RECIPROCATION SESSION

Titin: A Myofibrillar/Cytoskeletal Protein of Gigantic Proportions

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Titin is not only the largest molecular weight muscle protein known, but together with nebulin they are the third most abundant myofibrillar/cytoskeletal proteins, being about 15% of the total. Also, they have been proposed as the major protein components of an elastic cytoskeletal lattice, a third filament, within the sarcomere of skeletal muscle. Moreover, titan and nebulin may be the most important proteins in muscle as a food – meat.

If titin and nebulin are purported to be so important in muscle biology and meat science, then why haven't we known about these proteins of consequence long before now? Why have they been concealed ostensibly in obscurity? Very simply, in early muscle protein extraction studies, using common extracting agents (O.6M KCl or KI), titin and nebulin were insoluble and were disposed of as an insoluble residue. Wang and coworkers (1979) were intrigued by the insoluble residue, and by some very ingenious and painstaking research involving the use of sodium dodecyl sulfate, electrophoresis and chromatography, they discovered titin and nebulin. After learning of titin's insolubility in nondenaturants and developing high-porosity electrophoretic gels to accommodate titin's low mobility and resistance to entry, Wang and coworkers (1979) discovered titin as a pair of closely-related megadalton polypeptides. Parenthetically, titin is a name derived from "titan," meaning anything of large size. Furthermore, other complications of titin being very susceptible to proteolysis, inconsistently solubilized and also being an aggregate former, slowed progress.

Initially, three protein bands having large molecular weights were observed by using high-porosity sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The first two bands were referred to collectively as titin because they were chemically and immunologically similar and probably a precursor product pair. The band at the top of the gel was referred to as titin-1 ($T_1M_r = 1.4 \times 10^6$), and the second band titin-2 ($T_2M_r = 1.2 \times 10^6$). Recently Kurzban and Wang (1988) carried out studies to more accurately determine the molecular weight of titin, and they reported that the average molecular weight of titin polypeptides is 2.4 to 2.6×10^6. The third band, originally called band 3 (Wang et al., 1979), was purified and found to have a molecular weight of 5.5×10^5 (Wang and Williamson, 1980). By using immunofluorescent localization techniques, it was further shown to be associated with the N_2 line of the sarcomere. Consequently, band 3 was named nebulin for the nebulous structure of the I-band (Wang and Williamson, 1980). The localization of the N_2-line as a transverse structure in the I-band, where the thin-filament array changes from a square (tetragonal) lattice at the Z-line to a hexagonal pattern near the A/I-band junction, suggested that the N_2-line regulated thin-filament geometry (Franzini-Armstrong, 1970). Wang and Williamson (1980) proposed that nebulin and the N_2-lines were attached directly or indirectly to elastic titin filaments within the myofibril. Interestingly, Locker and Wild (1984) have concluded there are at least seven N-lines: an N_1 line always near the Z-line, four N_2-lines in the mid I-band and two N_3-lines at the extremity of the I-filaments or in the "gap." Seemingly, the history of the N lines has been shrouded also in doubt and mystery.

Titin and nebulin have been located in the myofibril with monospecific antibodies (Wang et al., 1979; Wang and Williamson, 1980). Wang et al. (1979) observed that titin antibodies labeled a wide zone at the A-/I-band junction and also at the Z-line, M-line and, possibly, throughout the entire A-band. Wang et al. (1979) concluded that transverse structures (M-line and Z-line) contained titin, that the titin-containing structure was attached to the ends of the thick filaments and that titin had variable affinity to antibody labeling, depending on sarcomere length. LaSalle et al. (1983) examined titin localization with polyclonal antibodies to bovine skeletal muscle titin in isolated skeletal myofibrils. Immunofluorescence labeling showed intense labeling in the region of the A-/I-band junction with some labeling extending into the A-band.

