AN ELECTRON MICROSCOPE STUDY OF THE INTERACTION BETWEEN FRUCTOSE DIPHOSPHATE ALDOLASE AND ACTIN-CONTAINING FILAMENTS

D. J. MORTON, F. M. CLARKE, and C. J. MASTERS

From the Commonwealth Scientific and Industrial Research Organization, Division of Food Research, Meat Research Laboratory, Cannon Hill, 4170, Australia, and the School of Science, Griffith University, Nathan, 4111, Australia

ABSTRACT

The interaction of fructose diphosphate aldolase with F-actin, F-actin-tropomyosin, and F-actin-tropomyosin-troponin has been studied by using negative staining. In the absence of troponin, minor aggregates of aldolase and the F-actin filaments are formed. A well-ordered lattice structure is only formed in the case of the fully reconstituted filament when the filament-to-filament spacing is 18 nm, and the cross-bridge spacing is 38.7 nm. Evidence is presented that the lattice is due to an interaction between troponin and aldolase. The minimum subunit structure of troponin, still capable of giving rise to a lattice, is the troponin-IT complex, which indicates that troponin-C is not involved in aldolase binding.

KEY WORDS actin filaments · aldolase · tropomyosin · troponin

The interaction of aldolase and other glycolytic enzymes with the structural proteins of the I-band of skeletal muscle is now well established (1–4, 6, 27), although the biological significance of this association remains elusive (7). The probability that it may have biological significance can be inferred from the binding of aldolase and other glycolytic enzymes to actin-containing filaments at ionic strengths and protein concentrations, approximating those within the muscle fiber (6).

The structure of aldolase is a four subunit protein molecule, and electron microscope examination of the negatively stained enzyme has served to confirm its tetrameric structure (20). At the same time, the application of electron microscope techniques has proven to be of great value in elucidating the structure of actin-containing filaments (12, 15, 29, 30). It was anticipated that these techniques would be of value in providing information on the nature of the complexes formed between aldolase and actin-containing filaments. In addition, it seemed plausible that electron microscope examination of enzyme-filament complexes might surmount one of the difficulties of the study of enzyme morphology, specifically the presentation of randomly oriented profiles on the grid. In this paper we describe the formation of a two-dimensional lattice structure between aldolase and filaments reconstituted from F-actin, tropomyosin, and troponin. The evidence presented suggests that the lattice is due to interactions of aldolase with troponin.

MATERIALS AND METHODS

Protein Preparations

Aldolase was purified from beef skeletal muscle by using the procedure of Penhoet et al. (21). F-actin and the tropomyosin-troponin complex were prepared from an acetone powder of beef skeletal muscle by the methods of Spudich and Watt (28), and the acetone powder

was prepared as described by Briskey and Fukazawa (5). Tropomyosin, troponin, and the troponin subunits, troponin-T, troponin-I, and troponin-C, were prepared as described by Clarke et al. (9). Myosin was prepared from rat or beef skeletal muscle by the method of Briskey and Fukazawa (5).

The F-actin-tropomyosin and F-actin-tropomyosin-troponin complexes were prepared by using the reconstitution procedure of Ishiwata (18). Complexes were also formed between F-actin-tropomyosin and the troponin subunits, troponin-T and troponin-I, as well as with the troponin subunit complexes, troponin-IT, troponin-IC, and troponin-TC. The troponin subunit complexes were made by mixing the isolated subunits in approximately equimolar ratios in the presence of 8 M urea, followed by dialysis against the buffers used in the reconstitution procedure. For reconstitution, the F-actin, tropomyosin, and the troponin subunits or subunit complexes were mixed in a solution containing 10 mM imidazole, 0.1 M KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol (DTT), at pH 6.8, with a molar ratio of actin to tropomyosin of 7:1; the troponin subunits were added in equimolar amounts to tropomyosin. Due to the insolubility of troponin-I and troponin-T at low salt concentrations (13, 16), additional KCl was included in these mixtures to a final concentration of 0.35 M. In the case of the troponin-IC complex, 0.1 mM Ethyleneglycolbis[β-aminoethyl ether]N,N'-tetraacetic acid (EGTA) was added because troponin-IC binds to F-actin-tropomyosin only in the absence of calcium (22). 0.1 mM $CaCl_2$ was added to the troponin-TC complex, as this complex is only soluble in the presence of calcium (11).

