

IMMUNOFLUORESCENT AND HISTOCHEMICAL LOCALIZATION OF AMP DEAMINASE IN SKELETAL MUSCLE

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ABSTRACT

Fluorescent antibody staining experiments with both isolated myofibrils and muscle fibers grown in culture show that AMP deaminase is bound to the myofibril in the A band. The strongest staining occurs at each end of the A band. The approximate width of the fluorescent stripes and their relation to the A band remains constant as a function of sarcomere length. Removal of enzyme from the myofibrils leads to loss of staining, and readdition of purified enzyme restores the original staining pattern. A histoenzymatic method for the detection of AMP deaminase activity in cultured fibers gives comparable localization. The results are consistent with the previous observation (Ashby, B. and C. Frieden. 1977. *J. Biol. Chem.* **252**:1869-1872) that AMP deaminase forms a tight complex in solution with subfragment-2 (S-2) of myosin or with heavy meromyosin (HMM).

KEY WORDS immunohistochemistry · AMP deaminase · myofibril · myosin · protein-protein interaction

AMP deaminase (AMP aminohydrolase, E.C. 3.5.4.6.) catalyzes the deamination of AMP to produce IMP and ammonia. The enzyme is present in many tissues but its level is particularly high in skeletal muscle. Various lines of experimental evidence suggest that the physiological role of the enzyme may be related to energy mobilization during muscle contraction. First, the onset of extensive and coordinated contractile activity in muscle during development is correlated with a sharp increase in the specific activity of AMP deaminase. Enzyme activity in the rabbit diaphragm increases rapidly at birth whereas in the leg muscles the onset of extensive muscle activity and the increase in enzyme activity occur ~10 d later (19). Second, it has recently been observed that lack of the enzyme may be relatively common

in individuals who experience muscle cramping or weakness following exercise (7). The same authors showed that while normal individuals during exercise exhibit a linear correlation between plasma lactate and plasma ammonia levels, individuals with the enzyme deficiency can produce lactate but not ammonia. One can speculate, for example, that the ammonia so produced would normally activate muscle phosphofructokinase, a key enzyme in glycolysis, and thus exert a regulatory effect on the production of ATP for muscle contraction (16). Furthermore, AMP deaminase itself shows many of the characteristics of a regulatory enzyme since its activity is specifically affected by compounds binding to nonsubstrate sites (27), and its kinetic properties can deviate from normal Michaelis-Menten behavior (2, 23).

Another reason for postulating that the enzyme may participate in reactions of the contractile apparatus is that it is a contaminant of myosin preparations, and Ashby and Frieden (1) have

found that in solution purified AMP deaminase binds to a specific region of the myosin molecule. A stoichiometry of 2 mol of enzyme (mol wt ~240,000 and containing 4 subunits) was found per mol of myosin (1). A protein-protein complex can be observed in the analytical ultracentrifuge between AMP deaminase and heavy meromyosin (HMM) or subfragment 2 (S-2) but not between the enzyme and light meromyosin (LMM) or subfragment 1 (S-1). S-2 represents the α -helical portion of HMM adjacent to the hinge region that is thought to allow the cross-bridges to project laterally from the thick filament (8, 17, 26). Further, kinetic experiments have shown that GTP inhibition of the enzyme is prevented in the presence of S-2 (2).

In the present paper, we use immunostaining methods to show that some of the muscle AMP deaminase binds to specific regions of the sarcomeres both in glycerol-extracted myofibrils and in muscle fibers formed from presumptive myoblasts in tissue culture. With both types of material, the staining pattern is consistent with binding studies using purified proteins in solution. The enzyme binds to the lateral edges of the A band in a region believed to contain accessible S-2 sites.

MATERIALS AND METHODS

AMP Deaminase

AMP deaminase was prepared from fresh chicken breast muscle by the procedure developed by Smiley et al. (22) for purification of the enzyme from rabbit skeletal muscle using cellulose phosphate chromatography. Chicken AMP deaminase prepared by this method gave a single major band with several very minor bands on gel electrophoresis (Fig. 1) in the presence of sodium dodecyl sulfate performed according to the method of Weber and Osborn (24). Enzyme concentration was determined approximately using an $A_{280}^{1\%}$ value of 10 compared with a value for the rabbit enzyme of 9.13 (22). Enzyme activity was measured as described previously (1).

Myosin and HMM

Myosin was prepared from fresh chicken breast muscle by the method of Holtzer and Lowey (9). HMM was prepared from chicken breast myosin by the method of Lowey and Cohen (12).

Sedimentation Velocity Experiments

Sedimentation velocity experiments were performed at 20°C and 52,000 rpm in a Beckman Spinco model E ultracentrifuge. (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resulting Schlieren patterns

were measured on a Nikon two-dimensional comparator (Nikon Inc., Instrument Div., Garden City, N. Y.). The sedimentation coefficients given are $S_{20,w}$ values.

Preparation of Myofibrils

Breast muscle or leg muscle from a freshly killed chicken was cut into longitudinal strips ~1 cm in diameter, tied with string at each end and suspended with weights pulling down the fiber axis in a medium of 10 mM imidazole/HCl, 0.15 M KCl, 2 mM ethylene glycol-bis(β -aminoethyl ether(N,N,N',N'-tetraacetate (EGTA), 50% glycerol, pH 6.5 at 4°C. Application of tension up to 400 g was found to be necessary to obtain well-stretched myofibrils. After soaking for at least 2 d in the glycerol-containing medium, the muscle strips were transferred into 50 vol of buffer consisting of 10 mM imidazole/HCl, 0.15 M KCl, 2 mM EGTA, pH 6.5, and blended for 15 s in a small Waring blender. The homogenate was centrifuged at 800 g for 5 min and the supernate discarded. The resuspended pellet was again blended, centrifuged and resuspended in the same buffer. After two more washes in buffer (without blending), the resulting myofibril suspension was stored at 4°C and used within a day.



