Tropomyosin Paracrystals Formed by Divalent Cations

Abstract. *Rabbit tropomyosin exhibits a polymorphism dependent on divalent cations and* p*H. Above a critical divalent cation concentration fibers with a period of about 400 angstroms are formed. Below this concentration, and near the isoelectric point, lattices are formed. Implications for the morphology and function of striated and smooth muscle are discussed.*

Tropomyosin is a fibrous protein found in all muscles, and was first isolated and characterized by Bailey (*1*). Classified as an α-protein by its wide-angle x-ray diagram (*2*), tropomyosin is a rod-shaped molecule whose polypeptide chain conformation is the α-helical coiled coil (*3*). This protein is found to be almost fully α-helical by optical rotatory dispersion, and, in this respect, it resembles two other muscle proteins, paramyosin and light meromyosin fraction 1 (the helical "subunit" of myosin) (*4*). Tropomyosin is highly charged, binds nucleic acid, and although very soluble, tends to aggregate under certain ionic conditions (*1*). Similar to paramyosin and light meromyosin fraction 1, tropomyosin is resistant to denaturation, and this stability is attributed to side-chain interactions in the coiled coil (*5*). Early estimates gave the molecular weight of rabbit tropomyosin as 53,000 (*6*). More recent measurements indicate a weight of about 70,000 (*7, 8*) and a light-scattering radius of gyration of 142 Å (*7*), figures corresponding with a two-chain α-helical molecule about 490 Å long.

Tropomyosins from skeletal and cardiac muscle form macroscopic crystals (*1*), and in this respect appear to be unique among fibrous proteins. The electron microscopic appearance of sections of rabbit tropomyosin crystals, and of crystal fragments stained with phosphotungstic acid, shows a rhombic lattice (*9*). The similarity of this structure to the appearance of the Z line in sections of striated muscle was first pointed out by Huxley (*10*), who suggested that part of the tropomyosin may be located in the Z line. Another possible location for tropomyosin which has been suggested is in association with the actin filaments (*11*). By cocrystallizing with actin, tropomyosin could provide a length-determining mechanism for the actin filaments (*10*). Specific interactions in solution between actin and tropomyosin have been reported (*12*).

Here we describe the formation of a highly ordered fibrous form of vertebrate striated muscle tropomyosin, produced by divalent cations. Rabbit tropomyosin, when dialyzed in tris buffer (0.05*M*) to about *p*H 7 to 9 in the presence of 0.01 to 0.1*M* $MgCl_2$, yields a precipitate of tactoids (Fig. 1); $CaCl_2$ also produces this form. These fibers show a period of about 400 Å in the electron microscope when stained with 1 percent uranyl acetate (Fig. 2). The fibers have a symmetrical nonpolar band pattern (that is, there are twofold axes perpendicular to the fiber axis).

In contrast, when dialyzed near the isoelectric point [*p*H 5.4 acetate buffer and 0.12*M* $(NH_4)_2SO_4$] rabbit tropomyosin forms crystals (*1*). Net structures with a spacing of about 200 Å are seen in preparations of such crystals in the electron microscope. Nets with a spacing of about 400 Å have also been observed. Dialysis of rabbit tropomyosin against 0.05*M* KH_2PO_4, *p*H 4.8, produces nets as well as small tactoids which sometimes have a repeat of about 45 Å.

Preliminary studies with chicken breast tropomyosin have yielded similar results, the band patterns in the fibers closely resembling those of rabbit tropomyosin.

Thus striated muscle tropomyosin exhibits a polymorphism dependent upon the divalent cation concentration and the *p*H. Above a critical divalent cation concentration, fibers with a large period are formed, and below (near the isoelectric point), crystallization into lattice structures occurs.

Tropomyosins from vertebrate smooth muscles have been reported to form needle-shaped paracrystals rather than crystals (*13, 14*). For comparative purposes, therefore, some experiments were done with chicken gizzard tropomyosin prepared essentially by the method of Bailey (*1*). Dialysis of this tropomyosin against the crystallization buffer used for rabbit tropomyosin generally yields fibers having either of two periods, about 400 Å or twice this length (see Figs. 3 and 4). Again, both types of fiber show a symmetrical nonpolar band pattern. The period of one form of these paracrystals is similar to that of the striated muscle tropomyosin fibers produced by divalent cations, although the band pattern is different. Tsao *et al.* (*15*) have reported both the 400- and 800-Å periodicities in paracrystals of tropomyosin from duck and goose gizzard.

