

Role of Proteinases and Protein Turnover in Muscle Growth and Meat Quality

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Role of Proteinases in Meat Quality

Even a cursory review of the role of proteases in both muscle growth and in meat quality would require a lengthy treatise that could easily become too discursive to be either informative or easily understood. Besides, the role of proteases in meat quality (i.e., meat tenderness) has been clarified significantly during the last 15 years. A large amount of evidence has accumulated to indicate that the Ca^{2+} -dependent proteinases (hereafter, called the calpains; μ -calpain for the proteinase requiring micromolar Ca^{2+} concentrations for activity and m-calpain for the proteinase requiring millimolar Ca^{2+} concentrations for activity) are responsible for most, if not indeed practically all, of the tenderization that occurs during postmortem storage of muscle at 2° to 4°C (Table 1). This evidence was reviewed a number of years ago by Goll et al. (1983), and the more recent studies in this area have been summarized in an excellent review by Koochmaraie (1991). Perhaps the most compelling evidence indicating that the calpains are responsible for postmortem tenderization is the unvarying finding that very little proteolysis occurs in muscle during postmortem storage; functional actomyosin can be prepared from muscle after 13 days of postmortem storage at 2° to 4°C, and electron micrographs show that muscle retains its characteristic A- and I-band structure after long periods of postmortem storage (Table 1). Consequently, the proteases involved in postmortem tenderization must be very specific and limited in their ability to degrade muscle proteins. The calpains are unique among the known proteases in that they do not degrade actin, myosin, or α -actinin (Goll et al., 1991a). SDS-PAGE of postmortem muscle samples have consistently shown that neither actin, myosin, nor α -actinin are degraded during postmortem storage (see Bandman and Zdanis, 1988, as an example of one of these studies). It has been well documented that skeletal muscle Z-disks are degraded to varying degrees during postmortem storage (Table 1 and Henderson et al., 1970), and it is surprising, therefore, that α -actinin, a major Z-disk protein, is not degraded during

postmortem Z-disk disintegration. The cathepsins are very active proteases that, in addition to degrading Z-disks, rapidly degrade myosin, actin, and α -actinin (Goll et al., 1989). These electron microscope and SDS-PAGE studies, therefore, clearly show that, contrary to the concept commonly accepted 10 to 15 years ago, the cathepsins have little or no effect on the myofibrils and myofibrillar proteins in muscle during postmortem storage at 2° to 4°C. The changes observed in myofibrils during postmortem storage, however, are virtually identical to those caused by treatment with the calpains, and it is sagacious to conclude that 90% or more of the tenderization that occurs during postmortem storage at 2° to 4°C can be attributed to the calpains (Table 1).

Postmortem storage at 25°C or higher results in significant degradation of myosin, actin and α -actinin, and it seems likely that postmortem storage at these higher temperatures results in catheptic degradation of the myofibrillar proteins. Storage at these higher temperatures also results in a soft or "mushy" texture, and it is probable that degradation of myosin and/or actin in postmortem muscle causes a mushy tenderness, such as results from over tenderization with meat tenderizers containing papain, which also degrades myosin and actin.

Role of Muscle Protein Degradation in Muscle Growth

Three factors ultimately determine the rate at which skeletal muscle mass accumulates in living animals (Table 2). Although a large number of treatments or substances, such as nutrition, hormone administration, proper management practices, etc., affect the rate of muscle growth, these treatments or substances exert their effects by acting on one of the three factors listed in Table 2. For example, certain growth factors such as FGF may during embryogenesis alter the number of cells that ultimately constitute the mature muscle. Hormones may affect the rate of muscle growth because they alter the rates of muscle protein synthesis or the rate of muscle protein degradation (e.g., the β -agonists) or both (e.g., insulin).

Most of the effort in attempting to increase the rate of muscle growth in domestic animals has involved studies on the number and type of muscle cells or attempts to increase the rate of muscle protein synthesis by using genetic selection or improvements in nutrition and management practices. Little attention has been given to devising ways to improve the rate of muscle growth by decreasing the rate of muscle protein degradation. This lack of attention to muscle protein degradation is probably due to a large extent because: 1) it has been and remains difficult experimentally to measure the rate of muscle protein degradation in living animals; and 2)

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Abbreviations used are: μ -calpain, the micromolar Ca^{2+} -requiring, Ca^{2+} -dependent proteinase; m-calpain, the millimolar Ca^{2+} -requiring, Ca^{2+} -dependent proteinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1. Some of the Evidence Indicating That the Calpain System Has the Primary Role in Normal Postmortem Tenderization.

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- A. Very little degradation of myosin and actin occurs during postmortem storage at 2°-4°C.
1. The calpains are unique among the known proteolytic enzymes in that they do not degrade myosin and actin.
 - a. The known cathepsins all degrade myosin and actin.
 2. Degradation of myosin leads to a "mushy" texture.
- B. Very little degradation of α -actinin, a major Z-disk protein, occurs during postmortem storage at 2°-4°C.
1. Although several of the cathepsins (and many other proteases) degrade the Z-disk structure, this degradation is accompanied by degradation of α -actinin; the calpains are unique in that they degrade the Z-disk structure, but they *release* α -actinin without degrading it.
 2. Degradation of myosin, actin, and α -actinin occurs during postmortem storage at 37°C, and this degradation probably is due to the cathepsins.
- C. There is very little proteolysis of muscle during postmortem storage at 2°-4°C.
1. The sarcomeric structure of muscle remains largely intact, with the exception of a variable degree of Z-disk degradation.
 2. Functional actomyosin and α -actinin can be isolated from muscle even after 13 days of postmortem storage.
 3. Many studies have failed to find significant increases in free amino acids, even after very long periods of postmortem storage.
 - a. The cathepsins cause extensive degradation of proteins.
- D. Several studies have shown that increasing Ca^{2+} in postmortem muscle results in increased tenderness.
- E. A number of studies have indicated that tenderness increases to a greater extent during postmortem storage in muscles that have increased calpain (especially μ -calpain) and decreased calpastatin activities than in muscles that have lower calpain or higher calpastatin activities.
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Table 2. Three Factors That Control Rate of Muscle Growth.

