PR-1 International Equipment Company (Needham Heights, Mass.) centrifuge using the 840 rotor at 4000 g for 20 min. The myofibrils were washed several more times by allowing the suspension to stand in the cold for 20 min before each centrifugation. Finally, the suspension was allowed to stand overnight in the cold. The following day the myofibrils were centrifuged (4000 g, 20 min), resuspended, and rehomogenized. After centrifugation, the layer which floated off carefully with the supernatant and discarded. Two layers could be distinguished in the packed material. The top layer consisted almost entirely of myofibrils, and the bottom layer contained heavy debris. The myofibrils were scooped out leaving the heavy debris behind. (If this separation of the packed material into two layers did not occur, the tissue was rewashed and rehomogenized.) The myofibrils were then washed repeatedly until the supernatant was free of trichloroacetic acid (TCA) precipitable protein. This usually occurred by the 12th–15th wash. A final wash of 0.05 M KCl, 2.5 mM EDTA pH 7.0 was followed by centrifugation, and the packed fibrils were left in ice in the cold overnight.

The packed myofibrils were then extracted in 10 volumes of 5 mM Tris-HCl (tris[hydroxymethyl]aminomethane hydrochloride) pH 8.0 for 15–30 min in the cold. The extraction was terminated by centrifugation in a Spinco model L centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using the 30 rotor at 29,000 rpm for 20 min at 2°-4°C. The extraction was performed in batches of about 20 ml of packed myofibrils. After centrifugation, the supernatant was poured into an ice-packed flask in the cold. The extracted protein was recovered by isoelectric precipitation using dilute HCl. Precipitation at pH 6.5 resulted in removal of all the protein from the supernatant. Precipitation at pH's above 7.5 resulted in a fraction rich in the heavy component (A). A fraction rich in the lighter component (B) was obtained by further reduction of the supernatant pH to 5.8–5.2. An intermediate fraction was often obtained by precipitation at pH 6.0. The relative amounts of the two components seem to vary from preparation to preparation, and the amount of A in the total extract usually exceeded the amount of B. The precipitates were packed by centrifugation at 2°-4°C in a model PR-1 International Equipment Company centrifuge using the 269 rotor at 1200 g for 10 min. The packed precipitates were dissolved in 5 ml Tris-HCl pH 8.0 and dialyzed against a large volume of that buffer in the cold. M band protein was stored in 5 ml Tris-HCl pH 8.0 containing 50% glycerol at −24°C. The glycerol had previously been passed through Amberlite MB-1 ion exchange resin (Mallinckrodt Chemical Works, St. Louis, Mo.). The yield of M band protein from this preparation varies from 100–200 mg of protein/100 g of fresh ground muscle.

Partial Separation of the Two Components

The tendencies of the two components to complex to each other and of the heavier component to aggregate made separation of the two components difficult. Both of the methods attempted met with limited success. One technique utilized the difference in isoelectric point between the two components; the other was based on their differing mobilities in discontinuous 7% polyacrylamide gel electrophoresis. Several stock solutions of M band protein were prepared as follows: Protein fractions which were isolated by precipitation at pH 7.5 or higher after extraction were pooled and labeled "A-rich stock." No further purification of component A was achieved in this study. Protein fractions which were isolated by precipitation between pH 6.0 and pH 5.2 were pooled and labeled "B-rich stock." The B component was further purified by two methods. The B-rich stock could be separated into two fractions by carefully adjusting the pH to 5.8, removing the resulting precipitate by centrifugation, and then lowering the pH to 5.2 where recovery of a second precipitate was possible. These two fractions were labeled "5.8 precipitate" and "5.2 precipitate." The degree of purification of component B by this method was improved if the pH was raised to 9.5 or 10.0 for several minutes before the isoelectric precipitation.
The B-rich stock was also electrophoresed on a 7% polyacrylamide slab in an Ortec preparative cell (Ortec, Inc., Oak Ridge, Tenn.) until the fastest moving component had migrated into the collection chamber under the dialysis membrane. The solution in the collection chamber was then recovered and labeled “component B.” M band protein which was concentrated in solution after extraction rather than isoelectrically precipitated was labeled “extract stock.” The term “total M band protein” was used to describe extracted protein which was totally recovered by isoelectric precipitation at pH 6.5, and thus contained both components. A schematic outline of the methods used to obtain the various fractions described in this section is presented in Table I.

