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Transgene-Coded Chimeric Proteins as Reporters of Intracellular Proteolysis: Starvation-Induced Catabolism of a *lacZ* Fusion Protein in Muscle Cells of *Caenorhabditis elegans*

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Abstract The product of an integrated transgene provides a convenient and cell-specific reporter of intracellular protein catabolism in 103 muscle cells of the nematode *Caenorhabditis elegans.* The transgene is an in-frame fusion of a 5′-region of the *C. elegans unc-54* (muscle myosin heavy-chain) gene to the *lacZ* gene of *Escherichia coli* [Fire and Waterston (1989): EMBO J 8:3419–3428], encoding a 146-kDa fusion polypeptide that forms active β-galactosidase tetramers. The protein is stable in vivo in well-fed animals, but upon removal of the food source it is inactivated exponentially ($t_{1/2}$ = 17 h) following an initial lag of 8 h. The same rate constant (but no lag) is observed in animals starved in the presence of cycloheximide, implying that inactivation is catalyzed by pre-existing proteases. Both the 146-kDa fusion polypeptide ($t_{1/2}$ = 13 h) and a major 116-kDa intermediate ($t_{1/2}$ = 7 h) undergo exponential physical degradation after a lag of 8 h. Degradation is thus paradoxically faster than inactivation, and a number of characteristic immunoreactive degradation intermediates, some less than one-third the size of the parent polypeptide, are found in affinity-purified (active) protein. Some of these intermediates are conjugated to ubiquitin. We infer that the initial proteolytic cleavages occur in the cytosol, possibly by a ubiquitin-mediated proteolytic pathway and do not necessarily inactivate the fusion protein tetramer. J. Cell. Biochem. 67:143–153, 1997. © 1997 Wiley-Liss, Inc.

Key words: *C. elegans; E. coli;* immunoreactive degradation intermediates; affinity-purified protein; ubiquitin

INTRODUCTION

Intracellular proteolysis has a crucial role in the biochemical economy of cells, but we know relatively little about which individual proteolytic enzymes are responsible for inactivating or degrading particular target proteins in vivo. Although regulation of proteolytic processes is clearly an important element in many kinds of physiological adaptations, in development, and in some pathologies, we still know little about the detailed mechanisms that govern the in vivo stability of particular proteins or the activities of individual proteases.

Proteolysis in vertebrate muscle has received close attention because of the clinically important phenomena of muscle atrophy in response to disuse or denervation [Fernandez, 1988]. Muscle cells contain both lysosomal proteases and a variety of cytosolic proteases, including the nonlysosomal Ca^{2+}-activated neutral proteases (calpains) [Pontremoli and Melloni, 1986] and the ubiquitin-dependent proteolytic system [Jentsch, 1992; Wilkinson, 1995] in which the actual proteolysis is catalyzed by an ATP-dependent complex variously termed "multicatalytic protease" or "proteasome" [Ciechanover and Schwartz, 1994; Rivett, 1993]. Each system can degrade myofibrillar proteins in vitro, but their roles in vivo are incompletely defined.

Fasting or denervation increase the rate of protein catabolism in muscle [Li and Goldberg, 1976; Fulks et al., 1975; Furuno et al., 1990;

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Medina et al., 1991; Attaix et al., 1994] and promote the autophagy of cytosolic materials into vesicles that subsequently fuse with lysosomes [Seglen and Bohley, 1992], but several inhibitor-based studies of muscle proteolysis after fasting and/or denervation have suggested that the ubiquitin-proteasome system may be primarily responsible for enhanced proteolysis. Levels of polyubiquitin mRNA [Medina et al., 1991], ubiquitinated protein [Medina et al., 1991], and a ubiquitin-conjugating enzyme [Wing and Banville, 1994] have all been reported to increase in muscle upon fasting and/or denervation. The ubiquitin-proteasome system, once thought to act only on abnormal or short-lived proteins, also has a role in degradation of long-lived proteins [Rock et al., 1994; Stancovski et al., 1995].

Although some studies of muscle proteolysis have focused on well-defined substrates, (such as muscle glycogen phosphorylase) [Leyland *et al.*, 1990], myosin heavy chains [Jakubiec-Puka et al., 1990], and acetylcholine receptor [Salpeter and Loring, 1985; Shyng et al., 1991; Shyng and Salpeter, 1990; Retzler et al., 1991; Caroni et al., 1993], proteolysis is often measured by release of labeled amino acids from total cellular protein. Biochemical analysis would be more straightforward if a more defined substrate were examined. In our view, it would be advantageous to use simpler model systems, particularly those that engage the experimental power of genetics.

We have been engaged in a genetic and biochemical analysis of proteolytic processes, using as a model the soil nematode *Caenorhabditis elegans*. This choice was made because of the ease with which mutant strains can be isolated and characterized [Herman, 1988] and the exceptional cellular simplicity of the animal [White, 1988]. In contrast to vertebrate muscle, *C. elegans* muscle is composed of relatively few muscle cells (e.g., 95 body-wall muscle cells) of known developmental origin [Sulston et al., 1983], some of which have well-characterized innervation by only a few neurons. The fact that the circuitry of the nervous system has been completely determined at the anatomical level [White et al., 1986] is also a potential advantage.