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- A. Number and type of muscle cells
1. The number of muscle cells is determined prenatally during embryonic development in domestic animals (Stromer et al., 1974); the greater the number of muscle cells, the greater the muscling potential.
 2. Three physiological types of muscle cells exist: 1) fast-twitch, glycolytic (FG); 2) fast-twitch, oxidative-glycolytic (FOG); and 3) slow-twitch, oxidative (SO); SO muscle cells are generally smaller than the other two.
- B. Rate of muscle protein synthesis
1. Sarcoplasmic proteins—30%-35% of total proteins; intracellular.
 2. Myofibrillar proteins—50%-55% of total protein; intracellular.
 3. Stroma proteins—15%-20% of total protein; over 50% of the stroma protein fraction is extracellular.
- C. Rate of muscle protein degradation
1. Sarcoplasmic proteins—probably degraded by lysosomal and the multicatalytic proteases (MCP; macropain).
 2. Myofibrillar proteins—highly organized structure places constraints on mechanism of turnover.
 3. Stroma proteins—Extracellular proteases, many of them are probably metalloproteases.
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very little is known about the mechanism of intracellular protein degradation and how this process is regulated in cells. Nevertheless, it is unfortunate that rate of muscle protein degradation has not received more attention in growth studies because decreasing rate of muscle protein degradation would produce two direct beneficial effects: 1) it would increase the rate of muscle protein accumulation; and 2) it would reduce the amount of feed (energy) required per

unit of gain because less feed would be needed to replace degraded muscle protein (i.e., for the animal to just "stay even"). Because it would reduce the amount of feed required to replace degraded muscle protein, a decrease in the rate of muscle protein degradation would directly increase the efficiency with which ingested nutrients are converted into edible muscle. Increasing the number of muscle cells or the rate at which muscle protein is synthesized would not directly

Table 3. Potential Effect of Muscle Protein Turnover on Rate of Growth in Swine.

100 kg (220 lb) animal	Gaining 0.8 kg (1.76 lb)/day
Approximate carcass weight - 70 kg (70%)	Adding 0.56 kg carcass/day
Approximate muscle weight - 31.5 kg (45%)	Adding 0.252 kg muscle/day
Approximate protein content - 6.3 kg (20%)	Adding 0.0504 kg protein/day
If this animal turns over muscle protein at the rate of 4.0% per day, which is average for swine (Mulvaney et al., 1985), then it would degrade $0.04 \times 6.3 = 0.252$ kg of muscle protein per day. This animal would need to synthesize $0.252 + 0.0504 = 0.3024$ kg of muscle protein per day to gain 0.8 kg live weight/day.	
If the rate of muscle protein turnover in this animal were lowered by 10% to 3.6% per day, then only $0.036 \times 6.3 = 0.2268$ kg of muscle protein would be degraded each day. If this animal continued to synthesize protein at the rate of 0.3024 kg/day, it would now be adding $0.3024 - 0.2268 = 0.0756$ kg protein/day net, and this would result in a live weight gain of $0.0756/0.2 \times 0.45 \times 0.7 = 1.2$ kg (2.64 lb)/day (50% increase), assuming constant body composition.	

increase feed efficiency, although it may reduce management costs because animals would be fed for shorter periods of time.

Altering the rate of muscle protein degradation can have enormous effects on rate of muscle growth. Table 3 shows that reducing rate of muscle protein degradation in a market-size pig by only 10% would increase the rate of live-weight gain in that animal by 50%, if rate of muscle protein synthesis and body composition remained unchanged. This increase in rate of gain would occur at the same (probably less) rate of feed consumption because rate of muscle protein synthesis is assumed to remain constant. Similar calculations can be made for other species (see Goll et al., 1989).

The question arises as to whether rate of muscle protein degradation can be changed by 10% (or by any amount) without causing detrimental physiological changes in the animal. The answer seems to be, yes. Measurements done thus far indicate that rate of muscle protein degradation varies significantly among different animals of the same species and that, in many instances, treatments designed to alter the rate of muscle growth are mediated primarily by altering the rate of muscle protein degradation with little or no effect on rate of muscle protein synthesis. For example,

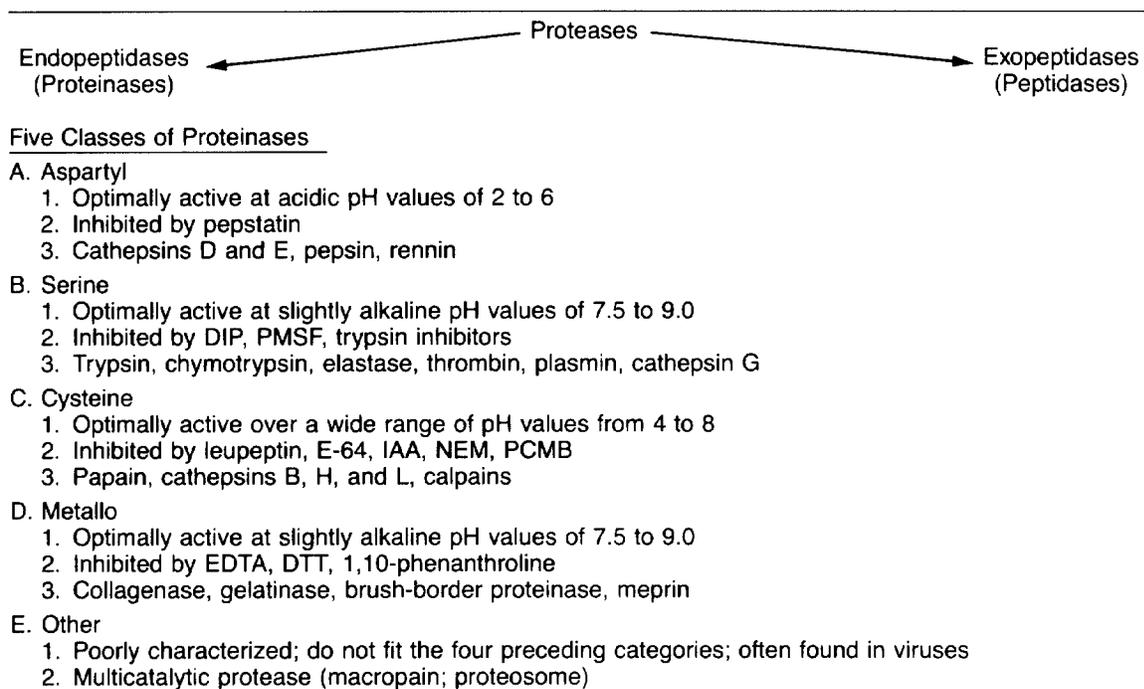
reducing the rate of growth in chicken breast muscle by lowering the amino acid level, the lysine content or the total energy content of rations was accompanied by a marked increase in the rate of muscle protein degradation with little or no change in rate of muscle protein synthesis (Table 4). Similarly, denervation of rat soleus muscle resulted in muscle atrophy (-6.6% fractional growth rate) because of a large increase in rate of muscle protein degradation with little change (or a slight increase) in rate of muscle protein synthesis (Table 4). The increase in soleus muscle mass caused by overloading was due to an increase in rate of muscle protein synthesis, whereas rate of muscle protein degradation also increased slightly (Table 4). It has often been observed that an increase rate of muscle growth is accompanied by an increase both in rate of muscle protein synthesis and in rate of muscle protein degradation (see Millward et al., 1978 for a review). This finding illustrates the importance that muscle protein degradation has in muscle growth; if this increase in rate of muscle protein degradation during rapid growth could be prevented, growth rates would increase by 30% to 50% without actually reducing rate of muscle protein degradation below its "basal" level.