**Preparation of Antibody**

Two albino rabbits were given three initial injections of total M band protein. A solution of 2.5 mg of protein in 0.15 m NaCl, 0.05 m phosphate buffer, pH 7.0 was injected intraperitoneally. An identical solution was injected intravenously in the lateral ear vein. Another identical solution was emulsified with an equal volume of Freund's adjuvant (complete, Difco Laboratories Inc., Detroit, Mich.) and injected subcutaneously at the back of the neck. Two wk later each rabbit was injected intraperitoneally with 3.5 mg of protein and subcutaneously with 3.5 mg of protein emulsified with adjuvant as above. Intraperitoneal injections of 6 mg of protein were continued once a wk for the following month, and the rabbits were bled twice a wk from the lateral ear vein during the last 2 wk of the injection period. The antiserum was pooled, and the γ2-globulin (IgG) fraction was isolated by ethanol fractionation (17) and stored frozen in standard salt solution (0.1 M KCl, 1 mM MgCl₂, 0.01 m phosphate buffer, pH 7.0). A portion of the immune globulin was labeled with fluorescein isothiocyanate using the technique described by Fothergill (18) with the following modifications: (a) the fluorescein isothiocyanate to protein ratio was 3 mg fluorochrome to 20 mg protein; and (b) the incubation of the fluorochrome and protein lasted for 3 hr. Unreacted fluorochrome was removed by dialysis against 200 volumes of standard salt solution followed by gel filtration using Sephadex G-25, medium. The purified conjugates were dialyzed against buffered glycerol (25% glycerol containing 0.075 M KCl, 0.75 mM MgCl₂, 0.0075 m phosphate buffer, pH 7.0) and stored at -24°C. The degree of conjugation attained was 3.8 mole of fluorochrome/mole of protein. The final concentration of the conjugated immune globulin was 4-5 mg/ml. The fluorescent antibody which stains both I bands and M bands used in this work is identical to the “antiactin” used by Pepe in earlier studies (2). This antibody will henceforth be referred to as “anti-I,M” since it consists pre-
dominantly of antibody against proteins other than actin (2). The concentration of this solution is 10-15 mg of protein/ml.

Preparation and Staining of Myofibrils

Glycerinated myofibrils prepared as described earlier by Pepe (2) were used for fluorescent anti-M band and anti-I,M staining. In one experiment, myofibrils prepared from fresh muscle were stained with fluorescent anti-M band. A sample of the fibrils extensively washed in preparation for M band extraction was used for this experiment. For anti-I,M staining, 1 drop of the fluorescent antibody was used to stain 1 drop of the myofibril staining, 1 drop of the fluorescent antibody was extensively washed in preparation for M band extraction. A sample of the fibrils additionally contained 0.1% DTE.

Polyacrylamide Gel Electrophoresis

Two different methods were used. The first method is that described by Davis and Ornstein (19, 20) with the exception that no large pore or sample gels were used. The samples in 50 mM Tris-HCl pH 8.0 containing 25% sucrose and lightly colored with bromphenol blue tracking dye were layered on the tops of the separation gels just before the start of the electrophoresis. The separation gels containing 7% polyacrylamide were made as described by Davis (19) except that 0.1% dithioerythritol (DTE, Sigma Chemical Co., St. Louis, Mo.) was added before polymerization. Resolution was improved by refrigerating the separation gels overnight wrapped in parafilm. The proteins were electrophoresed at a constant current of 1 mA/gel tube for 10-15 min at which time the current was increased to 3 mA/tube. The progress of the electrophoresis was monitored by watching the migration of the blue tracking dye disc. Running time was usually about 75 min. The tank buffer was identical to that of Davis (19) except that it additionally contained 0.1% DTE.

The second polyacrylamide gel electrophoresis method used has been described by Weber and Osborn (21). Proteins are denatured in the presence of SDS to form negatively charged rods (22) which migrate in an electric field at rates inversely related to the logarithms of the molecular weights. Runs were made on 5 and 10% polyacrylamide gels containing 0.1% SDS. Proteins used to standardize the gels were rabbit myosin heavy chains (220,000), \( \beta \) galactosidase (130,000, purchased from Worthington Biochemical Corp., Freehold, N. J.), Limulus hemocyanin (80,000), bovine serum albumin (68,000, purchased from Sigma Chemical Co.), rabbit actin (46,000), ovalbumin (43,000, purchased from Worthington Biochemical Corp.), tropomyosin (36,000), and hemoglobin (15,500, purchased from Nutritional Biochemicals Corp., Cleveland, Ohio). The Limulus hemocyanin, rabbit actin, and tropomyosin were made available by the faculty of the 1971 physiology course at the Marine Biological Laboratory, Woods Hole, Massachusetts.