All filament preparations were sedimented by centrifugation at 120,000 *g* for 60 min in a 60 Ti rotor in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The pelleted filaments were resuspended in 5 mM imidazole, 50 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, at pH 6.8, and stored on ice in the presence of a crystal of thymol or 2–3-day periods (23). The stock suspensions had a protein concentration of 10–15 mg/ml.

Assays

Aldolase activity was measured by the procedure of Richards and Rutter (24), and protein concentrations were estimated by the method of Lowry et al. (19). The actomyosin adenosine triphosphatase (ATPase) activity was measured as previously described (9).

Electrophoresis

Polyacrylamide-gel electrophoresis, in the presence of sodium dodecyl sulfate was performed as previously described (9).

Electron Microscopy

For examination in the electron microscope, mixtures of aldolase (0.05–0.1 mg/ml, final concentration) and the filament preparations (0.1–0.2 mg/ml, final concentration) were made in 5 mM imidazole, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, at pH 6.8, with the ratio of aldolase to filament usually 1:2 on a weight basis. A drop of the mixture was applied to a 400-mesh carbon-coated grid and allowed to remain on the grid for 3 min, and the excess was then removed with a filter paper. A drop of freshly prepared, aqueous 1% uranyl acetate was then applied to the grid and immediately removed with filter paper. Fixation with glutaraldehyde before staining did not lead to superior preparations. The grids were air-dried and examined in a Philips EM 300 electron microscope at an accelerating voltage of 60 or 80 kV.

The method of Dickson (10) was used for calibration of the microscope, in which the lens excitation is held constant between specimen and the calibration standard of catalase crystals. More than 100 measurements were taken for each lattice spacing on several different preparations of filaments.

Control Experiments

To test for aldolase absorption under the conditions used in the electron microscope studies, the mixtures of aldolase and filaments were centrifuged at 120,000 *g* for 60 min, and the resulting supernates and pellets were assayed for enzyme activity. These experiments showed that 99–100% of the enzyme was bound under these conditions. As the ionic strength of the buffer used in the electron microscope studies was relatively low in order to ensure aldolase adsorption (2), tests were also carried out to ensure the association of the tropomyosin and troponin components with the actin filaments under these conditions. To do this, samples of the stock filament suspensions were diluted to 0.2 mg/ml in 5 mM imidazole, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, at pH 6.8, and sedimented at 120,000 *g* for 60 min. The pellets obtained were analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate. Densitometric analysis of the gels revealed that the actin, tropomyosin, and troponin components were present in the same relative proportions as in uncentrifuged control samples, therefore establishing that tropomyosin and troponin remained associated with the actin filaments under the conditions used in the electron microscope studies. Full details of these binding studies will be published elsewhere.[1]

Filament Preparations

All filament preparations used in these studies were routinely analyzed by sodium dodecyl sulfate-gel electrophoresis, which showed that each preparation contained the pattern of components expected, and that the preparations were free of significant contamination. In addition, the presence of troponin on the fully reconstituted

[1] Walsh, T., F. M. Clarke, and C. J. Masters. Manuscript in preparation.

filaments was confirmed by its ability to confer calcium sensitivity on the actin-stimulated myosin ATPase activity.

RESULTS

Aldolase: F-Actin

The binding of aldolase to F-actin results in the formation of structures, as shown in Fig. 1. Single filaments were rarely found in the F-actin-aldolase mixtures, in contrast to the F-actin preparations alone, which appeared as single filaments similar to those previously described by Hanson and Lowy (15), and Huxley (17). Therefore, the presence of aldolase promotes the formation of complexes between the filaments which appear as side-by-side aggregates. While these complexes are interpreted as being a result of cross-linking of adjacent actin filaments by aldolase, the details of this arrangement have not as yet been clarified, although there are some indications of regularity.

Aldolase: F-Actin-Tropomyosin

As with F-actin, aggregates are also formed when aldolase and F-actin-tropomyosin are mixed, as shown in Fig. 2. As in the case of the F-actin-aldolase aggregates, the parallel arrangement of filaments, with some indications of regularity, suggests a cross-linking of the filaments by enzymes.