A number of studies (reviewed in Goll et al., 1989) have

Table 4. Role of Muscle Protein Degradation in Rate of Muscle Growth.

<i>Treatment</i>	<i>Fractional Rate of Muscle Protein Synthesis %</i>	<i>Fractional Rate of Muscle Protein Degradation %</i>	<i>Fractional Rate of Muscle Growth %</i>
In chicken breast muscle, Maruyama et al. (1978)			
Control	25.4	7.5	10.3
Control	25.6	9.9	8.0
75% of control amino acid level	26.8	13.8	6.4
75% of control amino acid plus 50% of control Lys	26.1	14.2	5.1
50% of control energy	17.6	15.1	1.6
35% of control Lys	17.6	16.4	0.7
50% of control Lys	24.3	22.0	0.6
In rat soleus muscle, Goldspink et al. (1983)			
Control	17.4	8.5	8.9
Control	19.6	9.9	9.7
3-day overloaded and 3-day denervated	20.9	27.4	-6.6
3-day overloaded	29.9	12.4	17.5

Figure 1
Classes of Proteolytic Enzymes



Schematic diagram summarizing the recommended nomenclature for proteases and the properties of the five classes of proteolytic enzymes.

shown that an increased rate of muscle growth in domestic animals occurs in many instances because the rate of muscle protein degradation has decreased while the rate of muscle protein synthesis has remained constant. Therefore, the current evidence suggests that genetic selection for rapid growth may unwittingly have been selection for those animals that degrade muscle protein less rapidly, and *not* for those animals that synthesize muscle protein more rapidly.

Proteolytic Enzymes Involved in Intracellular Protein Turnover

Because of the important role that muscle protein degradation has in rate of muscle growth, it is useful to examine what is known about the mechanism of muscle protein degradation and how it is regulated. Intracellular protein turnover clearly is mediated by proteolytic enzymes. Fig. 1 summarizes the properties of the five classes of proteinases and the recommended nomenclature for proteolytic enzymes. Protease is a generic term that refers to all proteolytic enzymes. Exopeptidases or peptidases refers to that class of proteases that specifically cleave one (or two or three) amino acids from the N- or C-terminus of a polypeptide chain (e.g., the carboxypeptidases, leucine aminopeptidase, cathepsin C, etc.). Endopeptidases (proteinases) refers to that group of proteases that cleave peptide bonds in the interior of a polypeptide chain (e.g., trypsin, chymotrypsin, cathepsin L, etc.). There are five classes of proteases depending on the nature of the amino acid side chain at their active site (Fig. 1).

These five classes of proteases are almost always distinguished experimentally by determining which type of compound inhibits their proteolytic activity (Fig. 1). For example, if a new proteolytic activity is detected, it is incubated with pepstatin, phenylmethylsulfonyl fluoride (PMSF), E-64, or 1,10-phenanthroline (or one or more of the other inhibitors listed in Fig. 1) to learn what kind (class) of protease is responsible for the activity. If the activity is inhibited, for example, by E-64 but not by the other inhibitors, this activity is probably due to a cysteine proteinase, etc. These tests can be done rapidly and on impure preparations and therefore are routinely used to identify the classification of a new proteolytic activity.

As indicated earlier, very little is known about the mechanism of intracellular protein turnover and how this mechanism is regulated. The current evidence indicates that at least three types of proteolytic systems are involved in intracellular protein turnover (Table 5). The lysosomal system has been studied for a number of years and the biochemical properties of the proteases belonging to this system are well characterized. It is still unclear, however, how activity of the lysosomal systems is regulated and the role this system has in degradation of proteins endogenous to the cell in which it is located. Numerous studies have shown that the lysosomal system is responsible for degrading those proteins that are endocytosed by the cell, and it is clear that once a protein enters a lysosome, it is doomed to degradation. Hence, control must occur at the level of entry of the protein to the lysosome. Muscle cells endocytose very few proteins and have a relatively low content of lysosomal enzymes. Several studies

(see Goll et al., 1989) have indicated that approximately 25% to 30% of total intracellular protein turnover in muscle cells can be attributed to lysosomes. This is approximately the percentage of total skeletal muscle protein that is constituted by the sarcoplasmic proteins. It is very unlikely that intracellular degradation of myofibrils could be initiated by lysosomal enzymes (see Goll et al., 1989; 1991b).

The error-eliminating system has been extensively studied, but the nature of the proteolytic enzymes involved in this system is completely unknown (Table 5). The error-eliminating proteases are probably located in the cell cytoplasm, and the available evidence indicates that they require hydrolysis of ATP and the presence of ubiquitin for activity. This system seems to be specific for proteins containing errors of translation (or other defects, such as oxidation). The system is studied by introducing radiolabeled amino acid analogs into proteins and monitoring rate of degradation of the abnormal proteins containing these analogs. This degradation occurs rapidly, a feature that would be advantageous for cells. Evidently, control of this system occurs at the level of recognition by the ubiquitin system, because once a protein containing an amino acid analog is tagged by ubiquitin, it is immediately degraded to amino acids, and it has been impossible to slow or inhibit this degradation to a point that intermediates can be detected. Although the enzymes involved in ubiquitination are reasonably well characterized (some have been cloned and sequenced), the mechanism it uses to recognize abnormal proteins is a complete mystery.

The "black-box" system (Table 5) is so-named because both the nature of the proteases involved and the mechanism used to regulate their activity is unknown. Based on studies with different protease inhibitors (see Fig. 1), it seems that this system includes enzymes from several of the different classes (Table 5). This system is responsible for turnover of the majority of intracellular proteins in cells (approximately 75% to 80% of the proteins in skeletal muscle cells; probably less than this in other cell types) and is able to identify

different proteins selectively so some of them are turned over with half-lives of minutes, whereas other adjacently located proteins are turned over with half-lives of hours or days. The mechanism by which this selectivity is mediated is unknown. Because these proteases are located in the cell cytoplasm, they must be under strict regulation to prevent continuous and indiscriminate degradation of cellular proteins. It seems likely that the two calpains and the multicatalytic protease are important proteases in the "black-box" system. It is unclear whether they are the major proteases involved, whether they are part of a limited number of major proteases involved, or whether they are part of a large number of different proteases involved.