Not until Wang et al. (1984) purified the leading band, titin-2 (T_2), of the titin doublet in native form by extraction with Guba-Straub solution followed by chromatography, was scientific information available to provide some accurate detail to the role of titin in the elastic filament of the myofibril. It was observed that T_2 chains self-assembled into extremely long (from 0.1 μm to more than 1.0μm) flexible and extensible slender strands (4-5 nm diameter) with axial periodicity. Furthermore, these strands tended to form filamentous bun-

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dles and meshworks. Independently, Trinick et al. (1984) reported results similar to those of Wang et al. (1984).

The name and the nature of the protein making up the third filament, however, has not been without controversy. Maruyama (1976) isolated a highly insoluble intracellular elastic protein by extracting actin, myosin and some regulatory proteins from myofibrils; then, the remaining insoluble residue was subjected to 0.1N NaOH or 6M urea and 1% SDS and finally treated with hot phenol. This heterogeneous and insoluble residue was called connectin. A more accurate term, however, would be connectin-residue. Furthermore, Maruyama (1976) and Maruyama et al. (1977) proposed that this elastic protein was responsible for structural continuity and tension transmission in skeletal muscles. Maruyama et al. (1977) reported that connectin constituted about 5% of the total myofibrillar proteins in rabbit *psoas* muscle and that it contained about 5% lipids and 1% sugars. King and Kurth (1980) concluded that connectin was a heterogeneous mixture of several proteins, including actin and collagen.

Maruyama et al. (1977) observed that when the SDS-soluble portion of connectin was subjected to SDS-PAGE, a large portion of connectin did not penetrate into a 10% gel, indicating that it was an extremely large protein. Maruyama et al. (1980) slowly electrophoresed connectin on 2.5% SDS-gels and reported a mobility similar to that of a tetramer of myosin heavy chains, suggesting a molecular weight of approximately 800,000 daltons. In addition, they found, by using immunofluorescence to localize connectin in myofibrils, that staining occurred strongly in the A-band regions and on the Z-line and, weakly, in the I-band. Thus, connectin was located throughout the sarcomere. Subsequently, Maruyama et al. (1981), by using more refined techniques, discovered that two forms of connectin exist, an alpha- and a beta-connectin, and that these two forms of connectin were identical to the T_1 and T_2 forms of titin. Consequently, Maruyama and coworkers concluded that titin and connectin are one and the same. Evidently, purified connectin is titin, and depending upon one's school of thought, the protein of the third filament can be called connectin or titin. Native beta connectin has been purified by Kimura and Maruyama (1983), and the molecular weights of connectin have been estimated at 2.8×10^6 and 2.1×10^6 for the alpha and beta forms, respectively.

Tremendous controversy has been also associated with the existence of a third set of filaments within the sarcomere. Indeed, it seemed that the early concept of a third filament was dead until R.H. Locker and coworkers revived the issue in the 1970's. It is generally accepted that the myofibril contains two distinct sets of parallel filaments, termed thick and thin filaments, which provide the interdigitated sliding-filament theory of muscle contraction as first proposed by Huxley and Hanson (1954). According to the sliding-filament theory, structural continuity between sarcomeres did not exist, and occurred only when actin and myosin were interacting during contraction. Experiments on continuity and extensibility, however, often suggested that there was a source of myofibrillar continuity and extensibility distinctly separate from ordinary actin-myosin crossbridging. Indeed, Huxley and Hanson (1954) demonstrated that myofibrils treated with various salt solutions to selectively remove thick or thin filaments did not fall apart but were instead held together and remained elastic. These observations were not easily explained by the two-filament model of the sarcomere, and, as a result, several authorities have recommended a three-filament model in which the additional filaments would function as an elastic element providing continuity between sarcomeres.

In an early three-filament model, Hanson and Huxley (1956) proposed that fine, elastic filaments joined the ends of the thin filaments within a sarcomere. These were named S-filaments because of their ability to stretch. Some doubts about this proposal were expressed (Huxley 1962) because thin filaments were observed to overlap in highly contracted sarcomeres, and thus this overlap would not accommodate the S-filaments. Reports on fine filaments spanning the nonoverlapped regions of highly stretched sarcomeres were published also in the early 1960's (Huxley and Peachy, 1961; Carlsen et al., 1961). These were named "gap filaments' by Sjostrand (1962), who also proposed that the ends of the thick and thin filaments were linked by these structures. The thickness of the gap filaments varied, measuring 30 Å more or less, which was thinner than the 70 Å of the I-band filaments.