We have observed lattice structures together with fibrous aggregates in these preparations of chicken gizzard tropomyosin. The spacing between the nodes of the nets was about 400 Å. Divalent cations also cause precipitation of chicken gizzard tropomyosin in a fibrous form having a 400-Å period.

The period in the fibers of rabbit tropomyosin is about 20 percent less than the molecular length of 490 Å estimated from solution studies. If recent molecular weights are an overestimate, however, the 400-Å period in the fibrous form may represent an end-to-end aggregation of the molecules. Alternatively, there may be an overlapping arrangement (about 20 percent of the period) similar to that postulated for collagen (*16*) and light meromyosin fraction 1 (*10*).

The spacing between the nodes in the rabbit tropomyosin nets in crystal preparations is about 200 Å (*10*). X-ray diffraction measurements of tropomyosin crystals (*17*) show that the crystal lattice is orthorhombic and that the body diagonal for the unit cell is about 400 Å —close to the period found in the fibers. If the molecules are placed along the cell diagonals, one projection of the lattice has about the same dimensions as the nets seen in the electron microscope.

The fact that all these aggregates have symmetrical nonpolar band patterns indicates that dimer relations exist between the molecules in these fibers. Divalent cations favor a compact association of these molecules, compared with the packing in the crystals. The presence of the symmetrical 800-Å period in fibers of tropomyosin from chicken gizzards implies that the 800-Å packing unit in the fiber has twofold symmetry and presumably is an end-to-end dimer of subunits about 400 Å long. Tsao *et al.* (*13*) have in fact reported a molecular weight of about 150,000 for duck gizzard tropomyosin.

The similar appearance of fibers of rabbit and chicken striated muscle tropomyosin reflects similarities in molecular structure. In contrast, fibers of tropomyosin from smooth muscle have

a different band pattern, which suggests a different amino acid composition. Although there have been no reports on the composition of chicken gizzard tropomyosin, analyses of other vertebrate smooth muscle tropomyosins (such as those from bovine uterus and urinary bladder) show differences from the composition of rabbit striated muscle tropomyosin (*14*). [These studies on tropomyosin may be compared with results on collagens and paramyosins from different sources, where proteins of somewhat different amino acid composition form aggregates of similar morphology (*18, 19*).]

Many fibrous proteins show polymorphic forms which are not related to structures seen in vivo. Caution should therefore be exercised in relating in vitro aggregates to cellular structures. Nevertheless, one should not overlook some possible implications of the results presented here for the morphology and function of muscle.

Divalent cations may initiate the formation of a fibrous aggregate of tropomyosin in the developing striated myofibril. Thus, a gradient of these cations, produced perhaps by the sarcoplasmic reticulum, could cause a crystalline lattice structure at the Z line and a fibrous form with about a 400-Å period in the I band. By microincineration experiments, Draper and Hodge (*20*) have demonstrated the presence of minerals (divalent cations) in the I band and their absence in the Z line. Although no large filaments of tropomyosin similar to those described here have been found in vivo, a period of about 400 Å is seen in the I band by electron microscopy (*9, 21*). Moreover, a 400-Å x-ray meridional spacing from vertebrate striated muscle has been reported (*22, 23*). This reflection could be related to the 400-Å spacing in the fibrous form of tropomyosin. However, our experiments do not establish that the fibrous form of tropomyosin occurs in the myofibrils, and the possible location of such a fibrous form is not restricted to the I band.

The difference in association properties of tropomyosins from vertebrate striated and smooth muscles may have implications for the morphology of these two kinds of muscle cells. Striated muscle tropomyosin forms either a crystalline or a fibrous aggregate, depending on divalent cation concentration and *p*H. Correspondingly, the Z line in the striated myofibril may have a structure related to that seen in the crystal; and a fibrous form can be postulated, possibly in the I band. Vertebrate smooth muscle tropomyosin, in contrast, while forming lattices under certain conditions, appears to form fibrous aggregates preferentially. Correspondingly, no Z lines have been observed in these smooth muscle cells. Differentiated structures called "dense bodies" have been noted (*24*), and it may turn out that they have an organization analogous to that of the Z line. Alternatively, however, the tropomyosin in these cells may exist for the most part in a form similar to the postulated fibrous portion of the tropomyosin in vertebrate striated muscle.