Although the cell cytoplasm contains a large number of different proteolytic enzymes, most of these proteases are associated with specific physiological functions such as the signal peptidase or insulinase, the protease that cleaves the newly synthesized insulin polypeptide into its mature form. These proteases often are found only in particular cells (e.g., the β cells in the islets of Langerhans in the pancreas) and have such restricted specificities that they could not be involved in general metabolic turnover of intracellular proteins. The calpains and the multicatalytic protease are the only proteases discovered thus far that seem to exist in all cells and are present in sufficient quantities that they could have a role in intracellular protein turnover. As indicated earlier, activities of proteases involved in cytoplasmic protein turnover must be under strict regulation, and it is possible that cells contain other proteases whose activity is blocked by an inhibitor or some other mechanism and is therefore undetectable in cell homogenates. Because the calpains and the multicatalytic protease are presently the only known proteases whose properties indicate that they could have a role in cytoplasmic protein turnover, and because these two proteolytic systems exist in skeletal muscle cells, their properties and potential roles in muscle protein turnover will be summarized briefly.

Table 5. Three Types of Proteolytic Systems Involved in Intracellular Protein Turnover.

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1. Lysosomes
 - a. Proteases are located in lysosomes and function at acidic pH (3 to 5).
 - b. Cathepsins are located in lysosomes.
 - c. Degrade endocytosed proteins, including some hormone receptors and exogenous proteins.
 - d. May cause bulk degradation of some cellular proteins but different protein half-lives would need to result from controlled uptake into the lysosome.
 - e. There is very little endocytosis in muscle cells, and muscle cells have few lysosomes.
 2. Error-Eliminating System (Cell Sanitation System)
 - a. Proteases are in the cell cytoplasm and are optimally active at pH 7.5-8.0.
 - b. Contains serine and cysteine proteases.
 - c. Specific for proteins containing errors of translation—very short half-lives.
 - d. Involves the ubiquitin system and requires ATP; nature of the protease(s) is unknown—may be the multicatalytic protease.
 3. The Black-Box System
 - a. Proteases are in the cell cytoplasm and are optimally active at pH 7 to 8.
 - b. Contains serine, cysteine, and perhaps some metalloproteinases.
 - c. Nature and number of proteases in this system are unknown; there may be many or only a few; they must be highly regulated.
 - d. The calpain system and the multicatalytic protease are two of proteases in this system (the major two?).
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Table 6. Some Properties of the Multicatalytic Protease (Macropain; Proteasome).

1. Found in all organisms from bacteria to man; amino acid sequences are highly conserved; found in large amounts probably in all tissues.
2. A very large complex (approximately 700 kDa) having a characteristic cylindrical shape with a hole in its center; composed of 13 *different* subunits ranging in molecular weight from 20-35-kDa.
3. Exists in a latent form that has very little activity; proteolytic activity is increased 10-fold or more by SDS, by polycations, and by some lipids; some investigators find that its activity is increased in ATP.
4. Has three kinds of proteolytic activities based on its ability to cleave synthetic substrates; a) trypsin-like; b) chymotrypsin-like; and c) peptidyl (general protease).
5. pH optimum is 7.0 (chymotrypsin), 8.0 (peptidyl), and 8.5 (trypsin).
6. It may form complexes with the ubiquitinating system and in this form may degrade ubiquitinated proteins.
7. Function and regulation are unknown; it may be the central protease responsible for nonlysosomal mediated protein turnover; it does not degrade myofibrils.

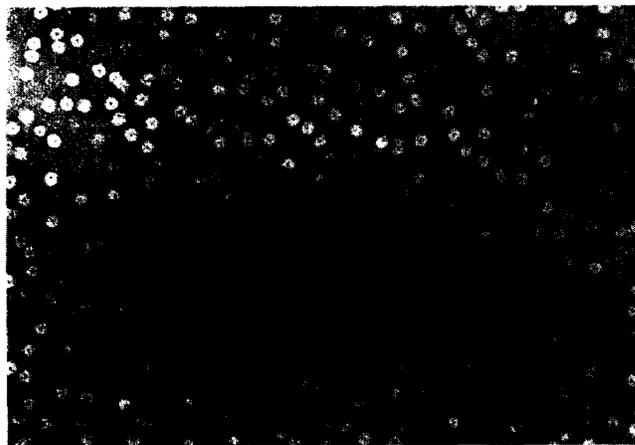
The Multicatalytic Protease (MCP)

Although the multicatalytic protease (MCP) was described in 1983 (see Goll et al., 1989 for a review; Wilk and Orlowski, 1983 for the original paper), it was not realized until late in the 1980's that a large number of different proteolytic activities identified by different investigators in different tissues all originated from the same enzyme (see Rivett, 1989, for a review). Some properties of this protease are summarized in Table 6. Failure to recognize that the proteolytic activities identified in different tissues originated from a common enzyme has led to a confusing nomenclature for the MCP (reviewed in Rivett, 1989). The protease was first named "multicatalytic protease" because it had three different proteolytic activities identified by its ability to hydrolyze three different synthetic substrates at different pH optima and different catalytic constants (Table 6). Other investigators have identified the same protease but have named it macropain, proteasome, ingensin, or ATP-stimulated protease. It has been recommended that the enzyme be named multi-catalytic protease, but the acceptance of this recommendation is still unclear.

The MCP is a large complex containing 13 different subunits (multiple numbers of at least some of them) ranging in molecular weight from 20- to 35-kDa (Table 6). It is most easily identified by its characteristic cylindrical shape in electron micrographs (Figs. 2 and 3). Five of the 13 subunits of the MCP have been cloned and sequenced; the sequences thus far have no homology to any known protein (including all known proteases), and it seems likely that the MCP represents a new class of proteolytic activity (see Fig. 1). The MCP is present in large amounts in all cells that have been examined for its presence, and it may be a major protease involved in intracellular protein turnover. It is difficult to detect the MCP in cells without actually isolating it and examining the purified preparation in the electron microscope, because it has almost no proteolytic activity in its "native" state. Incubation with SDS, certain unsaturated fatty acids, or certain polycations stimulates activity of the latent protease 10-fold or more. Obviously, proteolytic activity of the MCP must be under strict control *in vivo*, but the nature of this control is unknown; activation by SDS clearly is not a physiological mechanism.

