Urea Extraction of Z Bands, Intercalated Disks, and Desmosomes

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Specific extraction of Z bands, intercalated disks and desmosomes was accomplished with molar concentrations of urea. Thick and thin filaments and M line material were undisturbed, demonstrating that the Z band protein is not similar to actin, myosin, or the M-line protein. The literature indicates that the tropomyosins are the only other structural proteins present in sufficient amounts to account for the extracted Z bands. However, the evidence for a lipid-protein association in the Z bands is considered and is further supported by the close association of the dense material of the Z bands, intercalated disks, and desmosomes with the lipoprotein cell membranes.

The effects of urea on the configurational states of various isolated muscle proteins have been extensively investigated in the last 35 years (19, 23, 31, 38, 39, 42, 43, 44), and the mechanism of denaturation is now sufficiently understood to permit qualitative investigations of whole muscle. Numerous investigators have analyzed the physiological changes in muscle activity after exposure to various concentrations of urea (23, 31), noting especially the effects on myosin (23, 39, 42, 43) and actin (38, 42). In addition, the effects of urea on partially purified tropomyosin have been reported (1, 19, 40, 44), but in none of these reports has electron microscopic examination of the tissue been presented. We have therefore initiated a systematic electron microscopic and electrophoretic study of chick cardiac and striated muscle after varying periods of exposure to urea concentrations from 0.1 M to 8 M. Particular emphasis was placed on attempts to extract relatively pure tropomyosin without excessive actin or myosin contamination. We have thus been able to determine (a) the optimum concentration for the specific extraction of the Z bands, (b) the probable composition of the Z bands, and (c) the probable similarity in composition of the Z band protein to the densely staining material of the intercalated disks and desmosomes. In addi-

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tion, we have demonstrated a significant difference of the material of the M line of voluntary muscle from that of the Z band, in contradiction to data presented by Stromer et al. (36).

MATERIALS AND METHODS

Cardiac and striated muscles from embryonic and hatchling chicks were excised, rinsed in Sörensen's buffer (32), and extracted immediately. For comparison with published results of others, we have completed additional extractions after brief or prolonged storage in 50% glycerol (6, 11, 29), noting however, several possible sources of error introduced by this procedure (4, 8).

Extraction. Various concentrations of urea from 0.1 M to 8 M were prepared in a stock solution consisting of 0.05 M NaCl and 0.05 M Sörensen's buffer. In addition, the Kay (19) and Woods (44) 8 M urea media for tropomyosin characterization and various dilutions with and without β-mercaptoethanol were employed. After maceration and several rinses of buffer, extractions were attempted, exposure periods varying from 2 minutes to 6 hours in a single concentration or over a range of concentrations. Preliminary identification of extracted proteins was accomplished on a slab polyacrylamide apparatus, using 3 M urea in a buffer of Tris-EDTA-borate (pH 9.0). Gels were made with 7.5 g cyanogum 41 and run at 100 V for 3.5 hours. Amido black was used as the staining agent.

Electron microscopy. To ensure in situ fixation of denatured proteins and to reduce osmotic damage, 2.5% glutaraldehyde was added gradually. After 1 hour the tissue was rinsed twice in buffer, post fixed for 1 hour in buffered 1% OsO₄, rinsed in distilled water, and placed in 0.5% uranyl acetate for 16--20 hours before dehydration in successive concentrations of ethanol. Two rinses in acetone preceded embedding in plastic diluted with acetone. The final plastic mixture consisted of 10% Epon, 20% Araldite, 70% DDSA with 1 drop of DMP-30 accelerator added per milliliter of plastic. After polymerization at 80°, sections were cut on a Servall MT-1 ultramicrotome, mounted on uncoated 300-mesh copper grids, poststained for 1 minute in Reynolds' lead citrate (30), and examined on a Siemens Elmiskop I at 60 kV.

RESULTS

Initial experiments were undertaken to determine the effects of the unmodified Woods (8 M urea) medium on the ultrastructure of the myofibril. After only 2 minutes' exposure to 8 M urea with or without β-mercaptoethanol, the myofilaments, Z bands, intercalated disks, and desmosomes were completely disrupted. In order to reduce myofilament damage, the urea concentration was reduced to a level below

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**Fig. 1.** Three-hour extraction of chick heart muscle using 1 M urea. Z bands were completely removed, and ends of thick filaments partially disrupted. Thin filaments (arrows) are readily distinguishable. From macerated tissue. ×43,000.