Aldolase: F-Actin-Tropomyosin-Troponin

A well-defined lattice structure is formed when aldolase is added to filaments reconstituted from F-actin, tropomyosin, and troponin, as shown in Figs. 3 and 4. This lattice consists of a parallel alignment of filaments cross-linked at regular intervals. The cross-links are regarded as being the aldolase, as no cross-links are formed in the absence of enzyme. A lower magnification view of the lattice structure shows that nearly all the filaments in these mixtures are organized into the lattice structure (Fig. 4). These structures are quite extensive in length, and formations in excess of 20 μm are not uncommon. However, they are fairly limited in width, and occasionally reach a maximum of $\cong$12 actin filaments in parallel. These may be compared with the reconstituted actin filaments in the absence of aldolase (Fig. 5).

The well-ordered lattice structure formed between aldolase and the fully reconstituted filament is in marked contrast to the aggregates formed between aldolase and either F-actin or F-actin-tropomyosin. This suggests the involvement of troponin in the formation of the lattice structure. Measurement of the lattice dimensions gives a value of 38.7 nm (1.5 SD) for the center-to-center spacing of the aldolase cross-bridges, and 17.7 nm (1.2 SD) for the center-to-center spacing between adjacent filaments.

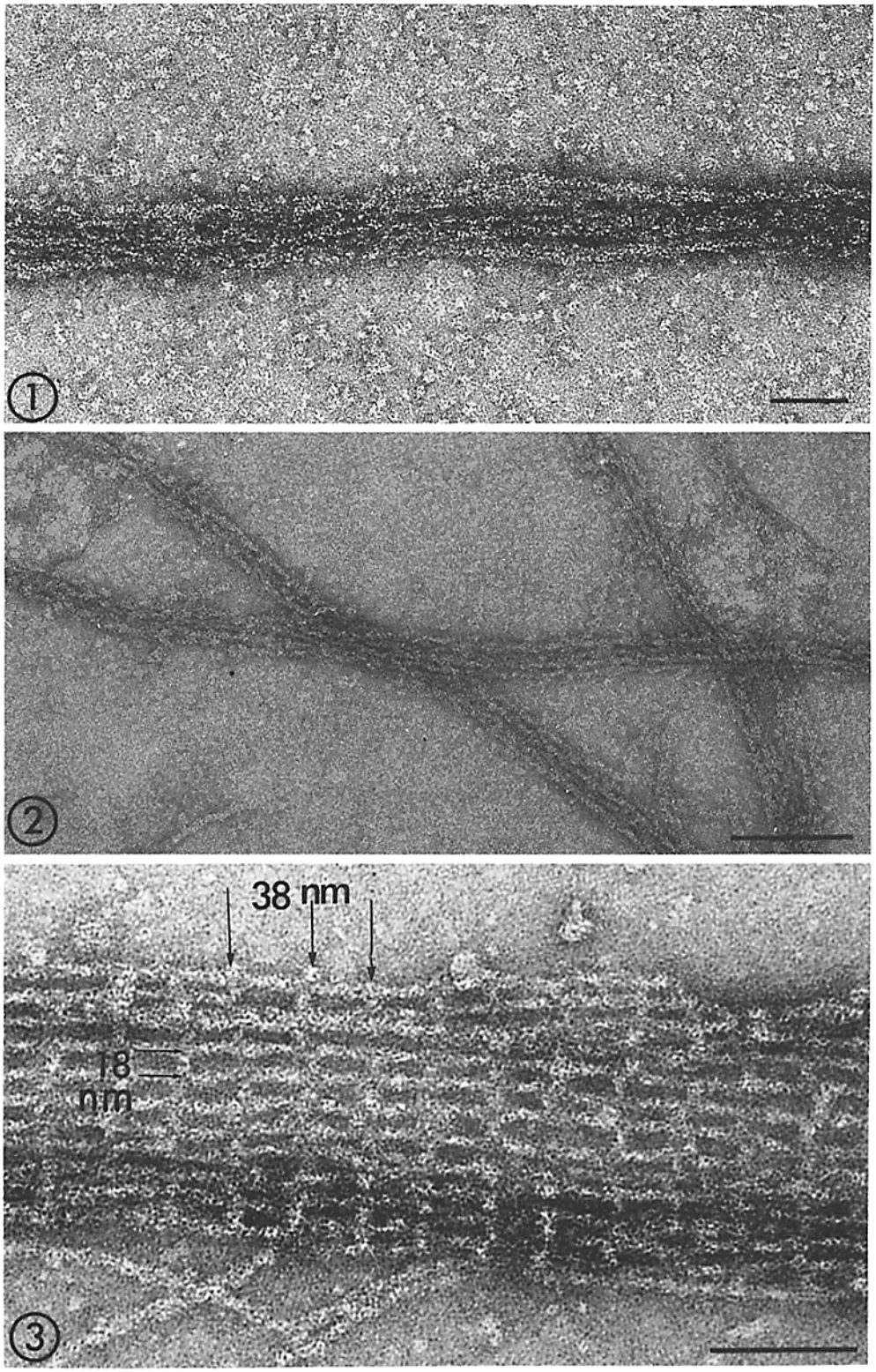
Using a goniometer stage, the specimen was tilted from $-42°$ to $+56°$ along the axis of the filaments of the lattice. The maximum filament separation was found at zero tilt, and there were no indications that the lattice was other than a two-dimensional structure.