Studies during the last year have suggested that the MCP may associate with the ubiquitinating system in cells to produce a very large complex with a molecular mass greater than 1,000-kDa. It has been difficult to isolate this complex in its intact form, but if it exists, its presence suggests that the MCP may be the protease involved in the error-eliminating system (Table 5). Other results have indicated that ubiquitin is involved in tagging normal as well as abnormal proteins for metabolic turnover and that the MCP has a role in ubiquitin-mediated turnover of normal cytosolic proteins (Table 5). The MCP, after activation, can also rapidly degrade ubiquitinated proteins, and some investigators believe that the MCP has a dual role in intracellular protein turnover; 1) degrading ubiquitinated proteins when it is associated with the ubiquitinating complex; and 2) degrading unubiquitinated proteins when it is not complexed with the ubiquitinating system. The MCP supposedly requires ATP hydrolysis (is activated by ATP) for activity when complexed with the ubiquitinating system but does not require ATP for activity when it is not complexed with the ubiquitinating system. This

Figure 2



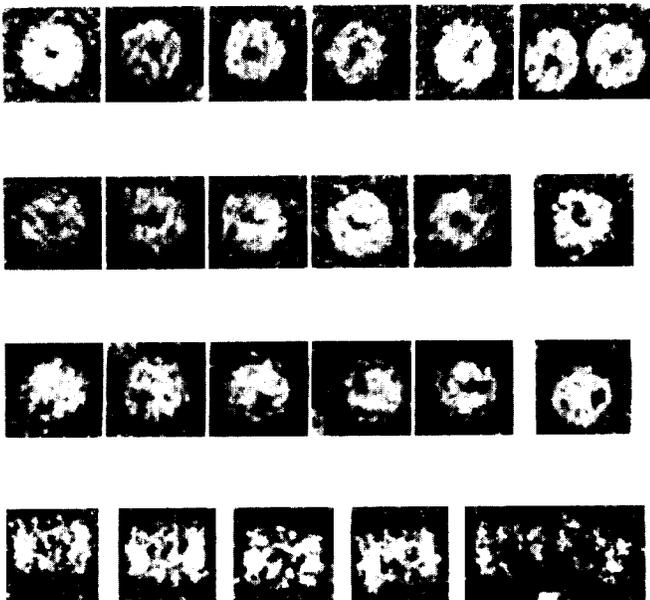
Electron micrograph of a preparation of the multicatalytic protease from rat liver. Most of the particles in the micrograph are seen end-on and show the small hole in the center of the MCP molecule. A few particles are seen from the side, showing their cylindrical structure. Bar is 1000 Å. Taken from Tanaka et al., (1988).

dual role would account for the conflicting results indicating that MCP activity is sometimes dependent on the presence of ATP and other times is unaffected by ATP. Clearly, a great deal remains to be learned about the MCP, how it functions in cells, and how its activity is regulated. Although it seems likely that the MCP has a major role in the "black-box" system of intracellular protein turnover, results thus far indicate that MCP has no effect on intact myofibrils. Consequently, metabolic turnover of myofibrillar proteins must involve some other proteolytic system, and the results summarized in the next section suggest that the calpain system is responsible for initiating myofibrillar protein turnover.

The Calpain System

The calpain system contains four known proteins: 1) μ -calpain, a proteinase that requires 5 to 50 μM Ca^{2+} for half-maximal activity; 2) m-calpain, a proteinase that requires 300 to 1000 μM Ca^{2+} for half-maximal activity; 3) a third proteinase identified in 1989 and still poorly characterized; it evidently requires 3000 to 4000 μM Ca^{2+} for half-maximal activity (Wolfe et al., 1989); and 4) calpastatin, a polypeptide that is specific for inhibiting the proteolytic activity of the three proteinases and that does not inhibit any other proteolytic enzyme with which it has been tested. Theoretical considerations indicate that the calpain system contains at least one more protein; this protein is not a protease but is an "activator" that is able in response to physiological demand to alter the Ca^{2+} concentration required for activity by μ - or m-calpain. The nature of this "activator" is unknown; it probably responds to Ca^{2+} fluxes in the nanomolar concentration range and may be a kinase, a phosphatase or a calmodulin-like molecule.

Figure 3

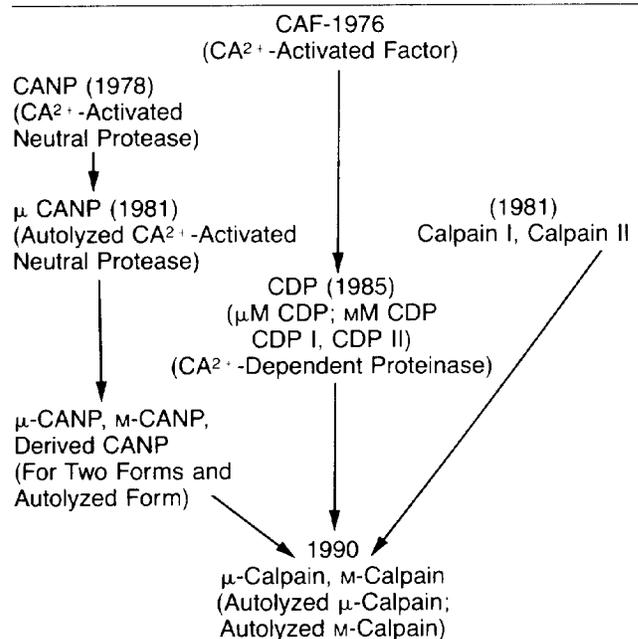


Electron micrographs of individual MCP molecules shown at higher magnifications. The top three rows show an end-on view of the MCP molecule, and the bottom row shows a side view of the cylindrical particles. Bar is 200 Å. Taken from Tanaka et al., (1988).

cDNAs for all four of the known proteins in the calpain system have been cloned and sequenced (see Suzuki, 1990; Maki et al., 1990), and the biochemical properties of three of the four proteins (excluding the third, high- Ca^{2+} -requiring proteinase) are well established. The μ - and m-calpain molecules each contain 80- and 28-kDa subunit polypeptides. The 80-kDa subunits of μ - and m-calpain are different polypeptides but share 50% to 60% amino acid sequence homology; the 28-kDa subunits are identical in μ - and m-calpain and originate from a single gene in humans. The amino acid sequence of the 80-kDa subunit of either μ - or m-calpain predicts four domains: 1) the sequence of the N-terminal domain containing approximately the first 90 amino acids has no sequence homology to any known protein; 2) the second domain, approximately from amino acid 90 to 300, contains a cysteine residue that evidently is the active site cysteine; 3) the sequence of the third domain approximately from amino acid 300 to 550 is not homologous to the sequence of any known protein; and 4) the sequence of the fourth domain approximately from amino acid 550 to 700 is homologous to the amino acid sequence of calmodulin and contains four sequences analogous to the E-F hand Ca^{2+} -binding sequences. All three proteinases in the calpain system are cysteine proteinases (Fig. 1), and the name, calpain, was devised from Ca^{2+} -dependent (analogous to calmodulin), cysteine protease (analogous to papain, a well-characterized cysteine proteinase). Although it has been reported that the amino acid sequence of domain II of the 80-kDa subunit (the domain having the active site cysteine) is homologous to the amino acid sequence of papain and other cysteine proteases such as cathepsin B, a closer comparison of these amino acid sequences shows that domain II of