**Fig. 2a.** Forty-five-minute extraction of chick heart muscle with 3 M urea. Z bands were extracted with reduced myofilament damage [cf. Huxley (17) pp. 635, 639]. ×53,000.

**Fig. 2b.** Cross section of myofibril extracted as in Fig. 2a, showing thick and thin filaments and patterns associated with A, I, and H zones. ×45,000.
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that known to denature actin (38) or myosin (39), yet hopefully sufficiently concentrated to solubilize tropomyosin. Exposure of cardiac muscle to 1 M urea with 0.01 M β-mercaptoethanol for 3 hours resulted in reduced myofilament damage, but in complete extraction of the Z bands, intercalated disks, and desmosomes. (The extraction of the desmosomes resulted in membranes similar in appearance to normal "bald" membranes.) The thick and thin filaments (Fig. 1) were easily recognizable, but the apparent partial disruption of the ends of the thick filaments was attributed to extended exposure to the 1 M urea to and β-mercaptoethanol.

Brief extractions in more concentrated urea (3 M) with or without β-mercaptoethanol were observed to greatly reduce myofilament damage, yet continue to remove Z bands, (Figs. 2a and 2b). After 45 minutes’ exposure to 3 M urea with β-mercaptoethanol, damage to the thick filaments was minimal. Note that in cross section (Fig. 2b) both thick and thin filaments are readily distinguishable. However, osmotic damage due to the concentrated urea is detectable in the mitochondria. Figure 3 reveals a similar 3 M extraction of breast muscle, the most obvious difference being the lack of extraction of the M line characteristic of voluntary muscle.

By reducing extraction time to 5 minutes in 3 M urea, we were able to produce extraction gradients in larger pieces of tissue (Fig. 4). In regions of incomplete Z band extraction, we consistently observed a similar and parallel degree of extraction of intercalated disks (arrows) and desmosomes, indicating that the material of the Z bands and the desmosomes and intercalated disks may be very similar or identical. (Partially extracted desmosomes usually are indistinguishable from areas of membranes with normally associated dense material.)

**INTERPRETATIONS**

The myofibril of cross-striated muscle has been demonstrated to consist of three major structural proteins: myosin, comprising at least 55% of the myofibril mass (14) and limited to the thick filaments (18); actin, comprising at least 25% (15) and limited to the thin filaments (14, 15, 18, 26); and tropomyosin, comprising at least 10–12 or 15% (3, 15, 29). The remaining few percent can be attributed to several minor structural proteins and to unextractable or residual actin, myosin, and tropomyosin.

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**Fig. 3.** Forty-five-minute extraction of chick breast muscle using 3 M urea. Note obvious Z band extraction without removal of M line. (The 3 subunits of the M line may be more easily visualized by viewing micrograph from extreme acute angle.) Identity of dense globular material in I regions (arrows) is unknown. × 37,000.

**Fig. 4.** Five-minute extraction of chick heart muscle using 3 M urea. Extraction gradients are obvious in tissue, with partial extraction of Z bands and intercalated disks (arrows). Adjacent cell with reduced Z band extraction. Note slightly distorted mitochondria resulting from osmotic damage. × 38,000.
Ashley et al. (2) and Guba et al. (12) have revealed that the Z bands are readily digested by proteolytic enzymes, while Hanson and Huxley (14) have revealed that the Z bands contain 12–15% of the myofibril mass. Thus, sufficient evidence is available to indicate that the Z bands must necessarily consist of appreciable amounts of protein, much more than can be attributed to the sum of the remaining minor proteins. Therefore, it can be assumed that actin, myosin and/or tropomyosin must occur in appreciable quantities in the Z bands. However, the obvious correlation that every tropomyosin extraction procedure has been reported to remove or severely disturb the Z bands (5, 6, 12, 29, 36) has not been emphasized, yet these data are consistent regardless of the other proteins extracted, whether actin, myosin or both. Indeed, sufficient evidence exists to reveal that neither actin (27) nor myosin (18, 27) is present in the Z bands. However, numerous investigators have assumed that each of the myofibrillar proteins could have more than one location in the sarcomere. Knappeis and Carlsen (22) and Kelley (20, 21) have suggested, contrary to Pepe (27), that actin alone may be responsible for the substructure of the Z bands, while Perry and Corsi (29), Samusodova et al. (33), and Hanson and Lowy (15) have suggested that tropomyosin may serve as a “backbone” for the thick or thin filaments. In addition, Moscona’s (24) evidence of muscle cell dissociation in hyaluronidase and trypsin has been interpreted by Franzini-Armstrong and Porter (8) as suggesting a mucoprotein component in cell contacts (intercalated disks and desmosomes), though they (8) and Bowman (4) favor a lipid protein association in the Z-bands and intercalated disks due to disruption in alcohols. Walcott and Ridgeway (41) and Garamvölgyi (11) have recently presented further evidence of a lipid component based on Z band removal with acetone and digestion by lipase. It should be noted, however, that others (40) have utilized the apparent low solubility in acetone in the preparation of tropomyosin, further supporting the suggestion that at least part of the tropomyosin may exist outside the Z bands (15, 29, 33).