A reversible binding of divalent cations by tropomyosin may have a regulatory function in muscle contraction. The hypothesis that these ions control prolonged tension maintenance ("catch") in molluscan muscles which contain much paramyosin has been advanced by Twarog (*25*). A role analogous to that of tropomyosin might be envisaged for paramyosin in the "catch" mechanism.

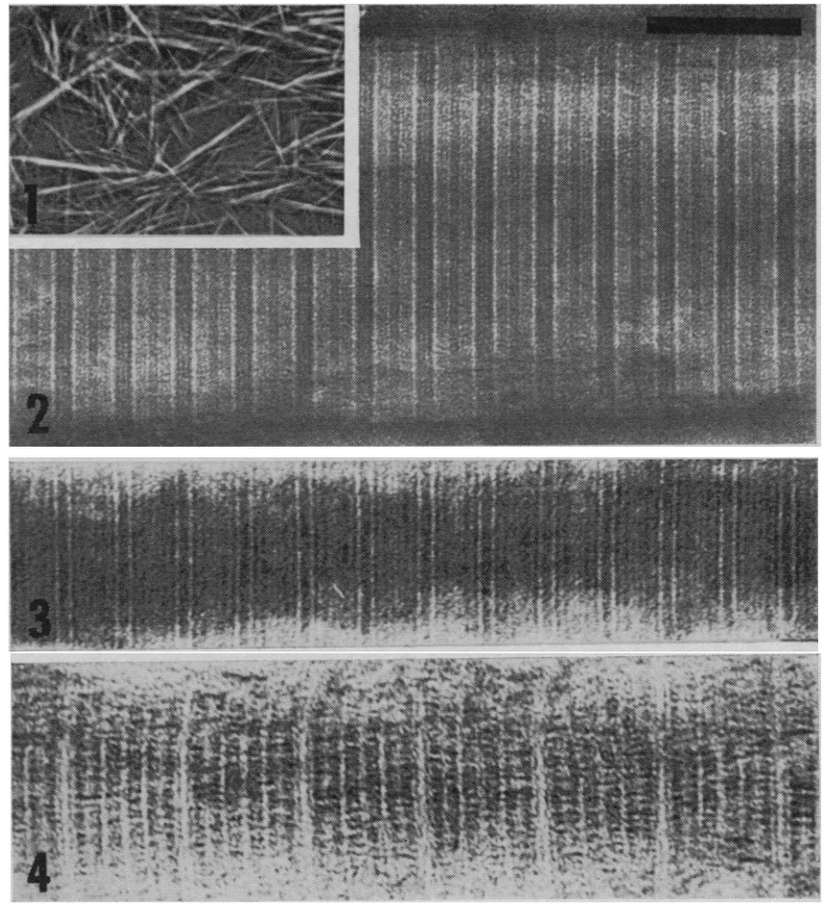

Fig. 1. A phase-contrast micrograph of tactoids produced by precipitating rabbit striated muscle tropomyosin with divalent cations (× 500). Fig. 2. An electron micrograph of a small tactoid of the type shown in Fig. 1, stained with uranyl acetate (scale, 1000 Å). Fig. 3. A tactoid of chicken gizzard tropomyosin, stained with uranyl acetate, prepared by dialysis against acetate buffer (*p*H 5.4) and 0.12*M* $(NH_4)_2SO_4$. The period (about 400 Å) is the same as that in Fig. 2 (magnification as Fig. 2). Fig. 4. A tactoid of chicken gizzard tropomyosin (prepared as in Fig. 3) showing the 800-Å period; uranyl acetate stain (magnification as Fig. 2).

Note added in proof: We have now obtained small-angle x-ray diffraction diagrams from rabbit tropomyosin fibers prepared with $MgCl_2$. These patterns show ten meridional orders of a repeat of 396 ± 8 Å.

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26. The electron microscopy was done in the Laboratory of Tissue Ultrastructure, and we thank its director, Dr. Betty G. Uzman, for assistance. We thank Dr. D. L. D. Caspar and Dr. Betty Twarog for discussion; Miss Ute Gallwitz for technical aid, and Dr. S. Farber for encouragement. Supported in part by NIH grants AM-02633 (CC) and CA-04696 to D. L. D. Caspar (W.L.).