FIGURE 1 SDS-polyacrylamide gel electrophoresis of purified chicken AMP deaminase prepared by the method of Smiley et al. (22). The gel was loaded with 100 μ g of enzyme and stained with Coomassie brilliant blue.

Extraction of Isolated Myofibrils and Addition of Exogenous AMP Deaminase

To remove AMP deaminase from isolated myofibrils, a fraction of the myofibril suspension was pelleted and resuspended in 10 mM imidazole/HCl, 0.15 M KCl, 2 mM EGTA, pH 6.5, containing 10 mM potassium phosphate. The washing procedure using phosphate-containing buffer was repeated ten times. This procedure was adopted since it was previously shown (1) that 10 mM phosphate is effective in dissociating AMP deaminase from purified myosin subfragments. The presence of AMP deaminase on the myofibrils was determined by pipetting an aliquot of the myofibril suspension into a cuvette containing 100 μ M AMP and using the assay system previously described (1). It was assumed that the activity is the same whether the enzyme is bound to myofibrils or in solution since HMM or S-2 does not markedly change the activity of the enzyme in solution (B. Ashby and C. Frieden, unpublished data).

To add back AMP deaminase to the phosphate-treated myofibrils, a fraction of the washed suspension was centrifuged and resuspended in 10 mM imidazole/HCl, 0.15 M KCl, 2 mM EGTA, pH 6.5. A sample of purified AMP deaminase in the same phosphate-free buffer was added, and the mixture was incubated for 15 min at room temperature. Unbound AMP deaminase was removed by washing twice with phosphate-free buffer.

Tissue Culture

Myogenic cultures were prepared as described by Bischoff and Holtzer (4). Pectoral muscle was removed from 12-d chick embryos, and the cells were dissociated with 0.1% trypsin (1:300) in Earle's saline. After trituration, the cell suspension was passed through a 10- μ m nylon filter (Small Parts, Inc., Miami, Fla.) to remove cell clumps and myotubes. The cells were grown on collagen-coated coverslips in 35-mm Petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) at an initial density of 7.5×10^4 cells/cm². The collagen was prepared from rat tail tendons, purified by three cycles of precipitation (3), and polymerized in the dish by exposure to ammonia vapor.

The culture medium was Eagle's minimal essential medium with 10% selected horse serum, 5% chick embryo extract, and 1% antibiotic-antimycotic solution. Embryo extract was prepared from 12-d chick embryos; all other culture reagents were obtained from Grand Island Biological Co., Grand Island, N. Y. The culture medium was replaced daily.

Antibody Staining

Rabbit serum containing antibodies against purified chicken muscle AMP deaminase was kindly donated by Dr. Oscar Chilson. The serum gave a single precipitin line in Ouchterlony immunodiffusion (16) when tested against highly purified chicken breast muscle AMP de-

aminase (Fig. 2, wells 1, 3, and 5) or against a crude extract of chicken breast muscle (Fig. 2, well 4). A precipitin line is also visible in well 2 of Fig. 2 which contained myofibrils solubilized in 0.5 M KCl. Chicken myosin prepared by the method of Holtzer and Lowey (9) showed no immunological reactivity with the rabbit serum (Fig. 2, well 6). This preparation contained a detectable amount of AMP deaminase estimated by activity measurements to be <1% of the total protein.

Control sera were obtained from nonimmunized rabbits and commercial pooled rabbit serum. The IgG fraction was purified by ammonium sulfate precipitation (25) and labeled with fluorescein isothiocyanate (FITC) isomer I on celite (Sigma Chemical Co., St. Louis, Mo.) according to Rinderknecht (20). The labeled globulins were diluted to 10–20 μ g/ml with 0.15 M NaCl for staining. In some cases, we used the indirect method in which specimens were first treated with unlabeled immune or control globulin at the same concentration, washed thoroughly, and stained with FITC goat anti-rabbit IgG (Pentex, Kankakee, Ill.). Both the indirect and the direct methods gave the same staining pattern.

Myofibrils were stained directly on the microscope

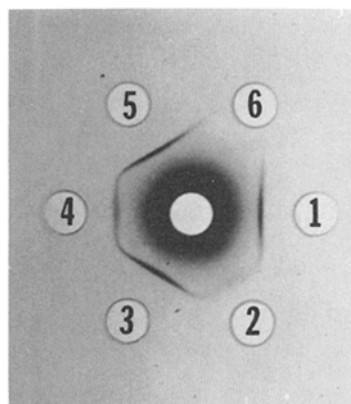


FIGURE 2 Double immunodiffusion test (14), performed in 0.7% agar in 0.5 M KCl, showing specificity of anti-AMP deaminase serum. Center well: anti-AMP deaminase serum (undiluted). Outer wells: (1, 3, 5) highly purified AMP deaminase (10 μ g representing 10 μ l of a 1 mg/ml solution); (2) supernate of a pelleted myofibril suspension extracted with 3 vol of 0.5 M KCl for 3 h; (4) supernate from centrifuged crude extract (50%) of chicken breast muscle, extracted with 3 vol of 0.5 M KCl for 3 h; (6) chicken myosin (30 μ g) prepared by the method of Holtzer and Lowey (9). After the appearance of precipitin lines, the gel was washed for 24 h with 0.5 M KCl and then stained with Coomassie brilliant blue. Destaining was performed in a solution of 5% acetic acid, 7.5% methanol in water. Examination of unstained plates or more thorough washing before staining failed to reveal additional precipitin lines.

slide in a chamber made by supporting a coverslip on slivers of broken coverslip. The fibrils were washed by pulling 0.15 M NaCl beneath the coverslip by capillary action. Treatment with each globulin preparation was carried out for at least 2 h at 4°C. Semipermanent preparations were made after the final washing by sealing the edges of the coverslip with sticky wax.