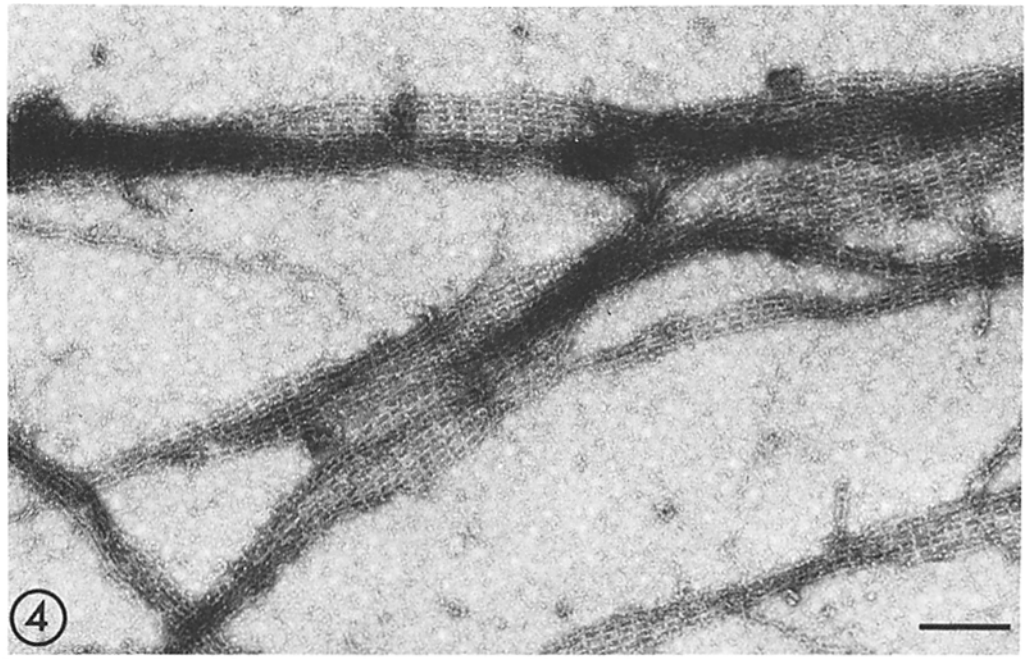
After the addition of aldolase to the F-actin-tropomyosin-troponin subunit complexes, a lattice-like structure was only observed with the F-actin-tropomyosin-troponin-IT filaments. This lattice is shown in Fig. 6. It is of interest that neither the F-actin-tropomyosin-troponin-I nor the F-actin-tropomyosin-troponin-T complex gave a lattice structure under the same conditions. These complexes, along with F-actin-tropomyosin-troponin-IC and troponin-TC complexes, formed aggregates of cross-linked filaments in which it was difficult to observe any regularity. Measurement of the lattice dimensions of the aldolase-F-actin-tropomyosin-troponin-IT lattice gave values of 38.7 nm (2.5 SD) for the cross-bridge repeat, and 18.0 nm (1.6 SD) for the interfilament spacing. These are virtually the same measurements as those for the lattice formed from filaments con-