Previous results seemed to only make the issue of gap filaments even more controversial and mysterious. One of the more convincing bodies of evidence for the existence of a third filament, however, was the work of Locker and coworkers who described gap filaments in highly stretched beef *sternomandibularis* muscle (Locker and Leet, 1975, 1976 a, b; Locker et al., 1977; Locker and Daines, 1980). These workers noted that, when the *sternomandibularis* muscle was stretched to five times its original pre-rigor length, gaps would be formed between the A- and I-bands of the sarcomere. Within this gap were very fine filaments, termed gap filaments, that appeared to be associated in some way with the thick filaments. During stretching, the A-band became broader due to stretching and dislocation of thick filaments, one half sliding in one direction and the other half sliding in another. No changes were noted in the I-band region. Locker and Leet (1975) explained these observations by postulating that the gap filaments secured the thick filaments only to one end of the sarcomere in an alternating fashion. During an excessive stretch, the gap filaments would bear most of the force, and the thick filaments would be dislocated from their original position in the A-band.

In a model proposed by Locker and Leet (1976a), based on their microscopic observations, gap filaments were described as a core to each thick filament, coming out only at one end and running through the Z-line, then ending as a core to another thick filament in the adjoining sarcomere. Also, this model was based on previous observations demonstrating that myofibrils remained elastic and were able to return to their original length after stretching, even after extraction of actin and myosin and disorganization of the thick and thin filaments. On the basis of this model, gap filaments formed the elastic component of muscle that provided continuity and elasticity of structure between sarcomeres.

Upon scrutiny, the Locker model came under severe criticism, especially for its simplistic approach to the structure and arrangement of the gap filaments within the sarcomere. Indeed, Locker (1987) recently proposed a new model for the

gap or third filament of muscle, this one being very complex, and renamed the third filaments T-filaments (T for ultrathin and titin). He proposed that gap filaments consist of six T-filaments positioned longitudinally on the surface of the A-filament. C-protein molecules overlie the T-filaments in transverse and axial rows, with their long axes following a helix. Each C-protein molecule binds a pair of T-filaments to one A-strand. Gap filaments arise from the coalescence of T-filaments in the I-band, and their elasticity comes from an unravelling of a beaded structure in the titin molecule. The elasticity of the beaded structure is evidently based on the report by Trinick (1981), who discovered structures of about 850 Å in length and a periodicity of 42 Å at the ends of thick filaments, termed end filaments.

Wang et al. (1984) and Wang and Williamson (1980) have suggested that titin and nebulin are the components of a third set of filaments providing continuity and support for thick and thin filaments within the sarcomere. Filaments of the proposed third set are continuous, connecting Z-line to Z-line and are elastic along their entire length, except where they interact with inelastic structures such as the thick filament. In addition, this titin-nebulin lattice may also have a role in the assembly and turnover of the thick and thin filaments. Indeed, Wang et al. (1984) reported that titin is ideally suited as a component of an elastic lattice forming a cytoskeleton that serves as an organizing scaffold or template for thick and thin filaments. In Wang's (1984) proposed endosarcomeric model, titin forms a helical strand around the thick filament and attaches to nebulin in the area of the N_2 line, and a longitudinal strand continues inward to the Z-line where it either connects to, or passes through, the Z-line, thus forming an elastic connection between the A- and I-bands. Although Wang's model is well designed, a number of unexplained parts remain. For example, the nature and composition of the strand extending from the N_2 to the Z-line, the connection, if any, of the strand in the Z-line and the organization of the strand(s) in the A filament region are not clearly understood.