Cultured myotubes were stained after first extracting the cells to remove soluble enzyme and to render the cell membrane and basal lamina permeable to antibody. In the method finally adopted, the cultures were washed with phosphate-buffered saline and treated for 1 h at 4°C with 10 mM imidazole/HCl, 0.15 M KCl, 1 mM EDTA, pH 6.5, to which was added 0.1% sodium deoxycholate just before use. The cultures were washed thoroughly with the same buffer minus detergent and stained in the dish overnight at 4°C. Coverslips were removed from the dishes after staining and mounted on slides with Aqua-Mount (Lerner Lab., Stamford, Conn.).

Several types of controls were done. First, specimens were examined for autofluorescence. Second, specimens were treated with nonimmune globulin using both the direct and indirect staining methods. Finally, specimens were stained after removal and readdition of AMP deaminase from isolated myofibrils as described under Materials and Methods.

Fluorescence Microscopy

Specimens were examined with a Zeiss WL microscope (Carl Zeiss, Inc., N. Y.) fitted with a IV F1 epifluorescent condenser for fluorescence and a substage pancreatic condenser for simultaneous phase contrast. Photographs were made on Kodak Tri-X film using Zeiss oil-immersion $\times 40$ and 100 objectives. All exposures were made for the same time unless otherwise noted. Magnification of the microscope was calibrated with a stage micrometer, and measurements of sarcomere band length were made from the finished prints using a micro-comparator with $\times 7$.

Activity Staining of Cultured Muscle

Cultured myotubes were stained for AMP deaminase activity by a method developed by Fishbein et al. (7) and obtained through personal communication. Coverslips bearing the cultured cells were incubated at 30°C for 1–3 h in 1.2 mM *p*-nitro blue tetrazolium, 3.25 mM dithiothreitol, 1 mM AMP, 0.2 M KCl, pH 6.1. After staining, the cultures were rinsed and mounted in Aqua-Mount. Controls for nonspecific staining were carried out by substituting 1 mM sodium citrate for AMP in the reaction mixture.

RESULTS

Sedimentation Velocity Experiments

Purified chicken AMP deaminase and chicken HMM were mixed in 10 mM imidazole/HCl, 0.15

M KCl, pH 6.5, in the presence and absence of 10 mM potassium phosphate. The mixture in the absence of phosphate gave rise to the sedimentation velocity pattern shown in Fig. 3 (upper pattern) in which a fast sedimenting peak ($S_{20,w} = 19$ S) is visible together with traces of two slower moving peaks. In the presence of 10 mM phosphate, the Schlieren pattern (Fig. 3, lower pattern) shows two peaks of 8 S and 11 S that correspond closely with values expected for homogeneous preparations of HMM and AMP deaminase, respectively. The results are essentially identical to those observed previously using AMP deaminase and HMM from rabbit skeletal muscle (1).

Amount of AMP Deaminase Bound to Myofibrils

Isolation of AMP deaminase from chicken breast muscle as described under Materials and Methods gives about 150 mg of pure enzyme per kilogram of wet weight of muscle, representing close to 100% yield based on enzyme assays performed on the crude homogenate. The extraction buffer used for enzyme purification contained 90 mM phosphate (22). Since we have shown that 10 mM phosphate is sufficient to completely dissociate the complex formed between AMP deaminase and HMM (Fig. 3, lower pattern), 3 vol-

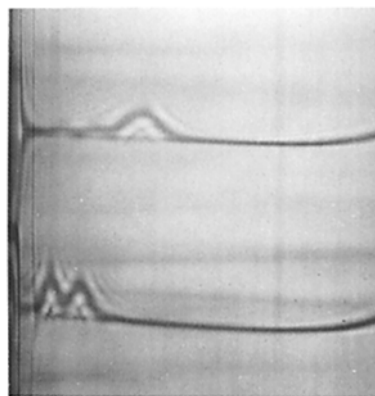


FIGURE 3 Sedimentation velocity patterns of chicken AMP deaminase and chicken HMM and the complex between the two proteins. The experiments were performed at 20°C in 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5 at a speed of 52,000 rpm, and the photograph was taken 20 min after reaching that speed. The phase plate angle was 55°. Upper pattern: AMP deaminase (1.0 mg/ml) and chicken HMM (0.7 mg/ml); Lower pattern: AMP deaminase (1.0 mg/ml) and chicken HMM (0.7 mg/ml) and 10 mM potassium phosphate.

umes of a phosphate-free buffer containing 10 mM imidazole/HCl, 0.15 M KCl, 2 mM EGTA, pH 6.5, were used to extract the enzyme in an attempt to determine the amount of AMP deaminase bound to myofibrils isolated from fresh muscle. The concentration of KCl used in this buffer was previously found to be optimal for formation of the AMP deaminase-HMM complex in solution (1). Under these conditions, 90–95% of the enzyme was extracted. The 5–10% of enzyme remaining attached to the resultant myofibril suspension (which was measured by direct assay of a suspension of myofibrils) could not be removed by repeated washings with phosphate-free buffer. The low level of bound enzyme observed in these experiments may be due to the release of high concentrations of endogenous nucleotides or other metabolites during extraction of the myofibril which could disrupt the complex. We have observed, for example, that 2 mM ATP, like 5 mM inorganic phosphate (1), will dissociate the solution complex between HMM and AMP deaminase (B. Ashby and C. Frieden, unpublished data). A calculation of the amount of enzyme bound is given in the Discussion.

In a separate experiment, myofibrils were prepared from 10 g of glycerinated muscle, and most of the endogenous AMP deaminase was removed from them by repeated washes with buffer containing 10 mM phosphate. After resuspending the myofibrils in phosphate-free buffer, 1.5 mg of pure AMP deaminase, corresponding to the amount of the enzyme in 10 g of native (wet weight) muscle, was added. After a 15-min incubation period, the mixture was centrifuged and it was found that 99% of the added enzyme was bound to the myofibrils. The added enzyme could not be removed by repeated washings with phosphate-free buffer but was readily removed by washing with 10 mM phosphate buffer or buffer containing 2 mM ATP.