In this report, we have sought to construct a simplified system for studying proteolysis in muscle cells by following the inactivation and degradation of a β-galactosidase fusion protein produced by an integrated transgene expressed in only 103 muscle cells. The fusion protein is stable in well-fed animals, but is inactivated rapidly in starved animals by a proteolytic system present before the onset of starvation. Thus, under starvation conditions the proteolytic system is activated and/or released from inhibition. The presence of ubiquitin-conjugated degradation intermediates in active fusion protein tetramers suggests a role for a ubiquitin-dependent pathway in the inactivation and/or degradation of the fusion protein.

MATERIALS AND METHODS

Growth of Nematodes

All experiments used *Caenorhabditis elegans* strain PD55 (see Results), kindly provided by Andrew Fire, Carnegie Institute of Washington [Fire and Waterston, 1989; Okkema et al., 1993]. Methods for culture of nematodes on NG agar with *Escherichia coli* strain OP50 (streptomycin-sensitive) as food source were as previously described [Sarkis et al., 1988], except that for enzyme purification and gel electrophoretic analysis experiments, we used as food source *E. coli* strain JF1001 (*thiA*, Δ*lacpro*) carrying a deletion of the entire *lac* operon, so as to preclude any contamination with *E. coli* β-galactosidase.

Worms growing on agar plates were roughly age-synchronized by washing the worms from the plates with BU buffer (70 mM potassium phosphate, 70 mM NaCl, pH 7), settling under gravity for 1.5–2 min, and withdrawing the small larvae from the upper portions of the suspension. These larvae were replated on bacterial lawns and grown (to early adulthood) for 36–42 h at 20°C prior to the initiation of experiments. To starve the animals, they were washed from their growth plates with BU buffer containing streptomycin (200 μg/ml), washed several times with the same buffer to remove bacteria, and replated on NG agar plates containing streptomycin but without bacterial lawns. Generally no bacterial growth was evident on such plates for at least 72 h; any experiments in which such contamination appeared were discarded.

Fluorometric Assay of β-Galactosidase Activity

Assays were conducted in a total volume of 50 μl in BU buffer containing 1 mM $MgCl_2$ and 0.4 mM 4-methylumbelliferyl-β-D-galactopyrano-

side. Samples were incubated at 25°C for 10 min to 2 h and the reactions stopped by the addition of 1 ml of 0.25 M Na_2CO_3. Control experiments showed that the assays were linear with time and linear with amount of enzyme added in this range. The product (4-methylumbelliferone) was quantitated fluorometrically in a Farrand Mark I spectrofluorimeter with λ_{ex} = 365 nM, λ_{em} = 450 nm. Measured fluorescence values were corrected for a blank reaction without enzyme. Under these conditions, a fluorescence signal of 3.15 units produced in 1 h corresponds to 1 femtokatal of enzymatic activity.

For assay of nematodes, 10 animals were picked into 20 µl of BU buffer containing 1 mM $MgCl_2$ and 0.1% Nonidet P-40. The samples were immediately frozen in liquid nitrogen and stored at −80°C. When all samples had been collected, the worms were lysed by six cycles of freezing in liquid nitrogen and thawing at about 10°C, then assayed as described above.

Affinity Purification of β-Galactosidase

Worms were suspended in starting buffer (50 mM HEPES, 0.5 M NaCl, 1 mM $MgCl_2$ 0.01% NaN_3, pH 7.3) and broken by sonication or by passage through a French press. The crude extract was centrifuged for 20 min at 35,000*g* at 4°C and the clear supernatant withdrawn for affinity purification. The purification method was modified from that used to purify β-galactosidase from *E. coli* [Steers et al., 1971]. A small 1- to 10-ml bed vol) column was packed with p-aminobenzyl-1-thio-β-D-galactopyranoside coupled to 4% agarose beads (Sigma Chemical A-0414) and equilibrated with at least five column volumes of starting buffer. The crude extract was applied to the column over about 1 h and the column was washed with about one column volume of start buffer, followed by one-half column volume of distilled H_2O. Bound β-galactosidase was eluted with 0.1 M borate, 500 mM NaCl, 1 mM $MgCl_2$, pH 10, and fractions collected into tubes containing an equal volume of 500 mM HEPES, 10% (w/v) glycerol, 1 mM $MgCl_2$, and 0.01% (w/v) NaN_3, pH 7.3. β-Galactosidase activity in the fractions was determined as described above and the active fractions stored at −80°C.

The results of an optimal purification are shown in Figure 1a. In some individual experiments, some unbound β-galactosidase emerged in the initial wash, particularly if the sample was run into the column too quickly or too large a sample of crude extract was loaded. The purified β-galactosidase is primarily active tetramer, as shown by gel permeation chromatography on Sephacryl S-300 (Fig. 1b).