M band protein was incubated for 2 hr at room temperature and at 37°C in a medium containing 0.01 M sodium phosphate, 1% SDS, and 1% DTE at pH 7.0. After incubation, the protein was dialyzed overnight against the same buffer containing only 0.1% SDS and 0.1% DTE. In one set of experiments, the DTE was omitted. Standards were prepared according to the procedure outlined by Weber and Osborn (21) except that DTE was substituted for \( \beta \)-mercaptoethanol. Gels with the normal amount of cross-linker were prepared as described by Weber and Osborn (21), and samples containing 25% sucrose, a small amount of bromphenol blue tracking dye, and a small amount of hemoglobin to serve as a marker after staining and destaining were layered on the gels. The electrophoresis was then performed at a constant voltage of 50 v until the tracking dye had traveled at least 0.75 of the length of the gel. The mobility was calculated by dividing the distance of protein migration by the distance of the hemoglobin marker migration. A graph was then constructed on semilogarithmic paper by plotting the polypeptide chain weights against their mobilities taking the mobility of the hemoglobin chain as 1.

Both types of polyacrylamide electrophoresis were performed in a Shandon Scientific Company, Inc. (Sewickley, Pa.) acrylamide electrophoresis apparatus. After electrophoresis, the disc and SDS gels were removed from the glass tubes, stained for 2 hr at room temperature in Coomassie brilliant blue as described by Weber and Osborn (21), and then destained by soaking in several changes of destaining solution (150 ml glacial acetic acid and 100 ml methanol diluted to a total volume of 2 liter with water). Gels were stored and photographed in Plexiglas boxes containing 7% acetic acid.

In one experiment, an Ortec preparative cell (cell No. 4217) containing a 7% polyacrylamide slab (no DTE) was used instead of the Shandon kit. The gel and sample were prepared as described above for disc gels, omitting the DTE and adding a 7% cap gel containing 0.075 M Tris-HCl pH 8.0, and electrophoresis was performed at a constant current of 35 mA.

Immunodiffusion

Immunodiffusion studies were conducted on 1 X 3 inch microscope slides. The slides were washed with soap and water, rinsed in distilled water, soaked in
ethanol, water rinsed, and dried before dipping in a 0.1% aqueous agar (Noble agar from Difco Laboratories, Inc.) solution for precoating. Precoated slides were dried in an oven at 70°-80°C and were then layered with 1-2 ml of 2% agar (water washed and acetone dried) dissolved in either Veronal buffer, pH 8.6 (0.075 ionic strength barbital-sodium barbital mixture) or Tris-glycine, pH 8.3 (5 mm Tris, 60 mm glycine). Ouchterlony patterns of six wells surrounding a central well were cut in the agar using a specially constructed template and the Buehler Instruments, Inc. (Fort Lee, N. J.) agar cutter. The wells were filled with antigens and antibodies using a Drummond 20 λ microtrol (Drummond Scientific Co., Broomall, Pa.). The slides were then allowed to diffuse for 48 hr at room temperature in a moist chamber. The slides were washed by pouring a 2% NaCl solution over the slides and allowing them to soak for 24-48 hr with several solution changes. The slides were then rinsed in distilled water for another 24-48 hr with several changes. The slides were then stained in Thiazine red R (1% aqueous solution) for 2-3 hr and destained in 10% acetic acid. Finally, the slides were rinsed in water and dried at room temperature under wet strips of lens tissue. Care was taken to avoid trapping air bubbles between the lens paper and the agar slides. The paper was removed under running water after drying was complete. Dried slides were used as negatives in a photomacrograph for printing the patterns.

**Sedimentation Velocity Studies**

Sedimentation velocity studies were performed on the A-rich stock fraction of the M band protein. The experiments were conducted during the post course session of the 1971 physiology course at the Marine Biological Laboratories in Woods Hole, Massachusetts, using a Spinco model E ultracentrifuge. Because the A component precipitates in 0.1 M NaCl at pH 7.0 and pH 8.0, the sample was prepared by dialysis against 0.1 M NaCl, 10 mm Tris-HCl, pH 10.0 both in the presence and absence of 0.1% β-mercaptoethanol. Two double sector cells were used in each run, one of which contained a 2° wedge window. Runs were conducted at 20°C at 48,000 and 52,000 rpm. Photographs were taken using Schlieren optics (Optics For Industry Inc., Milwaukee, Wis.) at 2- to 8-min intervals as the runs progressed. The plates were read on a microcomparator, the natural log of the radial distance traveled by the peak was plotted vs. the time, and the slope was determined by the least squares method. The sedimentation coefficient was determined using the equation, \( s = \frac{1}{\omega^2} \frac{d\ln r}{dt} \), at three