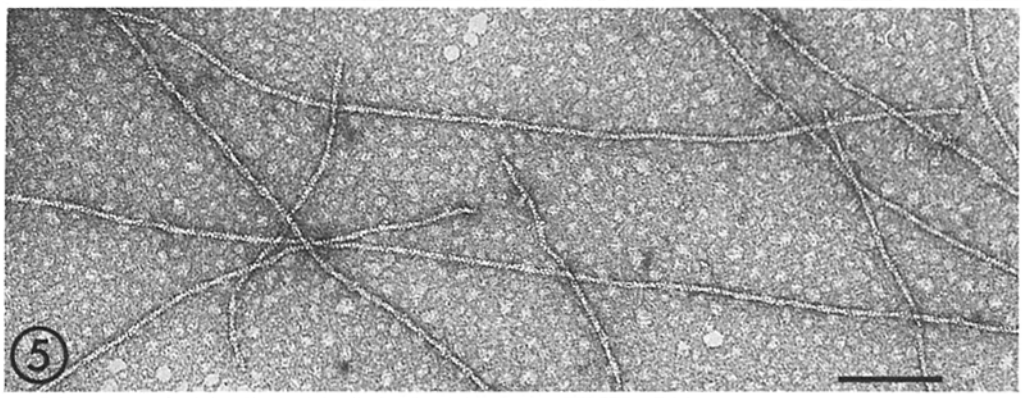
FIGURE 1 Mixture of pure F-actin and aldolase in the ratio of 2:1 by weight. Limited alignment of filaments occurs with indications of aldolase cross-linking the filments. Bar, 100 nm. × 123,000.

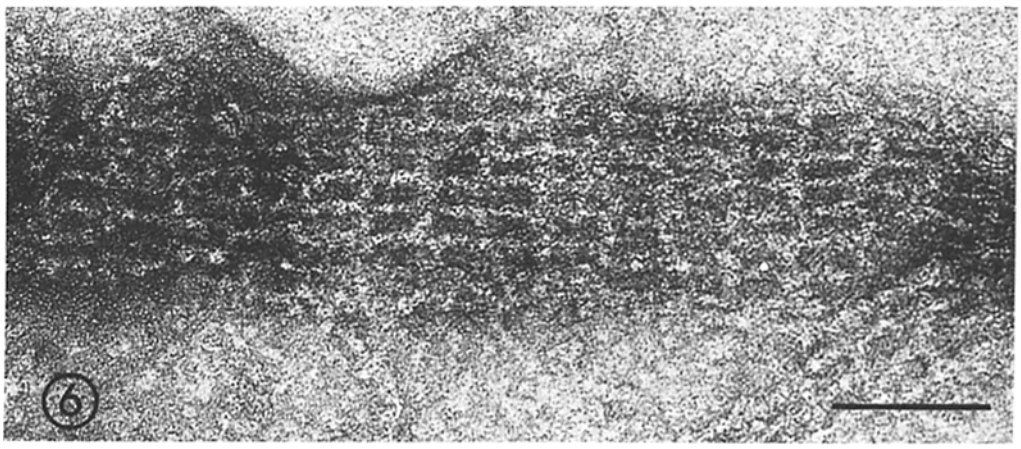
FIGURE 2 Mixture of F-actin-tropomyosin and aldolase in the ratio of 2:1 by weight. Although the aggregates tend to be larger than with F-actin and aldolase, the ordering is still not well defined. Bar, 100 nm. × 186,000.

FIGURE 3 Mixture of F-actin-trophomyosin-troponin and aldolase with the formation of a lattice structure. The dimensions of the lattice are indicated on the figure as center-to-center spacings. Bar, 100 nm. × 220,000.









taining the complete troponin complex. No lattice structure or aggregate is observed in the absence of enzyme.

A number of factors have been found to be important in lattice formation. For example, the integrity of the various components is essential. Actin filaments which are short, angular, or having lumpy irregularities are poor contributors to lattice formation. Similarly, proteolytic fragmentation of troponin (as evidenced by sodium dodecyl sulfate-gel electrophoresis) also leads to decreased lattice formation. Indeed, experience suggests that the formation of lattice may be employed as a simple and rapid test for the successful reconstitution of thin filaments.

DISCUSSION

The electron microscope data presented in this report describe the formation of aggregates of filaments when aldolase interacts with either F-actin or F-actin-tropomyosin. Although the precise structures of these complexes are not evident, it is clear that they arise from a lateral association of filaments cross-linked with aldolase. Although a detailed ultrastructural analysis of the interaction of aldolase with F-actin or F-actin-tropomyosin must await the formation of more ordered structures, it is evident from the present observations of cross-linking that aldolase must possess at least two sites for interaction with structural proteins.