Electrophoresis and Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were carried out as described [Jacobson et al., 1988], except that the polyacrylamide concentration was 7.5% and Immobilon-P membranes (Millipore) were used. After electroblotting, the membranes were blocked by overnight incubation in 3% bovine serum albumin (BSA) in TBST (10 mM Tris–HCl, 150 mM NaCl, 0.2% Tween-20, 0.01% NaN_3, pH 8). Bands with β-galactosidase immunoreactivity were detected by a 1-h incubation in a 1:2,000 dilution of monoclonal anti-β-galactosidase antibody (Promega Z378A) in TBST and by washing and secondary incubation (1 h) in alkaline phosphatase-conjugated goat antimouse IgG (Promega S372A) diluted 1:2,000 in TBST. Ubiquitin-conjugated proteins were detected with monoclonal anti-ubiquitin antibody (Vector Laboratories NCL-$UBIQ_m$) and the same secondary antibody. After washing 5 times in TBST, alkaline phosphatase activity was detected by incubating the membrane in detection solution (55 µg/ml 5-bromo-4-chloro-indolyl phosphate and 330 µg/ml nitroblue tetrazolium (NBT) in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) until the desired band density was attained. Development was stopped by rinsing in distilled H_2O and the membrane allowed to dry at room temperature. Bands were quantitated by reflectance scanning and integration of the digitized images with NIH Image software.

Histochemical Staining of β-Galactosidase Activity

Staining was performed by a modification of the protocol used by Fire [1992]. Oxidation buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgCl_2$ in 0.2 M sodium phosphate pH 7.5) was stored at −20°C. Immediately before use, X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside, 20 mg/ml in N,N′-dimethylformamide) was added, for a final concentration of 0.4 mg/ml. Worms were picked into 20-µl drops of BU buffer on slides. The slides were dried in a vacuum desiccator