**Figure 1**

(a) Glycerinated myofibril stained with fluorescent anti-M band and observed in phase contrast microscopy. X 4400  (b) Same myofibril as 1a but observed in fluorescence microscopy. X 4400  (c) Glycerinated myofibril stained with fluorescent anti-M band which had been previously absorbed with M band protein. X 7900  (d) Myofibril prepared from fresh muscle and stained with fluorescent anti-M band. X 4400.
different protein concentrations: 1.25, 2.50, and 3.75 mg/ml. The s values obtained were not corrected for solvent effects since the partial specific volume of the M band protein is unknown.

RESULTS

Antibody Staining

Figs. 1 a and 1 b show micrographs of a glycerinated myofibril stained with fluorescent antibody prepared against total M band protein. Fig. 1 a was obtained using phase contrast optics, and Fig. 1 b was obtained with dark-field fluorescence optics. The arrow shows that the fluorescent antibody stained band (light in Fig. 1 b) corresponds to the center of the A band (dark in Fig. 1 a). Note the crooked Z band separating the second and third sarcomeres at the left of Fig. 1 a. The absence of a similarly crooked fluorescent band in Fig. 1 b shows that the antibody is not staining the Z band. Fig. 1 c shows a glycerinated myofibril which was stained with anti-M band previously absorbed with total M band protein. No specific M band staining occurs. Fig. 1 d shows a myofibril prepared from fresh muscle and stained with fluorescent anti-M band. A sample of the myofibrils which had been washed extensively in preparation for M band extraction were used for this experiment. Note that only the M bands are stained specifically.

A glycerinated myofibril treated with antibody which stains both the M band and I band is shown in Fig. 2 a. Fig. 2 b shows a glycerinated myofibril stained with similar antibody which was first absorbed with total M band protein. The glycerinated myofibril in Fig. 2 c was stained with antibody absorbed with five times as much total M band protein as that in Fig. 2 b. In both cases, the M band staining only has been completely removed.

Polyacrylamide Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis patterns for A-rich stock and B component (isolated by preparative disc electrophoresis) are
shown in Figs. 3a and 3c. In Fig. 3a, the narrow upper band (labeled C) corresponds to the small amount of complex which could not be separated from the A component in the A-rich stock. The broader band (labeled A) in Fig. 3a corresponds to the A component. In Fig. 3c, only a single band (labeled B) corresponding to the B component is present. The gel shown in Fig. 3b contains a mixture of component B and the A-rich stock which was dialyzed overnight in 50 mM Tris-HCl pH 8.0

**Figure 3** Discontinuous polyacrylamide electrophoresis gels. Running time was 80 min. (a) A-rich fraction showing a heavy band due to component A and a light upper band due to a small amount of complex present. (b) A mixture of the A-rich fraction and component B showing enrichment of the band due to complex and a reduced intensity of the bands due to components A and B. (c) Component B showing a single rapidly migrating band due to component B.

**Figure 4** Discontinuous polyacrylamide electrophoresis gels. Running time was 75 min. (a) Freshly extracted M band protein overload showing the absence of impurities. The complex and the A component are not resolved at this high loading level. The lowest fast migrating band is due to free component B. (b) Freshly extracted M band protein showing a large amount of component B and a lesser amount of complex. A trace of free component B is also present but difficult to see.

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containing 0.1% DTE. Clearly, the band representing the complex (labeled C) has been enriched at the expense of the bands containing the separate components, A and B. The electrophoresis patterns shown in Figs. 3a, 3b, and 3c were obtained in the presence of DTE because component A forms large aggregates in the absence of DTE. These large aggregates do not enter a 7% polyacrylamide gel. Figs. 4a and 4b show disc electrophoresis patterns of freshly extracted M band protein. In Fig. 4a, which was overloaded to show the absence of any extra bands due to contaminating protein, the complex band cannot be resolved above the band of component A. In Fig. 4b, the bands for both complex (labeled C) and component A (labeled A) can be resolved, and a trace of the B component (labeled B) is visible. Fig. 4c contains twice as much protein as Fig. 4b.