Antibody Staining of Myofibrils

Nonspecific fluorescence was assessed in two types of control situations. Autofluorescence of unstained myofibrils was totally undetectable, even at the highest light intensity. Myofibrils stained with FITC-labeled nonimmune IgG directly or with nonimmune IgG followed by FITC-labeled goat anti-rabbit IgG gave a very weak fluorescence that failed to register on the photographic emulsion with the same exposure used for the immune globulin-stained fibrils (Fig. 4*a*). The

weak fluorescence was evenly distributed along the myofibrils with no distinct banding pattern.

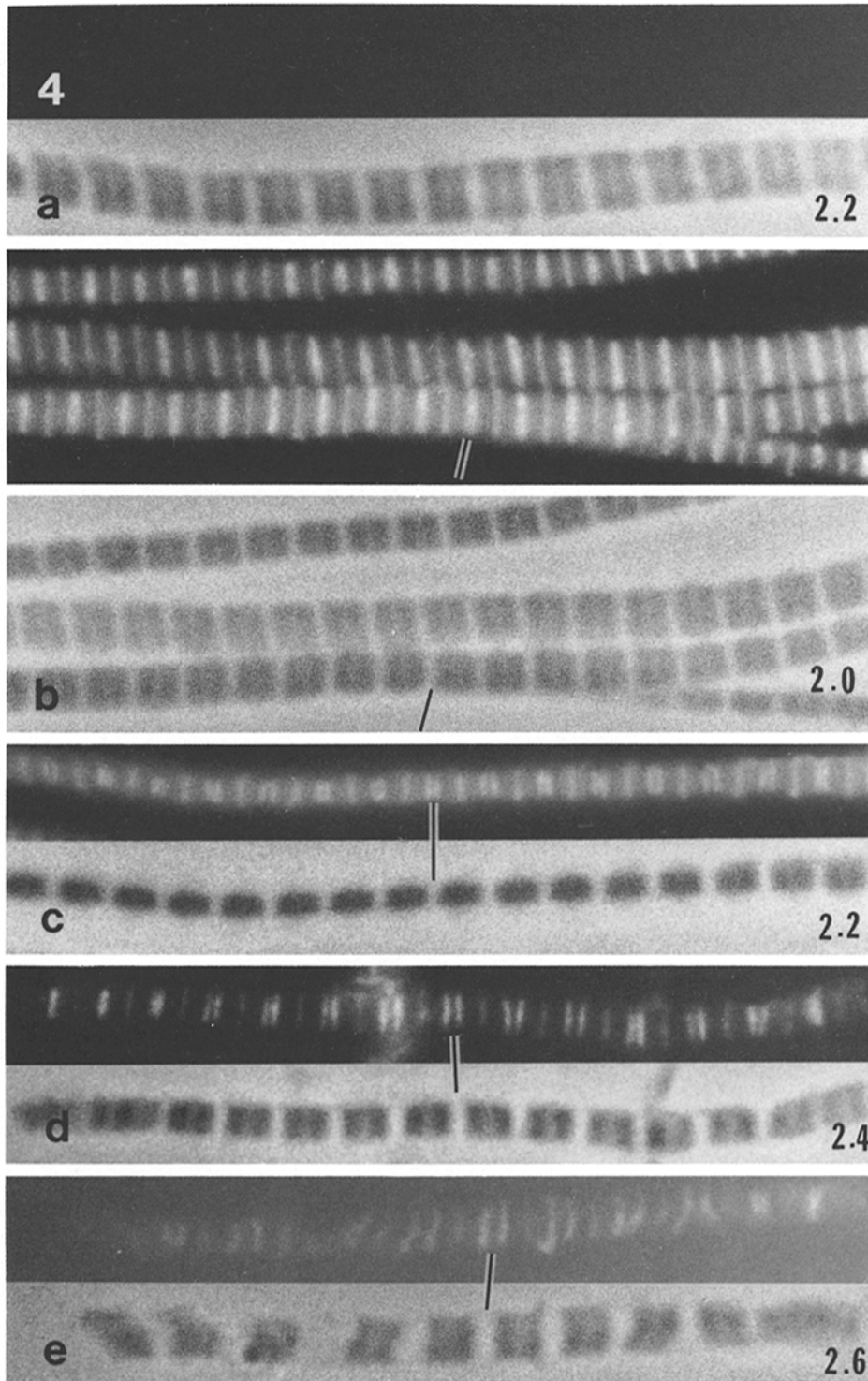
Anti-AMP deaminase staining of myofibrils prepared from unstretched glycerol-extracted muscle consistently produced strong fluorescence over the short I band (Fig. 4*b*). In addition, there was a thinner fluorescent line of more variable intensity in the center of the A band. In a few instances, the broad staining over the I band seemed to split into a closely spaced doublet.

To determine the effect of sarcomere length on the staining pattern, we examined myofibrils from several samples of muscle that had been glycerol-extracted while maintained under constant tension as described in Materials and Methods. In most preparations, myofibrils with a range of sarcomere lengths from ~2–3 μm could be found on the same slide and were stained simultaneously.

The staining in the middle of the A band was not affected by stretching. As the sarcomere length increased, however, the I band staining was clearly separated into two thinner lines, beginning at a sarcomere spacing of ~2.2 μm (Fig. 4*c*). No staining was observed over the Z line. With greater stretching, the separation between the doublets increased but the width of each individual line of the doublet remained constant (Fig. 4*d* and *e*).

Superimposition of the phase-contrast and FITC anti-AMP deaminase staining photographs showed the doublet staining to be localized over the lateral edges of the A band as visualized in the phase-contrast image and extending a short distance into the I band. The distance between the outer edges of the fluorescent stripes across the A band is somewhat greater than the width of the A band. Since the fluorescent staining appears to extend beyond the apparent edges of the A band in the phase-contrast image, we believe that the AMP deaminase is present at the extreme edges of the A band and perhaps extends from the ends of the thick filaments. The extreme ends of the A band would be indistinct in phase contrast because of the terminal tapering of the thick filaments.