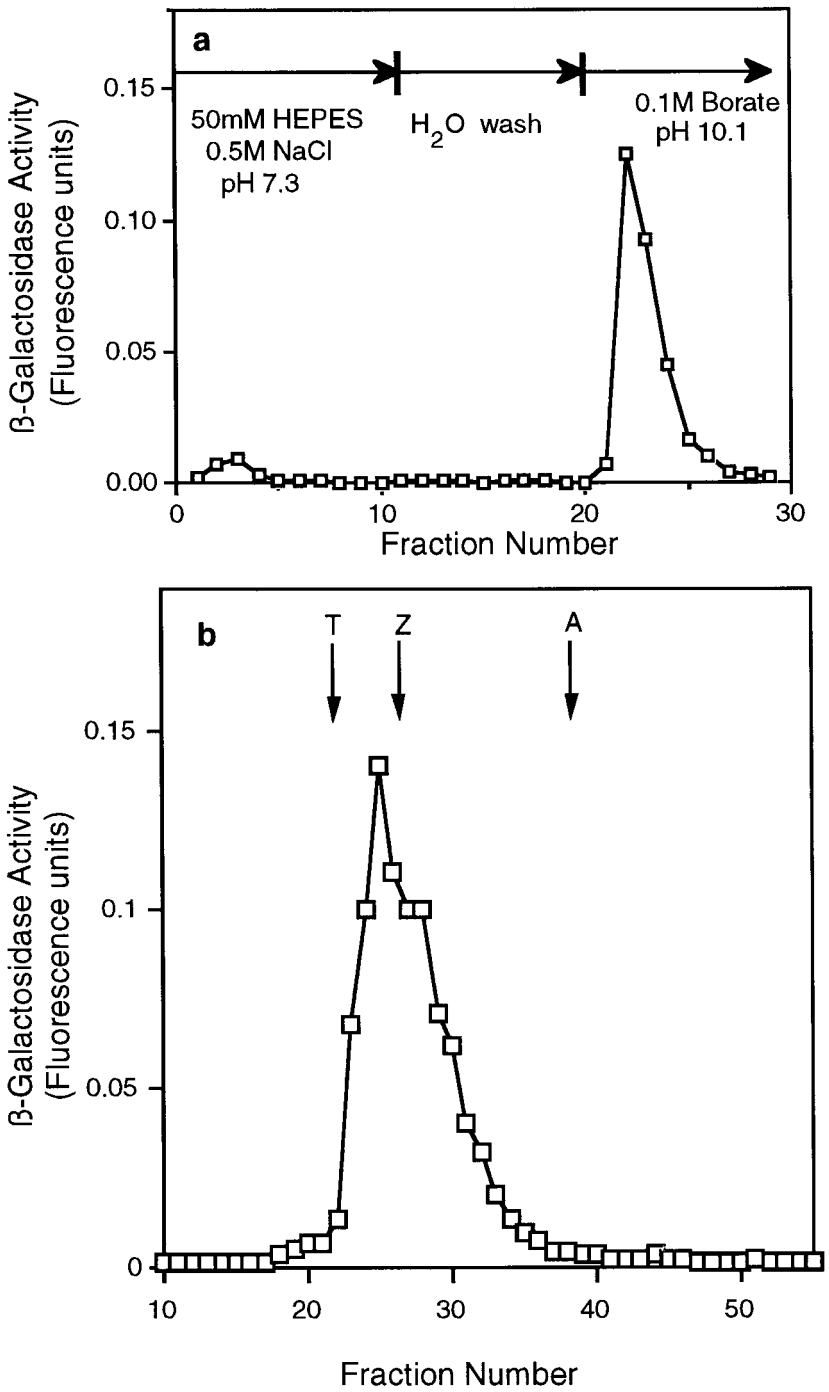

Fig. 1. **a:** Affinity purification of β-galactosidase fusion protein on immobilized p-aminobenzyl-β-D-thiogalactopyranoside. Details are given in Materials and Methods. **b:** Gel filtration chromatography (Sephacryl S-300) of affinity-purified β-galactosidase from partially starved *Caenorhabditis elegans* strain PD55. This material corresponds to that in Fig. 4, **lane 3.** Arrows, elution positions of bovine thyroglobulin (T, 669 kDa), *Escherichia coli* β-galactosidase (Z, 464 kDa) and sweet potato β-amylase (A, 200 kDa).

connected to a water aspirator for 10–20 min, then dipped in acetone (−20°C) for 3.5 min, and air-dried at room temperature. Staining solution (20 µl) was placed on each sample and the slides incubated at room temperature overnight in sealed Petri dishes lined with damp paper towels. Slides were photographed on Kodak Ektachrome EH-160 transparency film under bright-field illumination with a red suppression filter.

RESULTS AND DISCUSSION

Expression of the Transgene Product

All experiments used *Caenorhabditis elegans* strain PD55 [*tra-3*(e1107)IV; ccIs55V] [Fire and Waterston, 1989; Okkema et al., 1993]. The integrated transgene ccIs55 consists of 5′-portions of the *C. elegans* wild-type *unc-54* (muscle myosin heavy-chain) gene, with the fourth exon fused in-frame (with an intervening portion of the *E. coli trpS* gene) to the *lacZ* gene of *E. coli*, followed by a 3′-terminal portion of the *unc-54* gene (including a polyadenylation site). The fusion encodes a 146-kDa polypeptide.

The construct used for germline transformation also carries a mutant *C. elegans sup-7*(st5) allele that encodes an amber suppressor $tRNA^{trp}$ [Bolten et al., 1984], and the genetic background contains an amber-suppressible sexual transformer mutation *tra-3*(e1107)IV. Unpublished experiments in our laboratory indicate that the transgene is integrated on linkage group V and that the *unc-54::lacZ* fusion remains closely linked to *sup-7*. The resident wild-type copy of *unc-54* on linkage group I is not disrupted.

Wild-type *C. elegans* produces no endogenous β-galactosidase activity. In well-fed animals containing the *lacZ* transgene, the fusion product is expressed exclusively in muscle cells. Histochemical staining with X-gal (Fig. 2) revealed β-galactosidase activity in 95 body wall muscle cells that form four longitudinal bands of muscle, and 8 vulval muscle cells. No activity was observed in pharyngeal or uterine muscles.

Accumulation of the fusion product begins in embryos (data not shown) and continues throughout adulthood. During the time period of our experiments, well-fed young adult animals showed a continuous linear increase (Fig. 3a) in the amount of β-galactosidase activity per animal. The increase in activity was closely paralleled by the accumulation of immunoreactive 146-kDa β-galactosidase fusion polypeptide (cf. Fig. 3c) as detected on Western blots. The addition of cycloheximide (400 μg/ml) completely inhibited this accumulation in fed animals (Fig. 3a), but the existing β-galactosidase activity remained completely stable over 60 h. The best-fit slope to the cycloheximide data indicated an activity increase of 0.2% per hour, but Student's t-test showed a likelihood ($P \approx 0.8$) that any difference from a slope of zero was due to chance alone.

β-Galactosidase Inactivation In Vivo During Acute Starvation

When young adult animals were starved by removing the food source (*E. coli*), the β-galactosidase activity became unstable. Inactivation (Fig. 3b) was exponential (first-order) after a lag period of 8.8 ± 0.5 h. During the exponential phase of inactivation, the apparent half-life was 16 ± 0.5 h.

When similar animals were starved in the presence of cycloheximide to inhibit protein synthesis, exponential inactivation began immediately (calculated lag period = 0 ± 0.4 h) with an apparent half-life of 17 ± 0.6 h. Thus, inactivation proceeds at the same rate in the presence and absence of cycloheximide.

Three inferences can be drawn from a comparison of Fig. 3a and 3b:

1. The imposition of acute starvation increases the rate of β-galactosidase inactivation after an initial lag.
2. This starvation-triggered inactivation must be catalyzed by one or more proteolytic enzyme(s) that existed before the imposition of starvation, since the inactivation rate constant (the slope of each curve in Fig. 3b) was the same when cycloheximide was present to inhibit de novo protein synthesis.
3. The rate of β-galactosidase synthesis in the starved animals in the absence of cycloheximide must be zero or near-zero after the initial lag period, since the net rates of inactivation (synthesis minus inactivation) were the same with and without cycloheximide.