Molecular weights for components A and B were estimated using SDS-polyacrylamide gel electrophoresis on 5% and 10% gels. Figs. 5 and 6 show the standard calibrations for the gels. 5% gels containing samples of A-rich stock and 5.2 precipitate are shown in Figs. 7a and 7b. The mobility of component A is 0.38 which corresponds to a polypeptide chain weight of 100,000 daltons. Component B has a mobility of 0.71 corresponding to a polypeptide chain weight of 38,000 daltons. Figs. 8a and 8b show 10% gels containing samples of the 5.2 precipitate (mostly component B) denatured in the presence of DTE (Fig. 8a) and in the absence of DTE (Fig. 8b). These SDS gels contain a hemoglobin marker (labeled Hb). In Fig. 8a, a doublet is visible corresponding to polypeptide chains of about 39,000 and 40,000 daltons, in good agreement with the 5% gel estimate for component B. The major component in Fig. 8b, however, corresponds to a polypeptide chain of about 76,000 daltons, and this band can be resolved into a doublet on a 5% gel corresponding to chains of 76,000 and 81,000 daltons. The presence or absence of DTE during SDS denaturation makes no significant difference in the polypeptide chain weight of component A. Incubation at 37°C or room temperature caused no significant variation in either component.

**Immunodiffusion Studies**

Figs. 9 and 11 show typical patterns for the various M band fractions after immunodiffusion against anti-M band in 2% agar. The patterns can be explained if one assumes the anti-M band to be...
a mixture of two antibodies: one against each component. Diagrammatic representations of the patterns are shown in Figs. 10 and 12. Both the extract stock (Fig. 9 a, 4, 6, 8 o'clock positions; Fig. 9 b, 10, 12, 2 o'clock positions; and Fig. 11, 6, 8 o'clock positions) and the A-rich stock (Fig. 9 a, 10, 12, 2 o'clock positions; and Fig. 11, 10, 12 o'clock positions) show two precipitin lines. The outer line from the extract stock is much denser than the outer line from the A-rich stock. In Figs. 9 a and

**Figure 7** 5% SDS-polyacrylamide electrophoresis gels. Running time was 105 min. The lower band represents the internal standard hemoglobin (Hb). (a) The A-rich fraction showing a mobility of 0.38 corresponding to a polypeptide chain weight of 100,000 daltons. (b) The 5.2 precipitate showing a mobility of 0.71 corresponding to a polypeptide chain weight of 38,000 daltons.

**Figure 8** 10% SDS-polyacrylamide electrophoresis gels. Running time was 125 min. The lowest band represents the internal standard hemoglobin (Hb). (a) The 5.2 precipitate showing a major doublet with mobilities 0.46 and 0.48 corresponding to polypeptide chain weights of 39,000 and 40,000 daltons. (b) The 5.2 denatured in the absence of a sulfhydryl reagent showing a major band with mobility of 0.182 corresponding to a polypeptide chain weight of 76,000 daltons.
FIGURE 9 Immuno-diffusion patterns obtained in Veronal buffer, pH 8.6 containing 2% agar. Central wells were loaded with anti-M band while outer wells were loaded with different M-band fractions. The identities of the antigens placed in each surrounding well and of the antigens and antibodies responsible for each precipitin line are given in Fig. 10.

![Diagram of immuno-diffusion patterns](image)

FIGURE 10 Diagrammatic representation of the immuno-diffusion patterns shown in Fig. 9. The letters $a$ and $b$ represent the antibodies against component A and component B, respectively. The letters $A$, $B$, and $C$ represent the antigens: component A, component B, and a complex of A and B, respectively. The diagram is discussed in detail in the text.

FIGURE 11 Immuno-diffusion pattern obtained in Tris-glycine buffer, pH 8.3 containing 2% agar. The central well was loaded with anti-M band. The identities of the antigens placed in each surrounding well and of the antigens and antibodies responsible for each precipitin line are given in Fig. 12.

11, both the inner and outer lines from the A-rich stock fuse with their counterparts from the extract stock. There is an indication of spur formation in Fig. 9a where the outer line from the A-rich stock fuses with the outer line from the extract stock. In Fig. 9b, the single precipitin line from the 5.2 precipitate (4, 6, 8 o’clock positions) fuses with the outer line from the extract stock, and intersects its inner line with no interference. In Fig. 11, the inner line from the 5.8 precipitate (2, 4 o’clock positions) fuses with the outer lines from the A-rich and extract stocks, and the outer line from the 5.8 precipitate fuses with the inner lines from the A-rich and extract stocks.