With all the contradictory evidence, two apparently mutually exclusive interpretations of the data have arisen: (a) “discrete localization” of components [i.e., actin = thin filaments (15), myosin = thick filaments (18), tropomyosin = Z bands (18), minor proteins = M line, etc. (27)]; and (b) “diffuse localization” of component [e.g., tropomyosin forming the “backbone” of the thick or thin filaments, with various other components throughout the sarcomere (15, 20–22, 27, 29, 33)]. We suggest that the above urea extraction procedures facilitate a choice between certain aspects of these two hypotheses, based on the specific extraction of the Z band protein without detectable damage to the thick or thin filaments or the M-line. Several conclusions follow immediately from our data: Actin and myosin, as found in the thin and thick filaments, are not detectably denatured upon brief exposure to urea concentrations below 3 \(\text{M}\) (38), yet the Z bands were shown to be extractable even below 1 \(\text{M}\). We
assume this to indicate that the Z bands are relatively homogeneous and that they
cannot contain actin (cf. 33) or myosin (cf. 5, 13). The only other proteins present
in sufficient amounts to account for the mass of the Z bands are the tropomyosins
(cf. 9, 18). In support of this contention is the fact that our extraction procedures were
originally designed to extract tropomyosin without actin or myosin contamination.
The data, however, are not inconsistent with the interpretations of Garamvölgyi (11)
and of Stromer et al. (36) of lipid-protein association in the Z bands. This apparent
lipoprotein association, with its probable susceptibility to alcohols and acetone, may
explain the lack of labeling by fluorescent antibodies after glycerol pretreatment (27,
33) and the ease of actin extraction following possible disruption of Z band–I filament
attachments (36, 37).

Numerous investigators have noted the similar staining characteristics and the
physical continuity of the Z bands, intercalated disks, and desmosomes (7, 8, 10, 16, 25,
34), some suggesting that the three are homologous and intimately associated with
muscle formation (8, 10). Their parallel degrees of extraction over a wide range of
urea concentrations are assumed to indicate similarity of composition and appear
to support these earlier interpretations. The proposed association of lipid and pro-
tein in the Z bands, intercalated disks, and desmosomes is of particular interest be-
cause of the close associations with lipoprotein cell membranes.

In 1967, Stromer et al. (36) demonstrated similar rates of digestion of the Z bands
and the M line by trypsin and suggested this as indicating biochemical similarity.
However, the lack of M line extraction in 3 M urea provides sufficient evidence to
conclude that the M lines differ significantly from the Z band material. We therefore
support the conclusions of Pepe (27) that the M line exists as a discrete site for the
localization of one or more of the remaining minor proteins.

It should be noted that the probable deleterious effects of numerous reagents, in-
cluding alcohol and glycerol (4, 27, 29, 32), acetone (35, 36), Tris buffer (33), borate
buffer (5, 28, 29, 36), and EDTA (5, 11, 29), indicate that biochemical analysis of
muscle should be attempted without the use of these reagents and preferably on freshly
excised tissue.

In conclusion, we suggest that the above data from urea extractions are sufficient
to indicate the discrete localization of tropomyosin in the Z bands and possibly in
the intercalated disks and desmosomes. Preliminary spectrophotometric analysis of
purified Z band protein reveals the material to be deficient in tryptophan and tyrosine,
characteristic of tropomyosin. Definitive evidence of the biochemical composition of
the purified Z band protein may be provided by amino acid analysis in progress.

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