Intermediates in Fusion Protein Degradation

We observed that in well-fed worms (lysed by boiling in SDS buffer) the predominant form of

Fig. 2. Young adult *Caenorhabditis elegans,* strain PD55, stained with X-gal to show the location of β-galactosidase activity in body wall and vulval muscles. Control experiments with a strain lacking the transgene showed no staining.

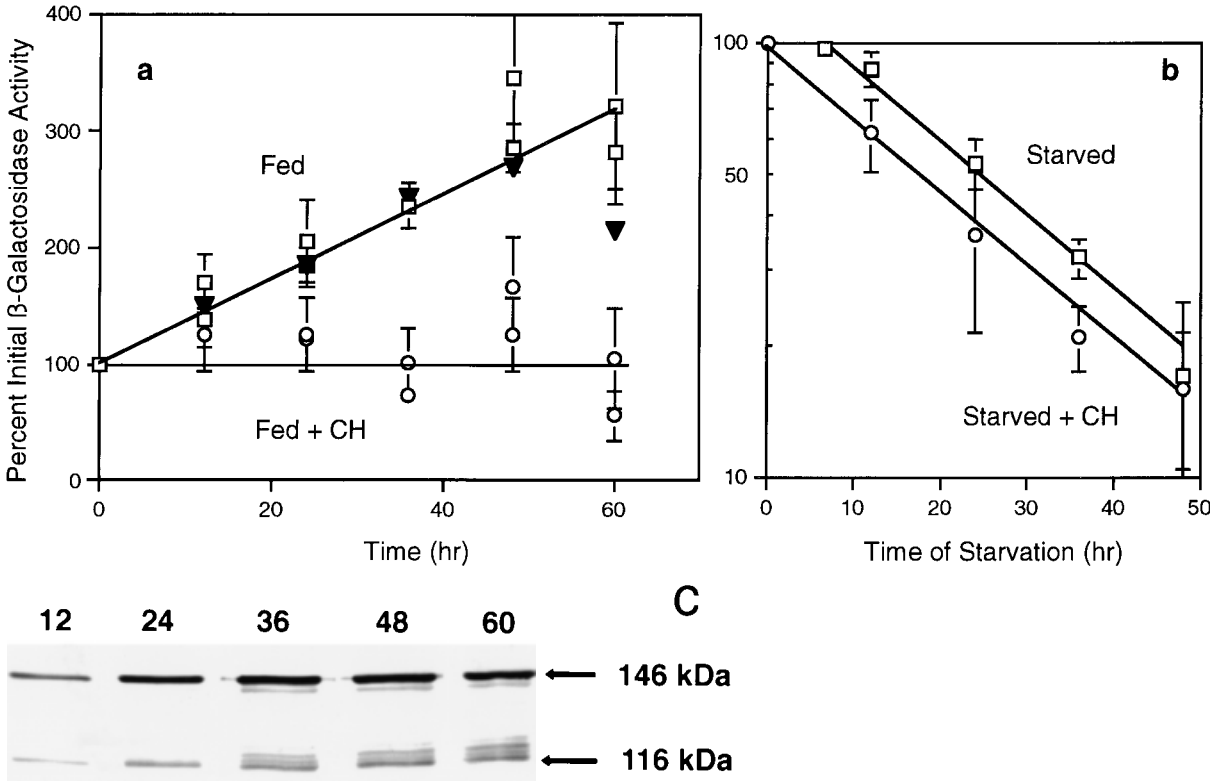

Fig. 3. **a:** Activity of the *lacZ* reporter protein in well-fed animals with (○) and without (□) cycloheximide (400 μg/ml) added at t = 0. Points are means ±SD of triplicate 10-worm samples in two independent experiments. Filled triangles (▼) show the relative amounts of immunoreactive 146-kDa β-galactosidase fusion protein in the fed animals, based on quantitation of the Western blot in c. **b:** Activity during acute starvation without cycloheximide (□, $t_{1/2}$ = 16 ± 0.5 h) and with cycloheximide (○, $t_{1/2}$ = 17 ± 0.6 h). Points are means ±SD of six independent experiments, each with triplicate 10-worm samples at each time point. Lines shown are least-squares fits to the equation $A(t) = A_o e^{-kt}$, where A(t) = percentage initial activity at time t and A_o = percentage activity at t = 0. The calculated intercepts on the time axis at A = 100% are □, 8.8 ± 0.5 h and ○, 0 ± 0.4 h. **c:** Western blot analysis of samples from fed worms (cf. a: ▼). Each sample of 30 worms was boiled in SDS sample buffer and the entire sample placed in a gel lane for electrophoresis. Detection was with monoclonal anti-β-galactosidase antibody (see under Materials and Methods). Densitometric data were normalized to the amount at t = 12 h.