Maruyama et al. (1980) proposed a model showing that connectin formed three-dimensional elastic nets that surrounded the entire sarcomere between the Z-lines. In a more recent model, however, Maruyama (1985) proposed that connectin nets were linked to other elastic nets, with nebulin being a possible protein candidate making up the other elastic nets and that then these nets were further connected to some other unknown elastic filaments attached to Z-lines to surround the ends of the thick filaments. His more recent model, although somewhat refined compared with his early one, seems to be based on observations similar to those of the Wang model.

What does an elastic third set of filaments and their proteins have to do with meat science? There may be three areas of significance. One is meat tenderness, another is water-holding capacity, and the third one is the growth and development (myofibrillogenesis) of skeletal muscle tissue.

The search for the mechanism of meat tenderization has been very productive in recent years. Locker's (1982) theory of meat tenderness invokes the gap filament as the major controlling factor involved in tenderness. He has proposed that gap filaments determine the tensile strength of the myofibril in both the raw and cooked state and that, therefore, they are important in meat tenderness. In our view, his results extend our earlier observations on myofibril fragmentation tenderness in which beef steaks with myofibrils fragmented at or near the Z-line and a 30,000 dalton component were tender (MacBride and Parrish, 1977). On the basis of our results, we believe tenderization occurs by the proteolytic action of calcium-activated factor (CAF), the natural protease in skeletal muscle (Olson et al., 1977; Parrish, 1977; Goll et al., 1983). Perhaps titin and nebulin in gap filaments are proteolytically degraded in postmortem aged beef, and as a consequence, beef steaks become more tender. Lusby et al. (1983) showed that both titin and nebulin exist in beef *longissimus* muscle and that these proteins are degraded during postmortem storage. Zeece et al. (1986) reported that titin and nebulin are highly susceptible to proteolytic degradation by CAF. Paterson and Parrish (1986) presented evidence to show that titin and nebulin in tender beef muscles were more highly degraded than titin and nebulin in tough beef muscles. Furthermore, the degradation of connectin has been proposed as a mechanism by which meat tenderness improves during postmortem storage (Takahashi and Saito, 1979; Young et al., 1980). These results indicate that titin (connectin) and nebulin have important roles in meat tenderness. Many researchable questions still exist in explaining tenderness on the basis of gap filaments; consequently, further research on the molecular explanation of meat tenderness promises to be productive.

The ability of fresh and processed meat products to bind inherent and added moisture is extremely important to product quality. Offer and Trinick (1983) have shown that water-holding capacity was improved when transverse structural constraint (cross bridges, M-line and Z-line) in the myofibril were removed at certain salt and phosphate concentrations, allowing the filament lattice to expand. Paterson et al. (1988) extended these observations by showing that the removal of titin and nebulin by salt and pyrophosphate extraction improved water-holding capacity and increased swelling of the myofibril. Hence, removal of these structural constraints (gap filaments) may play a very important role in improved water-holding capacity. Many challenging projects are in store for the innovative and ingenious researcher in the area of water-holding capacity.

Also, the phenomenon of myofibrillogenesis remains a very challenging area of research. It has been proposed that titin and nebulin play a scaffolding or template role for subsequent thick-filament alignment into A-bands, or thin-filament alignment into I-bands, and Z-line assembly and insertion of thin filaments into Z-lines. Much effort will be required to determine with certainty the role of titin in the assembly and organization of thick and thin filaments. Consequently, much opportunity exists for research in meat animal growth and development.

In summary, titin may serve as a myofibrillar/cytoskeletal protein in the third set of filaments in a:

(1) cytoskeletal role to stabilize and anchor thick and thin filaments,

(2) mechanical role to provide resting tension within the sarcomere,

(3) regulatory role for the assembly and turnover of myofibrillar/cytoskeletal proteins,

(4) contractile role to facilitate transition from

tetragonal arrangement of thin filaments to the hexagonal arrangement of thick and thin filaments,
(5) tenderization role as a consequence of proteolytic degradation of proteins in the gap filament,
(6) water-holding-capacity role by salt solubilization of structural constraints providing greater space for holding water, and
(7) myofibrillogenesis role in the assembly and arrangement of thin and thick filaments.