Preparations of myofibrils examined in the light microscope are not completely uniform, and individual fibrils vary in length, width, and state of contraction. The staining pattern described above was observed in the majority of myofibrils and varied only in intensity, probably as a result of different degrees of antibody penetration. In a small percentage of myofibrils, however, especially those that were highly stretched, a different



staining pattern was observed. The doublet staining over the ends of the A band was absent, and only the mid A band staining remained (Fig. 5a).

As a further test of the specificity of AMP deaminase binding to the A band, we examined the effect of enzyme removal and readdition on the staining pattern. Myofibrils from which most of the AMP deaminase activity had been removed by washing ten times with 10 mM imidazole/HCl, 0.15 M KCl, 2 mM EGTA, pH 6.5, containing 10 mM potassium phosphate showed very weak fluorescence at the ends of the A band and no staining at the middle of the A band (Fig. 5b). The weak fluorescence required prolonged exposure to record photographically. Addition of purified enzyme to the extracted myofibrils resulted in a striking increase in fluorescence intensity when stained with FITC anti-AMP deaminase (Fig. 5c). This enzyme was tightly bound to the myofibrils and could not be displaced by repeated washings with phosphate-free buffer. The added enzyme was located primarily at the ends of the A band, and the staining pattern was similar to that found with endogenous enzyme, except for the appearance of a weak staining over the Z line. Also, the fluorescent line in the middle of the A band was absent in many of the myofibrils. The width of the stained line at the ends of the A bands was more variable with added enzyme than with endogenous enzyme. In some cases, the staining extended from the ends of the A band medially for a distance of almost 0.6 μm (Fig. 5d).

Antibody Staining of Cultured Muscle

Preparation of myofibrils involves prolonged glycerol extraction and severe mechanical disruption. To study AMP deaminase localization under milder conditions, we used muscle fibers formed de novo from presumptive myoblasts in tissue culture (5, 14). >90% of the AMP deaminase

synthesized by myotubes in 8-d-old cultures has the catalytic and immunological properties of the adult muscle enzyme (21). The cells were stained after at least a week in culture or when the fibers exhibited spontaneous contractions and sarcomeres were aligned across the diameter of the fibers.

Preliminary experiments were carried out to devise a method for rendering the muscle plasma membrane and basal lamina permeable to macromolecules while maintaining the fibers in a relaxed state. Since only a fraction of the AMP deaminase (5–10%) is apparently bound to the contractile elements as described in an earlier section, the permeability barrier must be disrupted under conditions that leave any soluble enzyme free to diffuse out of the cell.

Treatment with various fixatives such as alcohol, acetone, and aldehyde alone or in combination resulted in strong but uniform fluorescence in the muscle fibers with no evidence of cross-banding even though sarcomeres were clearly visible under phase contrast. Evidently, these treatments cross-linked or precipitated the soluble enzyme, thus obscuring the banding pattern. Freeze-thawing was not useful in that it disrupted the alignment of sarcomeres.

Detergent extraction was more successful in fulfilling the criteria described above. Five detergents (Triton X-100, Emalphogene, Tergitol NP-40, sodium deoxycholate, and sodium lauroyl sarcosine) were tested in 7-d cultures at the lowest concentration that destroyed the cell permeability barrier within 5 min as evidenced by loss of cell refractility under phase-contrast optics or uptake of the vital dye nigrosin. Of these, 0.1% deoxycholate was the most useful in preventing shortening of the sarcomeres during extraction and in preserving their alignment. The direct antibody staining method was used with all cultured fibers since this procedure produced less cytoplasmic

FIGURE 4 Photomicrographs of myofibrils stained with fluorescent antibody. Each pair represents a myofibril photographed sequentially for fluorescence and phase contrast using the same optics. A line is drawn to connect the same Z line in each photograph for comparison. The numbers give the length in micrometers of the sarcomeres adjacent to the marked Z line. $\times 3,780$. (a) A myofibril showing the absence of staining with nonimmune serum. (b) A group of contracted myofibrils stained with anti-AMP deaminase showing heavy staining over the short I band and lighter staining in the middle of the A band. (c, d, and e) A series of myofibrils at increasing sarcomere lengths stained with anti-AMP deaminase. With increasing stretch, the I band staining observed in contracted myofibrils resolves into a closely spaced doublet located over the ends of each A band. The separation between the doublet bands increases with stretch, but the width of each band remains the same.