Figure 4

Nomenclature of the Ca^{2+} -Dependent Proteinases



Schematic diagram summarizing the history of different names used for the Ca^{2+} -dependent proteinases.

calpain shares very little sequence homology with the other cysteine proteases; indeed, the calpains have probably evolved from an ancestral gene entirely different from the ancestral gene of the other cysteine proteases (Fig. 5).

Like the multicatalytic protease, the calpain system has had a variety of names (Fig. 6). The protease initially purified in 1976, m-calpain, was called CAF, for Ca^{2+} -activated factor because, before it had been characterized, it was not clear whether the Z-disk-remaining activity was due to a protease or to some other activity (see Goll et al., 1990 for a summary). Suzuki and his colleagues purified the same protein (m-calpain) from chicken skeletal muscle in 1978, and they named their enzyme CANP (Fig. 6). In 1981, our laboratory in Arizona and Dayton's laboratory in Minnesota independently purified a second Ca^{2+} -activated protease that required micromolar Ca^{2+} concentrations for activity (μ -calpain). The same year, Suzuki found that very brief autolysis reduced the Ca^{2+} concentration needed for activity of his calpain (m-calpain). Suzuki originally assumed that his autolyzed calpain and the new Dayton/Goll protease were the same, and he named his second, autolyzed protease μ -CANP (for micromolar CANP). It was soon learned, however, that there were two different Ca^{2+} -dependent proteases and that both of them would autolyze in the presence of Ca^{2+} . These proteases became known as CDP I, CDP II, or

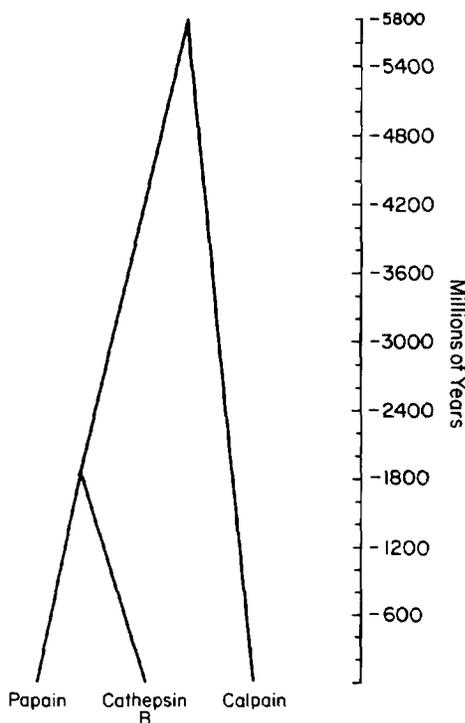
μ MCDP, mmCDP, μ -CANP, m-CANP, etc., depending on the laboratory. Although they did not purify the protease until later, Murachi and his colleagues in Kyoto proposed in 1981 that the two proteinases be named calpain I (for the micromolar form) and calpain II (for the millimolar form). This variety of names continued until 1988 when an effort was initiated to adopt a common nomenclature. Most investigators working in the area have now agreed to call the proteinases μ -calpain and m-calpain (or autolyzed μ -calpain and autolyzed m-calpain), and it is anticipated that this will be the adopted nomenclature for these proteinases (Fig. 6).

It was originally proposed in 1975 (Dayton et al., 1975) that the calpains acted to initiate metabolic turnover of the myofibrillar proteins by disassembling these proteins from the myofibril. Since that time, considerable circumstantial evidence has accumulated to support this original suggestion (see Goll et al., 1991b for a summary of this evidence). The calpains do not cause bulk degradation of the sarcoplasmic proteins (Goll et al., 1991b), so the calpains are *not* involved in metabolic turnover of the sarcoplasmic proteins. The calpains, however, do specifically degrade those structures and proteins that are responsible for maintaining the assembled myofibrillar proteins in the myofibril structure. The calpains remove Z-disks and degrade titin, nebulin, tropomyosin, troponin and C-protein. Titin and nebulin probably function as a scaffold that strengthens the myofibrillar structure, and Z-disks are necessary to keep adjacent sarcomeres together. Specific degradation of these structures would result in release of thick and thin filaments from the surface of the myofibril. Degradation of tropomyosin and troponin would facilitate disassembly of the released thin filaments to actin monomers, and degradation of C-protein would facilitate disassembly of the released thick filaments to myosin monomers. Because the calpains do not degrade actin or myosin monomers or α -actinin (released from the Z-disk), further degradation of these proteins to amino acids would require the action of at least one other protease (possibly MCP, which might also be involved in metabolic turnover of the sarcoplasmic proteins).

Consequently, it seems likely that metabolic turnover of the myofibrillar proteins, which constitute over half the total protein in skeletal muscle cells, occurs as follows: 1) thick and thin filaments are released from the surface of a myofibril by the specific action of the calpains; this release would progressively decrease the myofibril's diameter, a structural change characteristically observed in atrophying muscle; 2) the released filaments may either reassemble onto the surface of the myofibril (during periods of low myofibrillar protein turnover) or dissociate into monomers, either because of calpain-induced degradation of tropomyosin, troponin and C-protein or because of proteolysis by some other protease; and 3) the monomeric myofibrillar proteins are degraded to free amino acids, either by lysosomes, by the MCP, or by some other cytosolic protease or by all three of these routes (see Goll et al., 1989; 1991b, for schematic diagrams of this mechanism).

The mechanism outlined in the preceding paragraph accounts for many of the experimental observations made concerning metabolic turnover of the myofibrillar proteins. For example, rate of myofibrillar protein turnover (*not* rate of total muscle protein turnover which includes turnover of the

Figure 5



Schematic diagram showing time-scale of the predicted evolution of the genes encoding the cysteine proteases and the calpains. Based on a predicted rate of mutation, the genes for papain and cathepsin B evolved from a common ancestor approximately 1,800 million years ago. If the genes for the calpains and papain or cathepsin B had evolved from a common ancestor, this evolution would have started over 5,800 million years ago. Most estimates place the "big-bang" as occurring approximately 3,000 million years ago.

sarcoplasmic proteins and which is usually the experimental measurement made) to free amino acids sometimes is related to calpain activity and sometimes is not (degradation to free amino acids requires at least two proteolytic enzymes and release of free amino acids from myofibrillar proteins will be related to activity of the protease that catalyzes the rate-limiting step).