β-galactosidase in vivo was the intact 146-kDa fusion polypeptide (Figs. 3c, 4, lane 1), as detected by Western blotting with monoclonal anti-β-galactosidase antibody. Affinity-purified β-galactosidase (see under Materials and Methods) (Fig. 1) from well-fed animals (Fig. 4, lane 2) contained the 146-kDa fusion polypeptide, four large intermediate cleavage products with molecular masses between those of the 146-kDa fusion polypeptide and the 116-kDa *E. coli* β-galactosidase polypeptide, and only traces of degradation products smaller than 116 kDa. These four large cleavage products are present in minor amounts in vivo (cf. Fig. 4, lane 1, and Fig. 3c), but continue to form in vitro during the preparation of extracts and purification. Two observations suggest that these products are produced by processing proteases similar to the BLI-4 protease [Thacker et al., 1995], which cleaves at paired basic residues [Barr, 1991]. First, the in vitro conversion is strongly inhibited by Zn^{2+}, a known inhibitor of this class of proteases. (Unfortunately, Zn^{2+} has proved incompatible with our affinity purification method.) Second, the molecular masses of the four products correspond remarkably well to those predicted if cleavage occurs at paired basic residue sites (143 kDa, Ser-Lys-Lys in myosin exon 2; 127 kDa, Thr-Lys-Lys and 125 kDa, Asn-Lys-Lys in myosin exon 4; 115 kDa, Gln-Arg-Arg corresponding to residues 13–15 of β-galactosidase) in the N-terminal portion of the fusion polypeptide.

By contrast, we observed that affinity-purified and enzymatically active β-galactosidase from partially-starved animals contained little intact 146-kDa fusion polypeptide (Fig 4, lane 3). (It is difficult to achieve rapid onset of starva-

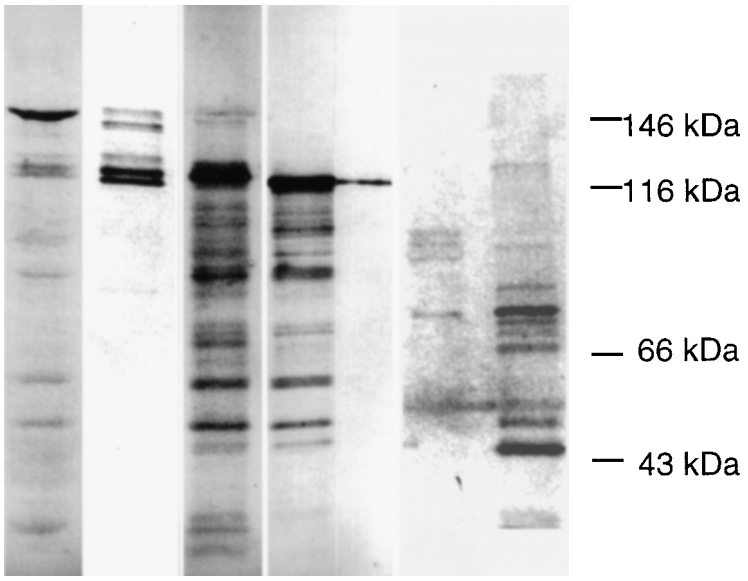

Fig. 4. Western blot analysis of intermediates in *lacZ* fusion protein degradation. Experimental procedures are given in Materials and Methods. **Lanes 1–5,** detection with monoclonal anti-β-galactosidase antibody: **lane 1,** well-fed worms boiled in SDS; **lane 2,** affinity-purified protein (cf. Fig. 1) from well-fed worms; **lane 3,** affinity-purified protein from starved worms; **lane 4,** affinity purified β-galactosidase from *E. coli;* **lane 5,** same as lane 4, but 1/20 of the amount loaded. **Lanes 6–7,** detection with monoclonal anti-ubiquitin antibody: **lane 6,** affinity-purified protein from starved worms; **lane 7,** unbound protein from affinity purification of lane 6. The lowest band in lane 6 (extends to lane 7) is an artifact. Additional size markers (scale right) are bovine serum albumin (66 kDa) and ovalbumin (43 kDa).

tion at the scale of culture required for protein purification. We estimate that these animals were effectively starved for the equivalent of 12–18 h.) There was a substantial amount of 116-kDa polypeptide, as well as significant amounts of smaller degradation intermediates. The fact that these various intermediates were present in affinity-purified, highly active enzyme implies that the proteolytic cleavages that produced them did not result in complete inactivation of the fusion protein tetramer.

It is interesting that a very similar set of intermediates was present in β-galactosidase from *E. coli* (cf. Fig. 4, lane 3 to lane 4). Since *E. coli* and *C. elegans* contain quite different sets of proteases, we surmise that the principal locations of proteolytic attack on the intact β-galactosidase molecule are determined primarily by structural factors such as the location of exposed loops on the surface of the tetramer.