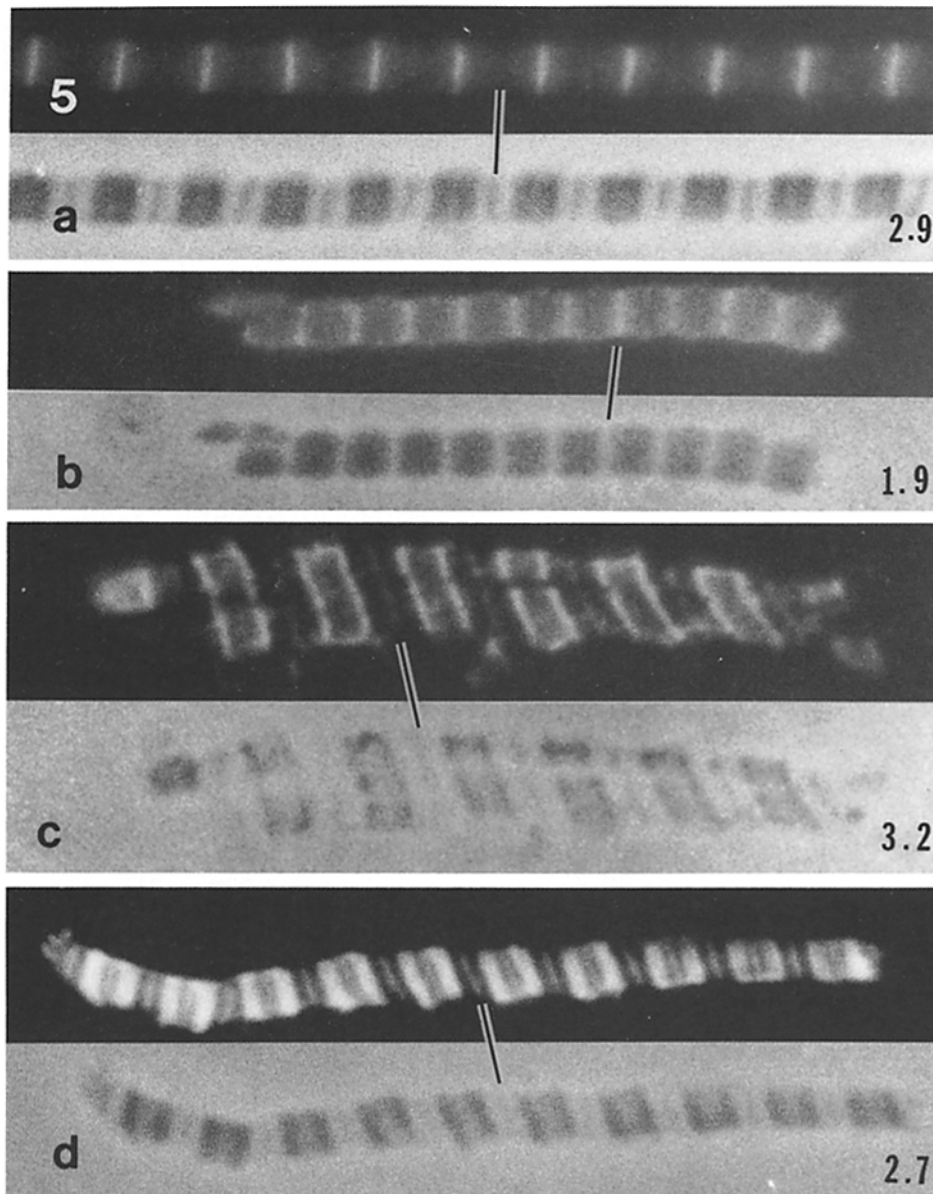


FIGURE 5 Fluorescence and phase contrast photomicrographs of myofibrils stained with anti-AMP deaminase. $\times 3,780$. (a) Highly stretched myofibril showing absence of staining at the edges of the A band. (b) Myofibril from which 80% of the AMP deaminase activity has been removed by treatment with 10 mM phosphate. The fluorescence photograph of this pair was made at twice the usual exposure to show the localization of the weak residual staining. (c and d) These myofibrils were extracted with 10 mM phosphate to remove endogenous AMP deaminase and were then treated with 1 mg/ml of purified enzyme and washed thoroughly before staining. The purified enzyme restores the original staining pattern of bright lines at the edges of the A band. In some myofibrils (d), the exogenous enzyme binds a greater distance medially in the A band.

background staining than did the indirect method.

When cultures extracted with 0.1% deoxycholate were stained with FITC anti-AMP deaminase, the myofibrils were brightly fluorescent and sharply delineated from the background cytoplasm (Fig. 6). The fluorescence in the fibers was located exclusively at the ends of the A bands,

with no evidence of staining at the Z line or center of the A band (Fig. 7). The fluorescent stripes corresponded more closely to the width of the A band and did not appear to extend into the I band as was observed with the isolated myofibrils. Some mononucleated cells in the culture showed moderately heavy staining located predominantly in