**Sedimentation Velocity Studies**

Fig. 13 shows a photograph taken with Schlieren optics during a sedimentation velocity run. The upper pattern shows A-rich stock in the presence of β-mercaptoethanol. A-rich stock in the absence of β-mercaptoethanol is shown in the lower pattern. The presence of a leading edge on the lower peak indicates self-aggregation in the absence of the sul-
hydral reagent. The A-rich stock is only slightly soluble in 0.1 M salt at pH 7.0 or 8.0 even in the presence of β-mercaptoethanol. At pH 10.0, however, the salt effect is reversed by the β-mercaptoethanol, and for this reason these sedimentation velocity studies were conducted at pH 10.0. $s_{20}$ values for the protein concentrations examined (1.25, 2.50, and 3.75 mg/ml) all fell between 8.9 and 9.0S. Sedimentation velocity studies were not conducted on component B due to the very small quantities available from preparative discontinuous polyacrylamide gel electrophoresis.

**DISCUSSION**

**Isolation Procedure**

The isolation procedure used in this work is based on the procedure reported by Kundrat and Pepe (9) for the specific removal of the M band by extraction of glycerinated fibrils for 15-30 min in 5 mM Tris-HCl. In addition, Kundrat and Pepe showed that although fluorescent antimyosin staining was unchanged, fibrils prepared in 0.1 M KCl, 5 mM EDTA pH 7.0 exhibited less uniform and less
intense I band fluorescent antibody staining than those prepared traditionally in standard salt containing 25% glycerol. This suggested that some of the antigenic I band material was removed during preparation of the fibril suspension in 0.1 m KCl, 5 mM EDTA pH 7.0. In this work, the following two major modifications of the original procedure for extraction of the M band material are made: (a) fresh muscle is used instead of glycerinated muscle, and (b) fibrils are washed repeatedly with 0.1 m KCl, 5 mM EDTA pH 7.0 until no further protein is found in the supernatant. The latter modification minimizes the possibility of contamination of the M band protein extract. The extract obtained by this method is highly opalescent, and a flocculant precipitate forms rapidly as the pH is lowered.

The identity of the protein extracted and isolated in this study is proven to be M band protein by the fluorescent antibody staining results. Antibody produced in response to injection of the protein stains the M band specifically (Figs. 1 a, 1 b, 1 d), and this specific staining is completely absent if the antibody is absorbed with M band protein before staining (Fig. 1 c). In addition, this protein can be used to absorb fluorescent antibody which stains both the I and M bands. Fibrils stained with such an absorbed antibody exhibit I band staining but no specific M band staining (Fig. 2). These three results: (a) specific antibody production, (b) total absorption of anti-M band, and (c) specific absorption of M band staining from anti-I, M, prove that the protein in this study is M band protein.

**Evidence for Two Components and Their Complex**

The existence of two different polypeptide chains as shown by SDS gel electrophoresis of M band protein suggests the possibility of a two component system. Differential isoelectric precipitation of the total M band protein results in partial separation of the two polypeptide chains indicating that they are separate proteins, not a two chained single protein. The component with the higher isoelectric point is called component A. Component B which has the lower isoelectric point can be resolved as a doublet (containing chains of about 39,000 and 40,000 daltons) on a 10% SDS gel (Fig. 8 a). If component B is denatured and electrophoresed in SDS in the absence of DTE, a doublet corresponding to 76,000 and 81,000 daltons appears. Because these weights are essentially twice those of each chain observed in the presence of DTE, it seems safe to conclude that the B component actually consists of two almost identical molecules, one with two identical polypeptide chains of about 39,000 daltons and the other with two identical polypeptide chains of about 40,000 daltons. Because these two molecules reacted as a single unit in all the other studies completed, it seems reasonable to consider them as a single component. The A component has only a single type of polypeptide chain of about 100,000 daltons in weight. This component forms large aggregates. In 0.1 m NaCl, pH 8.0, the protein is only slightly soluble. Even at pH 10.0 aggregation still occurs in the absence of β-mercaptoethanol as shown by sedimentation velocity studies (Fig. 13).

When a two component system separates into three bands during disc gel electrophoresis, the possibility of complex formation immediately is suggested. That such a complex does form in this system is shown quite dramatically in Fig. 3 b. Figs. 3 a and 3 c show the separated components which were mixed together to give the pattern in Fig. 3 b: The patterns are not additive. Instead the bands containing the two individual components are lower in intensity, and the band containing the complex is denser. The conclusion is that the two components must have reacted with each other to form additional complex.