A number of recent studies have indicated that some of the β -agonists (e.g., cimaterol or clenbuterol) increase the rate of muscle growth in several species of domestic animals by decreasing the rate of muscle protein degradation with little or no effect on rate of muscle protein synthesis. Muscle from animals fed these β -agonists have significantly lower μ -calpain and significantly higher calpastatin activities than muscle from control animals. These findings are consistent with the concept that these β -agonists, through some unknown mechanism, are down-regulating activity of the calpain systems in skeletal muscle and that this down-regulation results in a significant increase in rate of muscle growth. Interestingly, muscle from animals fed these β -agonists also is less tender than muscle from control animals, again a result that would be predicted if β -agonist administration down-regulated muscle calpain activity. As described at the beginning of this paper, decreased calpain activity would

almost certainly result in less tenderization during postmortem storage of muscle. Consequently, a number of findings suggest that the calpain system has an important role in muscle growth in addition to its well-documented role as the primary cause of postmortem tenderization. Information on how activity of the calpain system is regulated in living cells and how this activity might be manipulated in living animals and in postmortem muscle would be vitally important to the animal sciences.

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Discussion

L. Quinn: Question for Steve. When you do experiments with Myo D or other regulatory proteins, is there a titration effect or is it just like a switch? The other question is, has anybody done an anti-sense experiment with already differentiated muscle cells to see if they stop synthesizing contractile protein or Myo D? Do they need the Myo D after they are differentiated?

S. Konieczny: The answer to the first question is that there is a linear relationship. It's a concentration effect, so the more Myo D you put in, the more activity you will get from a reporting gene or the more muscle you get in a cell culture. But then there is a certain point where you reach a maximum level, and it doesn't seem to matter how much more you put in. So there is a titration effect. When we do the experiment, you want to try to not use too much Myo D because of the titration effect. In answering your second question, all I can tell you is that our lab and a lot of other labs have been trying anti-sense experiments. I don't think anti-sense is going to work because there are too many of these factors, and you have to really target your tissue very specifically. Another way of trying to do that is to knock the gene out. We currently are attempting these experiments and are in the process of making new animals, such as mutant mice. Unfortunately, those experiments are just ongoing and we don't have any data or results to tell you about.

S. Smith: Question for any one of the speakers: We now know that we can get meat from certain breeds of cattle and that meat simply won't age no matter how long you hold it. If you argue for the role of nebulin or titin in tenderness, have you run across muscle like that and found that the protein does not degrade?

R. Robson: We actually have not run across any like that, although I think that if you talk to Elisabeth Huff and visit her poster, she will show you that there are some differences in terms of the rate of breakdown of these proteins. Especially, say in bulls, for instance, you will find that there is a much slower rate of breakdown of these two proteins and that it relates to tenderness. It is actually rather surprising in some of the results she is getting just how well the correlation may hold up in terms of the rate of breakdown with tenderness of these muscles. I presume that relates back to the calpain system. Maybe Darrel wants to tell you about calpain in bulls.

D. Goll: No, I don't know whether sex has any effect on the calpain system.

R. Warner: Question for Darrell Goll. Is there an evolutionary or physiological reason for protein degradation such that if you decrease it, there is an effect on the protein quality or the meat quality?

Goll: There is no evidence for such a role. Obviously, protein degradation has to have some type of physiological significance or we wouldn't be undergoing such large amounts of it. One of the reasons for it, especially with muscle, is that it is a supply of amino acids to the other cells in the animal in between periods of feeding. Exactly what the significance is of the increased rate of degradation during periods of rapid growth is unclear. I used 10% decrease in rate of degradation, for example, because I suspect if one were to completely knock out protein degradation in an animal, it would probably not survive very long. I think it is probably reasonable that one could reduce it by 10%. I think

there is a role, but neither I nor anyone else I know of knows what it is.

Smith: Question for Steve. You talked about promoter regions within the genes for myofibrillar proteins. Do they know anything about enhancers that may be fairly far distant upstream?

Konieczny: It turns out that as far as I know, all contractile protein genes except possibly alpha actin utilize enhancers. The example that I used today for troponin I is actually an enhancer. It is located in the first intron and we can move that piece of DNA anywhere on that gene. We can move it to the 5' end or the 3' end or at a distance and we can rotate the orientation of this piece of DNA. We can put it on a different gene and now make this gene muscle-specific. Almost all contractile protein genes are utilizing these sorts of molecular mechanisms.

J. Price: Question for Dr. Goll. You seemed to imply, at least to me, that the multicatalytic protease couldn't possibly be involved in postmortem tenderization. Is that correct and, if not, why?

Goll: The multicatalytic protease has no effect upon myofibrillar proteins when they are assembled in the myofibril. That was the first thing a lot of people tested. Therefore, I don't think they would have much to do with postmortem tenderization. Now whether they will degrade myosin and actin in solution isn't exactly clear yet. If you isolate the multicatalytic protease from a mollusc it does, but if you isolate it from a couple of other species, the reports are that it doesn't. It is all pretty much an unsettled area right now.

R. Kauffman: This is a three-part question for Rich Robson, specifically. 1) You mention the fact that some of these proteins were related to water-holding capacity but you really didn't elaborate at all on exactly what you have in mind. 2) What is your evidence to suggest that titin stops at the M line rather than continuing from Z line to Z line? 3) Why don't you have a bow tie on?

Robson: To answer the last question first, I don't own one any more. Back to the first question, what is the evidence with regard to a role for some of these proteins in things like water-holding capacity? I think it has to do with the fact that in water-holding capacity, part of that is determined by the spacing, or the lattice spacing primarily in a transverse direction and opening up of the myosin and actin filaments. It also may, in fact, have something to do with how tightly the myofibrils are packed as they lie next to each other. Obviously, we have a lot of work to do there yet. But, I think we already have enough clues that this is a reasonable suggestion to make at this point. The other question had to do with how we know that a single titin molecule doesn't actually go all the way through the sarcomere instead of stopping at the M line. Basically, that evidence comes from the labeling patterns that several laboratories have seen. If you have a monoclonal antibody that recognizes a given titin epitope, it will always stain symmetrical lines around the M line. About the only way that you could have a titin molecule go the entire distance, would be to have a very strange molecule. It would probably have to be built in two halves that were absolutely identical and symmetrical around the middle. Actually, you could envision this possibility and, in fact, in the work from

Klaus Weber's laboratory, they do raise this possibility and then kind of argue against it; but they do, in fact, suggest that titin molecules have their heads right at the M line, and the tails kind of go off to the Z-lines. If you wanted to think about it as an antiparallel, two subunit protein, you also could use that argument. Marion may want to comment on that as well.