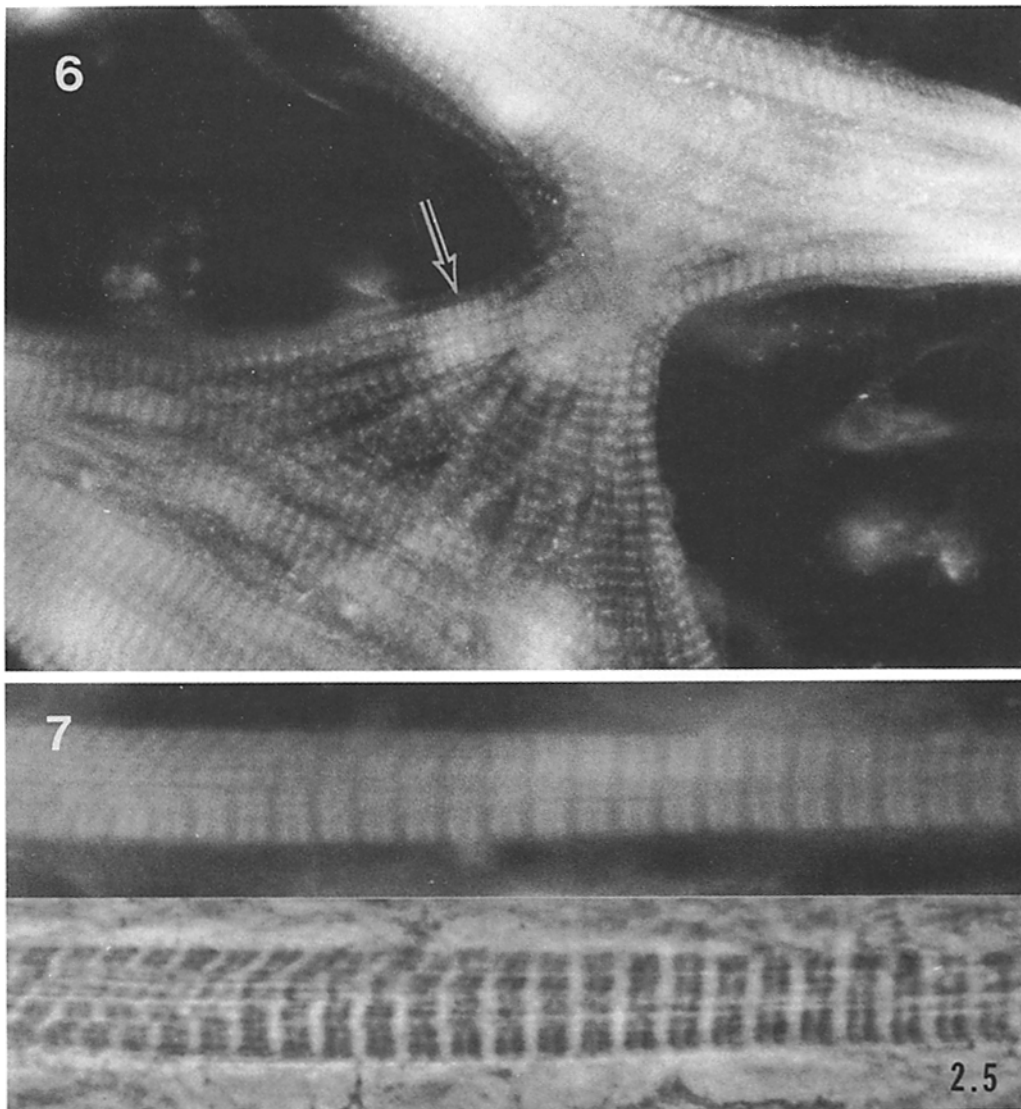


FIGURE 6 Fluorescence photomicrograph of whole mount of 11-d-old culture extracted with 0.1% deoxycholate and stained with anti-AMP deaminase. In the attenuated region of confluence between two muscle fibers, individual myofibrils are clearly delineated by the antibody staining. A doublet band can be seen in one stretched myofibril (arrow). $\times 1,164$.

FIGURE 7 Fluorescence and phase-contrast photomicrographs of a fiber from a 12-d-old culture extracted with 0.1% deoxycholate and stained with anti-AMP deaminase. The only periodic staining occurs at the ends of the A bands. $\times 2,360$.

cytoplasmic vesicles. These cells may be macrophages that have phagocytosed portions of degenerating myotubes.

Activity Staining of Cultured Muscle

To confirm the results of the fluorescent antibody staining and to determine whether the enzyme retains catalytic activity while bound to the A band, we used a recently developed method for histochemical staining of AMP deaminase (7). Muscle cultures were first extracted with 0.1% deoxycholate as before to wash out soluble enzyme. The cells were then incubated in the reaction mixture for up to 3 h or until sufficient formazan had accumulated. In general, younger cultures required longer incubation times.

Myotubes in the cultures showed a heavy accumulation of reaction product in the form of an amorphous blue deposit on the A bands of the myofibrils (Fig. 8). In some of the myofibrils, the heaviest staining occurred at the ends of the A band. In general, however, the localization was more uniform throughout the A band with the activity staining than with the fluorescent antibody technique. Mononucleated cells in the cultures gave a very light staining. There was also a moderate reaction in the nucleolus and nuclear envelope of the myonuclei. In addition to the amorphous reaction product in the cells, a coarse precipitate was present, particularly with long

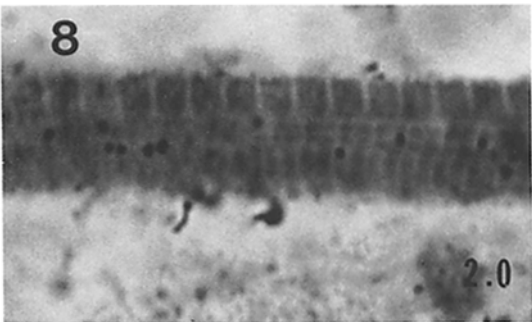


FIGURE 8 Brightfield photomicrograph of a fiber from a 10-d-old culture extracted with 0.1% deoxycholate and stained with a histoenzymatic reaction for AMP deaminase. The amorphous reaction product is located in the A bands. The staining of the myofibrils in the lower part of the photo appears to be strongest at the ends of the A bands, corresponding with the image obtained with fluorescent anti-AMP deaminase. The coarse, dense particles are from a precipitate that develops in the reaction mixture during incubation and are unrelated to enzyme activity. $\times 2,120$.

incubation times. This material appeared to form in the medium and bind indiscriminately to cellular and noncellular areas of the coverslip.

Several controls were carried out to determine the specificity of the reaction. Substituting citrate for AMP or omitting the dithiothreitol from the reaction mixture completely abolished staining. Staining was also essentially absent in cultures fixed in 4% formaldehyde or denatured by heating.

DISCUSSION

The aim of this investigation was to use immunostaining and histochemical techniques with myofibrils and cultured fibers to obtain morphological confirmation of the interaction between AMP deaminase and myosin, HMM and S-2 previously demonstrated by Ashby and Frieden (1) using purified proteins. Although the latter work was done with proteins prepared from rabbit skeletal muscle, identical results have been obtained with proteins prepared from chicken breast muscle, including the observation that chicken HMM forms a distinct complex with chicken AMP deaminase that can be observed using the analytical ultracentrifuge and that can be disrupted by 10 mM phosphate (Fig. 3). The specificity of the immunological techniques and interpretation of the labeling pattern obtained will be discussed below.

Specificity

The absence of autofluorescence and the weak binding obtained with the various control globulin preparations demonstrates that the staining pattern observed with immune globulin results from binding of specific antibodies. Since the immune globulin may contain minor antibodies in addition to those against AMP deaminase, it is important to determine whether the staining is specific for anti-AMP deaminase. The experiments involving removal and readdition of enzyme are the best evidence of specificity. Removal of AMP deaminase from myofibrils results in loss of antibody staining. When highly purified AMP deaminase is added back to myofibrils from which endogenous enzyme has been removed, the protein binds to the myofibrils to reconstitute the original localization at the ends of the A band when stained with antibody (Fig. 5c and d).

Specificity of AMP deaminase localization is also supported by the results of the histochemical staining. Enzyme activity is found in the A band,

primarily at the ends, and staining is eliminated by omission of substrate or by denaturation of the enzyme.