**Immunodiffusion**

Disc electrophoresis shows that the A-rich stock (Fig. 3 a) and the extract stock (Fig. 4 a) each contain mostly component A and complex. When these two solutions are diffused against anti-M band, each one shows two precipitin lines, one due to aA (where a represents antibody against component A and A represents the antigen, component A), and the other due to bC (where b represents the antibody against component B and C represents the antigen, complex between components A and B). The two precipitin lines formed by the A-rich stock can be seen at the 10, 12, and 2 o’clock positions in Fig. 9 a and at the 10 and 12 o’clock positions in Fig. 11. The two precipitin lines formed by the extract stock can be seen at the 4, 6, and 8 o’clock positions in Fig. 9 a; at the 10, 12, and 2 o’clock positions in Fig. 9 b; and at the 6 and 8 o’clock positions in Fig. 11. Diagrammatic representations of the immunodiffusion patterns shown in Figs. 9 and 11 are given in Figs. 10 and 12, respectively.

The identities of the two lines from the extract
stock can be established from the results shown in Fig. 9 b as follows. The wells at the 4, 6, and 8 o'clock positions contained the 5.2 precipitate (predominantly component B). The single precipitin line which formed from the 5.2 precipitate must therefore be due to \( bB \) (where \( b \) represents the antibody against component B and \( B \) represents the antigen, component B). Since this single line fuses with the outer line from the extract stock, these two lines must contain the same antibody. The outer line from the extract stock is thus identified as containing \( bC \). This means the inner line must contain \( aA \). This is consistent with the fact that the inner line intersects the single line from the 5.2 precipitate \( (bB) \) without interference since there is neither a common antigen nor antibody between them. The inner lines of the extract and the A-rich stocks fuse with each other as seen in Fig. 9 a (9 and 3 o'clock positions) and Fig. 11 (9 o'clock position). Therefore, the inner line of the A-rich stock must also contain \( aA \). The outer lines of the extract and A-rich stocks also fuse, so the outer line of the A-rich stock must contain \( bC \). The indication of a spur in Fig. 9 a can be explained by the inability of the low concentration of the complex in the A-rich stock to precipitate the excessive antibody \( b \) which then diffuses past the \( bC \) band, allowing formation of a spur with the complex from the extract stock. This type of spur formation and occasional line duplication is due to unbalanced serologically identical systems and is described by Ouchterlony (23). It is likely that the antigen wells were filled with more protein in Fig. 11, thus improving the balance of the system and preventing the spur from forming.

The 5.8 precipitate contains two major antigens, component B (\( B \)) and complex of A and B (\( C \)). In Fig. 11 (2 and 4 o'clock positions), two lines are present from the 5.8 precipitate diffusion. The outer line from the 5.8 precipitate is fused with the inner lines \( (aA) \) from the A-rich stock (1 o'clock position) and the extract stock (5 o'clock position) and must therefore contain the antibody \( a \). Component B cannot precipitate with the \( a \) antibody, so the outer line from the 5.8 precipitate must contain \( aC \). This means that the inner line must be due to \( bB \), and it is continuous with the \( bC \) lines from the A-rich stock and the extract stock as expected.

**M Band Structure and Sarcomere Length**

One of the most puzzling aspects of the M band is related to changes in sarcomere length. The distance between the thick filaments is known to increase as sarcomere length decreases during contraction (24), yet the M bridge patterns observed in cross sections using electron microscopy are the same at all sarcomere lengths (1). Thus the M bridges must become longer during contraction. This implies the existence of a dynamic M bridge having the ability to elongate and shorten as the sarcomere length changes. This could involve either a stretching of the molecules contained in the M bridge or a sliding of molecules over one another. There are two observations which do suggest that the M band structure is different at different sarcomere lengths. Kundrat and Pepe (9) found that their 15 min extraction procedure removed the M band completely only from fibrils with sarcomere length greater than 2.1 \( \mu \). Fibribs with sarcomere length less than 1.9 \( \mu \) did not lose their M bands even after much longer extraction, up to 3 hr. At these short sarcomere lengths, the thin filaments penetrate the M band region. It seems unlikely that their presence could inhibit the extraction of the M band since the increased space between the thick filaments would tend to compensate for any interference with the extraction caused by the thin filaments. This suggests that the solubility characteristics of the longer M bridges are different from those of the shorter bridges. An observation published by Frank et al. (5) also suggests a structural variation in the M band with sarcomere length. These investigators found that when muscle fixed in 10\% isotonic-buffered Formalin at pH 7.2–7.4 was homogenized and stained with fluorescent antibodies, the M band staining was absent from myofibrils which had contracted by 40\% of resting length. M band staining by fluorescent antibodies has been observed at all sarcomere lengths in unfixed glycerinated fibrils (4). The implication is that the longer M bridges found at short sarcomere lengths were antigenically altered by the Formalin fixation, while the antigenicity of the shorter M bridges at long sarcomere length remained unaffected by the fixation. The relationship between these observations of changes in M band properties at different sarcomere lengths and the two M band protein components and their complex reported in this work is unclear at present. One can speculate, however, that some type of complex-subunit transition may be involved in the lengthening and shortening of the M bridges as the sarcomere length changes.
Correlation with Other Reports of Isolated M Band Protein