Western blots of the same active, affinity-purified enzyme from partially starved animals, with detection by monoclonal anti-ubiquitin antibody (Fig. 4, lane 6), revealed the presence of ubiquitin-conjugated intermediates. The ubiquitin-conjugating system must therefore be able to act on active tetramers, and the ubiquitinated products must remain associated with active tetramers. These particular conjugated polypeptides were absent from the unbound fraction of the β-galactosidase affinity column (Fig. 4, lane 7), although other ubiquitin-conjugated polypeptides were detected. It is noteworthy that the electrophoretic mobilities of the principal ubiquitinated degradation intermediates did not correspond with those of the most abundant intermediates detected with monoclonal anti-β-galactosidase antibody (cf. Fig. 4, lanes 6 and 7), indicating that the principal intermediates are not themselves ubiquitinated. This suggests that ubiquitinated products are subject to relatively rapid further inactivation and/or degradation.

We measured the kinetics of loss of the 146-kDa fusion polypeptide and several principal degradation intermediates in starved animals by Western blotting with anti-β-galactosidase antibody. As shown in Figure 5, the rate of degradation of each form of β-galactosidase polypeptide was well represented by an exponential (first-order) process, but only after an initial lag period of 7–8 h. This lag period was essentially the same as that observed in the inactivation measurements (Fig. 3b) in the absence of cycloheximide. After the initial lag period, each polypeptide was degraded with a characteristic half-

life, ranging from 13 ± 1 h for the intact 146-kDa fusion polypeptide to 7 ± 0.1 h for the 116-kDa band. Thus, it appears that the rate of physical degradation of β-galactosidase polypeptides accelerates as proteolysis proceeds.

The fact that similar lag periods are observed before inactivation (Fig. 3b) or physical degradation (Fig. 5) begin after starvation implies that this lag reflects some fundamental feature of the physiology of acute starvation. Similar lags have also been observed by others [Avery and Horvitz, 1990] before the onset of starvation-induced changes in *C. elegans*. It seems likely that this period of about 8 h represents the time required for *C. elegans* to exhaust its internal reserves of energy-storage materials, whereupon physiological starvation actually commences. Some other proteolytic processes triggered more immediately by acute starvation (e.g., the degradation of cathepsin D in intestinal lysosomes) [Hawdon et al., 1989] must be regulated in a different way.

It is also noteworthy that the half-life of the intact 146-kDa fusion polypeptide and those of the degradation intermediates (Fig. 5) are all shorter than that of β-galactosidase activity in vivo (Fig. 3). At first, this seems a paradox: How can inactivation be slower than physical degradation? The most probable explanation is that even after initial proteolytic events, the cleavage products do not dissociate, but remain noncovalently associated with active tetramers. This explanation is strongly implied by our observation that degradation intermediates, some as small as one-third the size of the parent 146-kDa polypeptide, are found to be present in enzymatically active, affinity-purified β-galactosidase tetramers (Fig. 4). There is ample precedent for retention of activity of noncovalently associated fragments in the phenomenon of "alpha-omega complementation" in *E. coli* β-galactosidase [Ullmann et al., 1967; Langley et al., 1975].

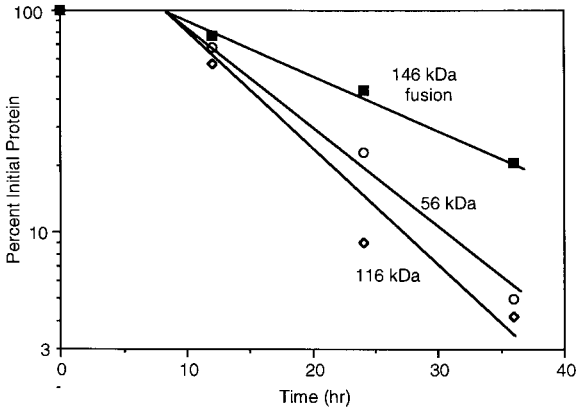

Fig. 5. Western blot analysis of the kinetics of physical degradation of *lacZ* fusion protein in *Caenorhabditis elegans* strain PD55 during starvation begun at t = 0. Data were obtained by densitometry of blots similar to that in Figure 4, lane 1, except that worms were starved for the indicated periods. The SDS extract of 10 worms was loaded in each gel lane. The 146-kDa band is the intact fusion protein. The 116-kDa band is the same size as for *Escherichia coli* β-galactosidase. The 56-kDa band is a major degradation intermediate (see Fig. 4, lane 3). Lines shown are exponential least-squares fits to the equation $P(t) = P_0 e^{-kt}$, where P(t) = percent of initial intensity of each band at time t and P_o = percentage intensity at t = 0. The calculated intercepts on the time axis at *P* = 100% are 8 ± 1 h.

Mechanism and Regulation of Muscle Proteolysis

Perhaps the most interesting aspect of our observations is that acute starvation increases the rate of β-galactosidase inactivation and degradation by a proteolytic pathway involving pre-existing enzymes. Thus, the imposition of starvation must promote net proteolysis by one of the following mechanisms:

1. An increase in the activity of enzymes required for proteolysis (e.g., ubiquitin-conjugating enzymes or proteases) without new synthesis
2. A decrease in the activity of one or more inhibitor(s) of such enzymes
3. An increase in the physical access of the fusion protein to a subcellular compartment (e.g., lysosomes) that contains proteolytic enzymes, for example by an increased rate of autophagy [Seglen and Bohley, 1992].