In contrast to the complexes formed with F-actin and F-actin-tropomyosin, the interaction of aldolase with filaments reconstituted from F-actin, tropomyosin, and troponin results in the formation of a well-ordered lattice structure in which the filaments are cross-linked at regular intervals along their length. It is proposed that the bridges of this lattice structure are formed by aldolase cross-linking troponins on adjacent filaments. The lattice spacing of 38.7 nm supports this interpretation. Evidence from X-ray diffraction studies of muscle fibers labeled with anti-troponin-C antibodies (25), and analysis of paracrystals of reconstituted filaments (12, 14) are in agreement with troponin repeats at about 38.5 nm along the actin filament. As previously stated, the characteristic lattice is not formed with filaments lacking troponin. Although some form of organization is apparent with both actin-aldolase and actin-tropomyosin-aldolase, neither the extent nor the level of ordering approaches the organization of the complete system.

Aside from these observations, other studies have established the binding of the tropomyosin-troponin complex to aldolase (8), although recent experiments have demonstrated that aldolase may directly interact with troponin.[1] Therefore, it seems reasonable to postulate that the cross-bridges are due to aldolase cross-linking the troponins on adjacent filaments.

Experiments with subunit preparations of troponin indicate that troponin-I and troponin-T subunits are required for the formation of the lattice. The evidence seems to exclude the participation of troponin-C in the cross-bridge formation. However, from the available evidence, it is not possible to conclude whether the troponin-I or the troponin-T subunit is involved in aldolase binding. For example, the role of troponin-T may be to bind with tropomyosin at specific sites to correctly position the troponin-I for lattice formation.

On the basis of the 18-nm lattice filament separation, it is doubtful whether the formation of links between troponins on adjacent filaments could occur in the I-band of muscle. The I-filament separation has been established as 22 nm in the tetragonal arrangement near the Z-disk, and depending on contraction, the hexagonal arrangement of filaments in the A-band is 20–25 nm (26). Of course, this does not preclude an association between aldolase and a single troponin. The possibility that such an association may occur within the muscle fiber is indicated by the histochemical localization of aldolase within the I-band (3, 27), by the binding of aldolase to actin-containing filaments under conditions believed to exist within

FIGURE 4 Lower magnification of a lattice preparation showing the extent of lattice formation. Most filaments are involved in lattice formation. Although there is no apparent limit to the length of these structures, this micrograph illustrates the usual distribution of lattice widths. Bar, 250 nm. × 50,000.

FIGURE 5 F-actin-tropomyosin-troponin filaments in the absence of aldolase. Bar, 100 nm. × 140,000.

FIGURE 6 Lattice formed between aldolase and filaments reconstituted from F-actin, tropomyosin, and the IT subunits of troponin. The lattice spacings are virtually identical to those formed with the fully reconstituted filament. Bar, 100 nm. × 212,000.

the muscle fiber (6), and by the marked influence which tropomyosin-troponin exerts on this binding (6, 7). Whether or not an association between aldolase and actin filaments is of significance in nonmuscle cells remains to be determined.

We thank Ms. S. Tucek for her skilled assistance.

This work was supported in part by the Australian Meat Research Committee.

Received for publication 17 December 1976, and in revised form 23 May 1977.