M. Greaser: In reality, the evidence is not there that it doesn't extend from one Z line to the other. You can envision a model in which you had one set of molecules extending from one Z line with the heads towards the other Z line and another set going the opposite way. With that kind of a system, you still have the symmetry and you get the two antibody bands stained per sarcomere. I think this is unlikely; nobody wanted to believe that titin was long enough to go from M line to Z line to begin with, and now you're just doubling the length, which makes it even worse. I think that the idea of it ending at the M line is best, but there are a number of other possibilities that can't be ruled out at present.

S. Mills: Question for Steve Konieczny: Perhaps you would like to speculate on the question that you leave us with as to the different roles of MRF4, myogenin and Myo D and secondly, you show us the potential for turning a non-muscle cell into a muscle cell which is obviously very inviting to this group. If you were to do that, would you choose one of these factors and what are the limitations or potential for doing that in a practical situation?

Konieczny: The role of these factors is very intriguing. Why are there four related proteins? For instance, it is interesting that there are no known muscle cell lines, such as ones that people in this audience use, like L6 or C2C12, that express all four factors. So, in fact, culture cells appear to behave normally even though not all four factors are there. What the individual roles of these factors are is obviously a major focus of my lab. Unfortunately, we really don't know what these factors do in the animal and therefore we are trying to devise assays to look at that. The other question about whether these factors may be helpful in increasing meat production, I think there is a lot of potential there. If I had to choose one of these particular four factors, I would concentrate on MRF4. The reason is that this is the major muscle regulatory factor in the adult animal. It is more abundant than any of the other three factors. Therefore, we think it may have a role in the maintenance of the adult musculature and may be involved in overall meat production. I should tell you, though, that there is another interesting factor out there that doesn't belong to this particular protein family, known as ski. Some elegant studies have been done by Steve Hughes at NCI making transgenic ski mice and also ski transgenic pigs. These animals are incredible when looking at them, even for someone like myself who is not a farmer. You can immediately recognize that these animals have a tremendous amount of increased muscle mass and a large decrease in the fat content. These are transgenic animals that are overproducing the particular protein known as ski. I think that for the future, this will be a very interesting area of research to follow up on.

J. Acton: Rich, you presented nebulin as being a parallel filament to actin and then both Marion and Darrel, Darrel in particular, mentioned the N_2 line. Will you straighten me out? What is the N_2 line composed of or can you place it correctly in the architecture?

Robson: I am not sure I can straighten you out as to exactly what the N_2 line is. The N_2 line has been floating around in the literature since about 1948. I actually saw an original electron micrograph where I could make out some densities that look like they were some of these N- lines in an article published back then. As the years have gone on, sometimes people will see them, sometimes they don't. There is a paper by Kuan Wang and Wright in the *Journal of Cell Biology* in 1988, the December issue, in which they actually go through very carefully and describe where they think the N_2 may come from. Their suggestion is that, generally speaking, when we see it, it is probably because there has been some alteration during fixation of the tissue and that, in fact, it is probably an artifact. Originally, everybody thought that the N_2 line was probably nebulin. In their recent paper, Wang goes through the argument and suggests that, in fact, it (N_2 line) could have some nebulin associated with it but it's probably actually titin. I can't really straighten you out completely because we really don't know yet. It is probably part of the titin molecule but there could be some nebulin there as well.

Greaser: There is a lot of confusion about this because nebulin was originally identified as being at the N_2 line. Kuan Wang has reversed his thinking on that. Another comment about the N_2 line and the N lines in general—they are much more prominent in tissue that is poorly fixed. If you go back in the early literature in animals like a rat which has a lot of protease activity, you see the N- lines much more prominently. I think I would put my marbles with the idea that they are probably an artifact band that is formed during fixation.

Sebranek: I think in the interest of time, we will entertain two more questions.

M. Hunt: Question for Marion or maybe others. A lot of what you have said has to do with proteolysis and enzymatic changes relative to degradation. Could you comment just on the postmortem conditions per se relative to some of the cytoskeletal changes that may occur? I am thinking specifically of the rapidly declining pH, high carcass temperatures and resulting softening of the tissue.

Greaser: Well, that is fairly broad. I don't know whether Rich or I are the right ones to answer that, or Darrel. Certainly, the pH goes down postmortem, the temperature goes down. pH going down will increase activity of the catheptic enzymes, but as Darrel mentioned, those may not be that important. The lowering of pH is going to reduce the activity of the calpains because they are most active at pH 7. We really don't understand what the exact proteolytic events are. We see a few changes in the titin and nebulin, desmin, troponin T, but we can't identify which proteases are responsible for those at this point.

Robson: I was just going to add that although the pH drops, temperature drops, and so on, and although certainly the conditions are not ideal for the calpains, you do still have a small amount of calpain activity even at reasonably acidic pH values and lower temperature. You remember, that with postmortem muscle, you are actually aging that thing for a long, long time, so you can still possibly get some degradation just because of the time factor, even though the specific activity has dropped a great deal.

Greaser: Like I stressed, I think you have to look at what does happen as opposed to what might happen. Cathepsins

might be active, calpains might be active. But what happens is that myosin and actin are not degraded.

D. Mulvaney: I will address the question to Darrel or perhaps some of the group from Clay Center about the regulators of calpastatin. Is there a relationship between activity of calpastatin and varying levels of rates of growth, or myofibril deposition, or protein accretion?

Goll: Yes, I think so. I was going to mention it, but I got too long-winded early in the talk. There is evidence now suggesting that some of the beta-agonists at least, influence rate of muscle growth primarily by reducing rate of protein turnover. Those same muscles, when you isolate them, tend to have a high activity, 50% greater, of calpastatin and sometimes

somewhat reduced contents of u-calpain. When you test them later on, they also tend to be tougher, all of which links the calpain system into all of these events. The difficulty with all of this is that there is enough calpastatin already in muscle to completely inhibit all the calpains. I don't know how this regulation works. One must feel that somehow the cells have a way to overcome this, and apparently there is a way to overcome it even in postmortem muscle. I think that is really the most interesting part of postmortem tenderization right now. I think it is reasonably well established that the calpains are responsible for postmortem tenderization and we ought to be focusing attention on how they do it.