Interpretation of Labeling Pattern

If the fluorescent antibody and histochemical staining accurately reflect the location of AMP deaminase in the myofibril, the question arises as to whether the periodicity of enzyme binding reflects some known underlying arrangement of structural proteins in the myofibril. The staining pattern observed could result either from enzyme's being located in the contractile apparatus or in some other periodic repeating organelle such as the sarcoplasmic reticulum. Survival of membrane elements is unlikely, however, after prolonged glycerol extraction or treatment with detergent. Only occasional membrane profiles are visible with the electron microscope after glycerination (10, 18), and the sarcoplasmic reticulum is completely dissolved after deoxycholate treatment (6).

The fluorescent region observed at the middle of the A band is the most variable component of the staining pattern and may represent spurious relocation of enzyme during preparation of the myofibrils. This stained band has the following characteristics: (a) The fluorescent intensity is always weaker than that observed at the ends of the A band in the same myofibril. (b) The staining is not detectable after removal of 80% of the enzyme activity from the myofibrils although weak staining at the ends of the A band persists (Fig. 5*b*). (c) When exogenous enzyme is added back to extracted fibrils, the enzyme binds predominantly to the ends of the A band (Fig. 5*c* and *d*). (d) The mid A band staining is not observed in cultured fibers (Fig. 7).

That the width of the stained doublets at the ends of the A band does not change as a function of sarcomere length suggests that enzyme binding is not dependent upon actin-myosin interaction. The enzyme stripe is approximately as wide at a sarcomere length of 2.2 μm where filament overlap is almost maximal as it is at 2.8 μm . Accessibility of myosin to specific antibodies varies with sarcomere length, indicating that the thin filaments can either mask or expose antigenic sites on myosin (19).

On the other hand, filament overlap may be necessary but not sufficient for enzyme binding. Although myofibrils with sarcomere lengths $>3 \mu\text{m}$ were rare in our preparations, many of the

highly stretched myofibrils that were found lacked the lateral A band staining (Fig. 5*a*). Filament overlap is small at 2.9 μm sarcomere length, suggesting that enzyme binding to the A band is not maintained in the absence of thin filaments. Exogenous enzyme added to extracted fibrils did bind to the ends of the A band, however, even at sarcomere lengths that almost completely eliminated filament overlap (Fig. 5*c*).

That the position of the fluorescent regions with respect to the A band remains constant at various sarcomere lengths indicates that the enzyme is attached to the A band. Although exact localization is not possible with the light microscope, the position of the enzyme along the A band seems to vary somewhat depending on the preparation. With isolated myofibrils the fluorescent stripes appear to extend beyond the apparent edge of the A band, while in cultured fibers both immunohistochemical and histochemical staining are more closely confined to the lateral portion of each half A band.

According to Pepe (18), the antigenic determinants for LMM are restricted to the lateral thirds of the half A band. It is unlikely that the enzyme is bound to LMM, however, since accessibility of LMM varies with stretching (18) and, more importantly, LMM fails to bind AMP deaminase under *in vitro* conditions (1).

The most likely attachment site for AMP deaminase is the S-2 portion of myosin. Lowey and Steiner (11) have shown that staining myofibrils either with antimyosin absorbed with S-1 or with antirod (LMM plus S-2) absorbed with LMM gives a bright stripe at either end of the A band. Although measurements of the width of these bands are not given, close examination of their photographs (Plate IX and Plate X of reference 11) shows that the location of AMP deaminase corresponds exactly with the antigenic sites of S-2 in glycerinated myofibrils. Since this localization is consistent with the biochemical evidence of interaction between AMP deaminase and S-2 (1), it seems likely that a fraction of the enzyme is firmly bound to S-2, perhaps near the antigenic site.

Significance of the Enzyme

Myosin Interaction

It is of interest to calculate the number of deaminase molecules bound per thick filament. If one assumes a myosin content of muscle of 7.2% (13), then 1 kg of muscle which contains ~ 150

mg of deaminase will yield 72 g of myosin. Using the molecular weight of a thick filament (~300 myosin molecules) as 170×10^6 and the molecular weight of the deaminase as 240,000, one calculates ~1.5 molecules per thick filament if all the enzyme is bound. While the results presented above indicate that only 5–10% of the enzyme is bound, we have observed that complex formation of HMM and AMP deaminase is pH sensitive (B. Barshop and C. Frieden, unpublished data). Even under these conditions (100% bound), it is unlikely that adenylate deaminase plays any structural role in myofibril organization but rather that the interaction influences the enzymatic properties of the deaminase.

A physiological role for the AMP deaminase-myosin interaction is suggested by the fact that the same enzyme localization observed with myofibrils is found in intact muscle fibers grown in culture (Figs. 6 and 7) though the nature of this role is unclear. Work by Zydowo et al. (28) demonstrated that AMP deaminase has no effect on the length or shape of synthetic thick filaments prepared from purified myosin. AMP deaminase does not affect the activity of myosin ATPase nor of actomyosin ATPase (B. Ashby and C. Frieden, unpublished data). However, Ashby and Frieden (2) have demonstrated that binding of S-2 to AMP deaminase slightly activates the enzyme as well as reversing the effect of the inhibitor GTP. It is possible that S-2 binds to a site on the enzyme which also binds a number of purine nucleotides that act as activators of the enzyme and that displace GTP from a separate inhibitor site on the enzyme (2). Since S-2 may be competing for the activator site with a number of nucleotides that may be present in relatively high (mM) concentrations in vivo, notably ATP, it is possible that the amount of AMP deaminase bound to the myofibrils may vary with the metabolic state of the muscle. Thus, interaction of the enzyme with the myofibril may be related to the physiological role of the enzyme. During muscle contraction, AMP deaminase may act to displace the myokinase equilibrium to generate ATP from ADP. The ATP produced would then be readily available to the myosin ATPase. Ammonia (the second product of the deaminase reaction) is a potent activator of phosphofructokinase (15) and could therefore effectively stimulate glycolysis. The above described mechanism is entirely consistent with the apparent clinical symptoms of a muscle AMP deaminase deficiency disease recently described

by Fishbein et al. (7). Typically, patients who have no detectable muscle AMP deaminase activity experience muscle weakness or cramping after exercise although the muscle histology is apparently normal.

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