The first report of the M band protein isolation was a preliminary note published in 1968 by Masaki et al. (12). The technique used was extraction of the entire A band followed by purification of the M band protein. The procedure has recently been modified in a second preliminary note (14). The more recently purified protein has two components of molecular weights of 155,000 and 94,000 daltons as estimated from SDS-polyacrylamide gel electrophoresis. The 94,000 dalton subunit (their component II) may be identical to the component A reported in this paper. The 155,000 dalton subunit (their component I) might correspond to the complex between our components A and B, although it is unclear why this should persist in the presence of SDS. Using this assumption, the fact that M band staining by fluorescent antibodies could be completely absorbed by their component I, but not by their component II can be explained. Their component I (corresponding to our complex) would contain both antigens but their component II would carry only the antigenicity of our component A and thus could not be expected to completely absorb the M band staining.

After the initial report (16) of the isolation of M band protein described in this paper, two additional reports have come to our attention. Palmer et al. (13) reported isolation of an M band protein which may form large aggregates in KCl. Since component A also has this property, it may be that Palmer et al. have found another isolation method for component A. It is unclear whether our component B is also present in their preparation. M band protein isolation has also been reported by Morimoto and Harrington (15), and it is extremely likely that their protein is identical to our component B. Their isolation procedure is also based on a specific removal of the M band, but their extraction is carried out by overnight dialysis at pH 7.7 in place of our brief extraction at pH 8.0. Component A begins to precipitate at pH 7.8, so it is likely that overnight dialysis at pH 7.7 could result in almost total loss of component A from the supernatant. They report a molecular weight of 88,000 daltons from equilibrium sedimentation experiments and a molecular weight of 43,000 daltons for the subunit obtained in 6 M guanidine HCl. The polypeptide chain weight estimated from SDS-polyacrylamide gels was 48,000 daltons. Additional physical properties of the protein were determined, and there are no obvious contradictions to the identity of their protein and our component B.

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SUMMARY

M band protein was isolated from myofibrils prepared from fresh adult chicken breast muscle. In order to obtain the M band protein specifically, exhaustive washing of the myofibrils with 0.1 M KCl, 5 mM EDTA pH 7.0 was necessary before brief extraction of the myofibrils with 5 mM Tris-HCl pH 8.0. Complete recovery of the extracted M band protein was obtained by isoelectric precipitation. Fluorescent antibody prepared against the isolated M band protein stained only the M band of the myofibril and that staining was completely absorbed by M band protein. M band staining was also specifically removed from fluorescent antibody against I and M bands by absorption with M band protein. These antibody studies in conjunction with polyacrylamide electrophoretic studies established the identity and purity of the M band protein.

The isolated M band protein gave three bands after discontinuous polyacrylamide gel electrophoresis. These bands were identified as two separate components of the M band protein (A, B) and a complex of the two components. Enrichment of the band containing complex occurred when the two partially purified components were mixed together. The polypeptide chain weights of the two components were estimated at 100,000 (A) and 40,000 (B) daltons using SDS-polyacrylamide gel electrophoresis. When the B component was denatured in 1 % SDS in the absence of a sulphydryl reagent, the chain weight observed was 80,000 daltons, suggesting that the B component actually exists as a molecule composed of two 40,000 dalton chains. Immunodiffusion data indicated that the anti-M band prepared in this study is a mixture of two specific antibodies, one against each component. The possible significance of a two component M band protein with respect to M bridge length at different sarcomere lengths was suggested. The relationship between M band protein prepared in this study and those reported recently by other investigators (12–13) was also discussed.

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