25 January 1966

Estrous Cycle in the Rat: Effects on Self-Stimulation Behavior

Abstract. *The performance of female rats, in pressing a bar for electrical stimulation of the hypothalamus, changes during the estrous cycle. Highest bar-pressing rates accompany the appearance of vaginal cornification. This increase is not an artifact of increased spontaneous activity at estrus, although the factors underlying these changes in activity may also mediate the changes in self-stimulation behavior.*

The rewarding properties of electrical stimulation of the forebrain in rats are influenced by motivational variables such as availability of food and water and systemic androgen levels, the onset of high-drive states usually being accompanied by increase in the rate of self-stimulation (see *1, 2*).

In one experiment, however, the hormonal induction of estrous behavior in ovariectomized rats had no effect on self-stimulation (*3*); lordosis was used to indicate the onset of estrus, but it is not certain that the hormonal treatment used had reinstated other aspects of the behavioral and physiological complex that is characteristic of estrus. For example, restoration of the high levels of activity seen in the normal estrous female seems to require more prolonged hormone-replacement therapy than was possible in the design of the experiment (*4*). The study I now report sought to determine whether changes in self-stimulation behavior accompany the normal course of the estrous cycle in intact rats.

Six female albino rats (200 to 300 g) were used; each had a monopolar 28-gauge electrode implanted in the lateral hypothalamus. Electrode loci were histologically verified as ranging between the level of the mammillary bodies and the region of the optic chiasm. The rats were maintained under controlled lighting: alternating 12-hour periods of darkness and light. The experiments were always conducted during the first 3 hours of darkness each day, with the apparatus illuminated by a red light. The course of the estrous cycle was followed by taking vaginal smears from each animal daily at the beginning of the dark period.

Anesthesia, surgery, or electrical stimulation interrupted the regular cycling of some rats. Only females whose normal cycle of cornification was not disturbed by the implantation of electrodes, or by the subsequent training and testing procedure, were used in the experiment. The animals were tested in a Skinner box having a floor 30 cm square. A sine-wave generator (50 cy/sec) delivered 0.5-second stimulation at the implanted electrode, on a continuous reinforcement schedule, when the rat depressed a bar. Four of the subjects were tested in a two-bar box in which operation of one of the bars was never reinforced. Food and water were available during all tests. Animals were trained to press for brain stimulation during 30-minute sessions on three consecutive days, during which time the electrical threshold for self-stimulation was determined; thereafter the stimulus intensity was set 15 μa above threshold for each animal, with the root-mean-square current values ranging from 40 to 125 μa. After training, the animals were tested daily throughout three complete estrous cycles.

Each test consisted of 15 minutes of acquisition (in which bar-pressing was reinforced by brain-stimulation) followed by a 30-minute extinction period with no stimulation. During the extinction period the following behavior patterns were recorded by use of a 5-second time-sample procedure: walking, rearing, grooming, sitting still, eating, and drinking. Testing did not begin on the same day of the cycle for every subject. There was no significant change in bar-pressing rates between the three estrous cycles.

Figure 1 summarizes the results for the group as a whole (*5*). In five of the rats, the highest mean score for self-stimulation occurred at estrus; in the sixth, on the day following estrus. For the group as a whole the self-stimulation score was significantly higher for the day of estrus than for the other days of the cycle, whether the other days were grouped as a single class ($p < .001$) or individually compared with the day of estrus [$p < .02$, $< .05$, and $< .05$, respectively (*6*)]. There was no significant difference ($p > .5$) between bar-pressing scores during the extinction period on the day of estrus and on any other day, although there was a significant tendency ($p < .05$) for the mean number of extinction responses to be lower at estrus, if the other days were considered as a single class. Responses on the no-reinforcement bar showed no significant change throughout the experiment. The behavior changes recorded during extinction were a guide to the activity changes accompanying the estrous cycle. The mean scores for the group show that on the day of estrus time spent sitting and grooming was less, and time spent walking and rearing was greater, than on other days of the cycle.

These observations agree with others obtained by different methods in indicating that female rats are most active at estrus (*7*). Although the