Five excellent reviews of the myofibrillar/cytoskeletal proteins and their possible role in living muscle and meat are recommended. They are: Robson and Huiatt (1983), Locker (1984), Wang (1984), and Maruyama (1985, 1986).

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Discussion

Session One

J. Price: Dr. Parrish, you said that an unknown protein hooks into the Z-line. Is that also susceptible to proteolytic degradation?

F. Parrish: I would say it is really unknown at this time. It seems that we know that titin and nebulin are very susceptible to proteolysis but we are not really sure what protein connects them into the Z-line.

Price: There is another reciprocation session going on right now where they are talking about whether or not we have an emulsion or a protein gel. Since titin and nebulin are soluble with phosphate, is it very possible that these proteins could be involved in gel or structure formation in processed meats?

B. Paterson: We have studied this question using beef tissue treated with certain quantities of salt and phosphate. We then examined the tissue with phase-contrast microscopy and SDS-gel electrophoresis to see what myofibrillar proteins were affected by these treatments. Titin was definitely extracted from the beef muscle in the presence of certain concentrations of salt or lower concentrations of salt when pyrophosphate was also present. So although we did not study actual emulsions, I believe it is a possibility that titin and nebulin play an important role in emulsion and gel formation and stability.

T. Gillett: Is titin a very heat-sensitive protein?

Parrish: We have worked with it under cooking temperatures of rare, medium and well done. We would then isolate myofibrils and examine them with SDS-gel electrophoresis. We did not find titin on the gels, but of course that raises the question: Is it denatured to the extent that it will not go into the gel or does heating actually cause it to dissolve? This is something that we are still evaluating. I believe that some Australian researchers have indicated that it is heat-sensitive and it will break down by heating. Also Dr. Locker reported some work which showed that when meat was cooked to 80°C and examined with the microscope, no gap filaments were present. At what point and how heat-sensitive it is, I would say is still unknown.

Gillett: But not as much as myosin?

Parrish: No, I do not think so. There is some titin still present on SDS-gels from tissue that has been heated to 60° internal and then it starts disappearing from the gels as the temperature increases at 10-degree increments.

B. Marsh: If titin is heat-sensitive at temperatures no higher than that at which meat is normally cooked, how can we say that it's got anything to do with tenderness? It seems that if we are going to destroy titin before we finish cooking the meat, I don't quite see its connection with tenderness.

Parrish: I do not know that you have to completely destroy the titin to have tender meat. In studies that we have done with tender meat, there is still T-2 present in the SDS-gels. I do not believe that our knowledge is solid enough to make a clear-cut statement as to what is happening to titin or the gap filaments during cooking. We have to also realize that meat usually is not cooked when it is stretched 2 to 5 times over its normal length. We are talking about meat usually at rest length, and maybe in some cases shorter than rest length, and the question then is: What are the protective effects of the other proteins on titin or the protective effects of the thick and thin filaments on the gap filaments? These are questions that we still need to answer.

T. Bidner: Fred, you gave the different titin/nebulin models that have been proposed but you did not really comment on which one you thought was most accurate. Would you care to share your opinion on that?

Parrish: I am a proponent of the Wang model. I believe that his model most represents current research evidence. However, I do know that he is still refining his model and the other researchers are also refining their models as more research is conducted. We are far from seeing the final model.

Session Two

C. Calkins: Dr. Parrish, you indicated that in order to solubilize titin, it required detergents such as SDS. Later you said something about salt and phosphate being involved with the solubilization of titin. Can you resolve that for us?

Parrish: First of all, I think we have come a long way in trying to understand something about the molecule and its interaction with the other proteins. Very definitely when Wang tried to purify titin, it was insoluble at .3M NaCl. I am not quite sure how that differs from our research, other than the fact that we treated myofibrils much like processed meat would be. The meat was aged two days before we isolated myofibrils and added to them various solutions of sodium chloride and pyrophosphate. So perhaps within that two-day aging period, proteolysis occurred to allow some solubilization of titin and nebulin in the presence of NaCl and pyrophosphate.