REFERENCES

1. ARNOLD, H., and D. PETTE. 1968. Binding of glycolytic enzymes to structure proteins of muscle. *Eur. J. Biochem.* **6**:163–171.
2. ARNOLD, H., and D. PETTE. 1970. Binding of aldolase and triosephosphate dehydrogenase to F-actin and modification of catalytic properties of aldolase. *Eur. J. Biochem.* **15**:360–366.
3. ARNOLD, H., J. NOLTE, and D. PETTE. 1969. Quantitative and histochemical studies on the desorption and readsorption of aldolase in cross-striated muscle. *J. Histochem. Cytochem.* **17**:314–320.
4. ARNOLD, H., R. HENNING, and D. PETTE. 1971. Quantitative comparison of the binding of various glycolytic enzymes to F-actin and the interaction of aldolase with G-actin. *Eur. J. Biochem.* **22**:121–126.
5. BRISKEY, E. J., and T. FUKAZAWA. 1971. Myofibrillar proteins of skeletal muscle. *Adv. Food Res.* **19**:279–360.
6. CLARKE, F. M., and C. J. MASTERS. 1975. On the association of glycolytic enzymes with structural proteins of skeletal muscle. *Biochim. Biophys. Acta.* **381**:37–46.
7. CLARKE, F. M., and C. J. MASTERS. 1976. Interactions between muscle proteins and glycolytic enzymes. *Int. J. Biochem.* **7**:359–367.
8. CLARKE, F. M., C. J. MASTERS, and D. J. WINZOR. 1974. Interaction of aldolase with the troponin-tropomyosin complex of beef muscle. *Biochem. J.* **139**:785–788.
9. CLARKE, F. M., S. J. LOVELL, C. J. MASTERS, and D. J. WINZOR. 1976. Beef muscle troponin: evidence for multiple forms of troponin-T. *Biochim. Biophys. Acta.* **427**:617–626.
10. DICKSON, M. R. 1974. Electron microscopy in enzyme analysis. *Lab. Pract.* **23**:392–395.
11. EBASHI, S., I. OHTSUKI, and K. MIHASHI. 1973. Regulatory proteins of muscle with special reference to troponin. *Cold Spring Harbor Symp. Quant. Biol.* **37**:215–223.
12. GILLIS, J. M., and E. J. O'BRIEN. 1975. The effect of calcium ions on the structure of reconstituted muscle thin filaments. *J. Mol. Biol.* **99**:445–459.
13. GREASER, M., and J. GERGELY. 1973. Purification and properties of the components from troponin. *J. Biol. Chem.* **248**:2125–2133.
14. HANSON, J. 1973. Evidence from electron microscope studies on actin crystals concerning the origin of the cross-striation in the thin filaments of vertebrate skeletal muscle. *Proc. Roy. Soc. London. Ser. B.* **183**:39–58.
15. HANSON, J., and J. LOWY. 1963. The structure of F-actin and of actin filaments isolated from muscle. *J. Mol. Biol.* **6**:48–60.
16. HARTSHORNE, D. J., and P. DREIZEN. 1973. Studies on the subunit composition of troponin. *Cold Spring Harbor Symp. Quant. Biol.* **37**:225–234.
17. HUXLEY, H. E. 1963. Electron microscope studies on the structure of natural and synthetic filaments from skeletal muscle. *J. Mol. Biol.* **7**:281–308.
18. ISHIWATA, S. 1973. A study of the F-actin-tropomyosin-troponin complex I. Gel-filament transformation. *Biochim. Biophys. Acta.* **303**:77–89.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**:265–275.
20. PENHOET, E. E., M. KOCHMAN, R. VALENTINE, and W. J. RUTTER. 1967. Subunit structure of mammalian fructose diphosphate aldolase. *Biochemistry.* **6**:2940–2949.
21. PENHOET, E. E., M. KOCHMAN, and W. J. RUTTER. 1969. Isolation of aldolase A, B, and C. *Biochemistry.* **8**:4391–4395.
22. POTTER, J. D., and J. GERGELY. 1974. Troponin, tropomyosin and actin interaction in the calcium regulation of muscle contraction. *Biochemistry.* **13**:2697–2703.
23. REES, M. K., and M. YOUNG. 1967. Studies on the isolation and molecular properties of homogeneous globular actin. *J. Biol. Chem.* **242**:4449–4458.
24. RICHARDS, O. C., and W. J. RUTTER. 1961. Comparative properties of yeast and muscle aldolase. Preparation and properties of yeast aldolase. *J. Biol. Chem.* **236**:3177–3184.
25. ROME, E. M., T. HIRABAYSHI, and S. V. PERRY. 1973. X-ray diffraction of muscle labeled with antibody to troponin C. *Nat. New Biol.* **244**:154–155.
26. ROWE, R. W. D. 1971. Ultrastructure of the Z-line of skeletal muscle fibers. *J. Cell Biol.* **51**:674–685.
27. SIGEL, P., and D. PETTE. 1969. Intracellular localization of glycogenolytic and glycolytic enzymes in white and red rabbit skeletal muscle. *J. Histochem. Cytochem.* **17**:225–237.
28. SPUDICH, J. A., and S. WATT. 1971. The regulation of rabbit skeletal muscle contraction. Biochemical studies on the interaction of tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**:4866–4871.

29. SPUDICH, J. A., H. E. HUXLEY, and J. T. FINCH. 1972. Regulation of skeletal muscle contraction II. Structural studies on the interaction of the tropomyosin-troponin complex with actin. *J. Mol. Biol.* **72:**619–632.

30. WAKABAYASHI, T., H. E. HUXLEY, L. A. AMOS, and A. KLUG. 1975. Three-dimensional reconstruction of actin-tropomyosin complex and actin-tropomyosin-troponin-T-troponin-I-complex. *J. Mol. Biol.* **93:**477–497.