We do not believe that lysosomal proteolysis contributes significantly to β-galactosidase inactivation in *C. elegans* muscles for several reasons. First, the muscle cells of *C. elegans* are extremely poor in lysosomes. Although lysosomes in other cells (e.g., intestine) are readily observed by loading with a lysosomotropic dye like acridine orange [Clokey and Jacobson, 1986], we have been unable to observe any lysosomes in muscle cells by this method. Second, among the lysosomal proteases of *C. elegans*, only cathepsin D [Jacobson et al., 1988] can inactivate intact β-galactosidase in vitro; partially purified preparations of other lysosomal proteases such as cathepsins Ce1, Ce2, and Ce3 [Sarkis et al., 1988] do not do so (J.D. Ashcom and L.A. Jacobson, unpublished observations). We have also found that double mu-

tants (*unc-52 cad-1*) with severe genetic deficiency of cathepsin D activity [Hawdon et al., 1989] do not inactivate the β-galactosidase fusion protein in muscle any more slowly than do wild-type animals and show no abnormal accumulation of degradation intermediates (S.J. Barmada and L.A. Jacobson, unpublished observations). Finally, we have found that pure β-galactosidase is very rapidly inactivated ($t_{1/2}$ = 1.5 h) in vitro by denaturation at pH 5. Thus, if the initial proteolytic cleavages had occurred only after entry into the acidic lysosomal compartment, it is very unlikely that we would have recovered the cleavage products in active tetramers. Although these considerations mitigate against the lysosomal compartment as the site of initial proteolytic attack on the fusion protein, they in no way exclude the possibility that later steps of fusion protein degradation may take place in the lysosomes.

On the other hand, we have detected ubiquitinated degradation intermediates in affinity-purified β-galactosidase from partially starved animals (Fig. 4), suggesting that a ubiquitin-mediated proteolytic pathway plays a role at some stage in the degradation of the fusion protein. However, this evidence does not prove that ubiquitin conjugation is necessary for inactivation, nor indeed whether ubiquitin conjugation precedes or follows initial proteolytic attack. A polyubiquitin gene [Graham et al., 1989] and genes for ubiquitin-conjugating enzymes [Zhen et al., 1993, 1996; Leggett et al., 1995] have been identified in *C. elegans*. So far as we know, there is no biochemical evidence for the existence of a proteasome complex in *C. elegans* that preferentially degrades ubiquitin conjugates, although unpublished *C. elegans* sequences homologous to proteasome subunits are in the GenBank database. If fusion protein inactivation and degradation involves a ubiquitin-mediated system, our data imply that starvation must upregulate ubiquitin conjugation and/or subsequent proteolysis at the level of enzyme activity, and not primarily at the level of enzyme synthesis. By contrast, the enhanced protein degradation induced in rat muscle by fasting or denervation is accompanied by increased levels of mRNA for both polyubiquitin [Medina et al., 1991] and 14-kDa ubiquitin-conjugating enzyme [Wing and Banville, 1994], although these have not been shown to be causally related to enhanced protein degradation.

Transgene Products as Reporters of Intracellular Proteolysis

Our experiments show that transgene-encoded fusion proteins are convenient "reporters" for studying the processes of intracellular proteolysis and their physiological regulation. The method has hitherto been exploited only rarely [Bachmair et al., 1986, 1993] but offers the significant advantage that appropriate transgenes may be constructed to limit expression to specific cell types, so that proteolytic processes in those cells can be studied in vivo. It must be borne in mind that such transgene-encoded fusion proteins are not normally found in these cells, and thus may not be "representative" of those proteins normally present. For example, we used a myosin-β-galactosidase fusion to study proteolysis in muscle, but there is no reason to suppose that proteolytic degradation of myosin, whether or not in myofilaments, proceeds by the same biochemical mechanism or is regulated in the same way. We also caution that the region immediately surrounding the point of fusion may have a relatively disordered structure and thus be unusually susceptible to proteolytic attack. There is some suggestion of this in the accumulation of a degradation intermediate (Fig. 4) corresponding to the intact *lacZ* portion (116 kDa) of our fusion polypeptide.

We will report elsewhere (J.J. Hartman, N.J. Szewczyk, M.J. Zirwas, and L.A. Jacobson, unpublished data) that the rate of β-galactosidase fusion inactivation in *C. elegans* is also controlled by neural inputs to the muscle cells. We find that β-galactosidase inactivation in body wall muscle is promoted by mutations that decrease presynaptic synthesis or release of acetylcholine, or that block postsynaptic response to acetylcholine. In such mutants, the body-wall muscle is genetically "denervated." Conversely, β-galactosidase is stabilized by agonists acting at postsynaptic nicotinic acetylcholine receptors in body-wall muscle. In view of the well-known effect of denervation in promoting proteolysis in vertebrate muscle, these observations suggest that transgene-encoded fusion proteins in *C. elegans* muscle may also be useful models for studying the regulation of proteolytic processes by nerve–muscle communication.

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