

Identification of a Putative Structural Gene for Cathepsin D in *Caenorhabditis elegans*

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ABSTRACT

Mutants of *Caenorhabditis elegans* having about 10% of wild-type activity of the aspartyl protease cathepsin D have been isolated by screening. Mutant homozygotes have normal growth rates and no obvious morphological or developmental abnormalities. The mutant gene (*cad-1*) has been mapped to the right extremity of linkage group II. Heterozygous animals (*cad-1/+*) show intermediate enzyme levels and animals heterozygous for chromosomal deficiencies of the right extremity of linkage group II have 50% of wild-type activity. Cathepsin D purified from a mutant strain has a lower activity per unit mass of pure enzyme. These data suggest that *cad-1* is a structural gene for cathepsin D.

PROTEOLYTIC processes are important in regulating enzyme levels, in reducing dietary proteins to constituent amino acids and in the maturation of primary translation products, but the particular proteolytic enzymes involved have been identified in very few instances. In most cases, it has even proven difficult to determine whether a particular proteolytic process occurs principally in the lysosomes, principally in the cytosol, or in some measure in each compartment (HOLZER and HEINRICH 1980; HERSHKO and CIECHANOVER 1982; PONTREMOLI and MELLONI 1986).

We are investigating this problem by the isolation of mutants deficient in individual, identified proteolytic enzymes, in the belief that single-gene mutations provide the most unambiguous route to determining the function(s) of particular proteases in various cellular processes.

For this analysis, we have chosen the small soil nematode *Caenorhabditis elegans*, because of its well-known advantages for genetic analysis (BRENNER 1974; STERNBERG and HORVITZ 1984) and the presence of a fully developed lysosomal system. Nematode lysosomes contain a variety of proteases and peptidases (SARKIS *et al.* 1988). In most cases these have close analogs in mammalian cells with respect to enzymatic mechanism and specificity, although some nematode proteases have specificity properties which clearly distinguish them from their mammalian an-

alogs or homologs. The complement of lysosomal acid glycosidases and phosphatases is comparable to that found in mammalian cells, with detailed similarities extending even to molecular properties of individual enzymes (BOLANOWSKI, JACOBSON and RUSSELL 1983). The lysosomes in the intestinal cells of *C. elegans* take up exogenous macromolecules by fluid-phase pinocytosis (CLOKEY and JACOBSON 1986), much as do the lysosomes in many types of mammalian cells.

SARKIS *et al.* (1988) showed that *in vitro* proteolysis by crude extracts of *C. elegans* was strongly inhibited (>95%) by the polypeptide pepstatin, which specifically inhibits the action of the aspartyl protease cathepsin D. It was suggested that cathepsin D might be responsible for initial proteolytic attack on many native proteins, prerequisite to subsequent cleavage by other proteases and peptidases.

Accordingly, cathepsin D was our first target for the isolation of protease-deficient mutants. We report here the isolation of mutants which contain about 10% of the normal amount of cathepsin D activity. The mutations lie in a gene which we designate *cad-1*, at the right extremity of linkage group II. Animals heterozygous for the mutant alleles show intermediate enzyme levels and mutant homozygotes produce cathepsin D which, when purified, shows a lower specific activity *in vitro* than the cathepsin D produced by wild-type animals. On the basis of these observations, we suggest that *cad-1* is the structural gene for lysosomal cathepsin D in *C. elegans*.

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MATERIALS AND METHODS

Nematodes: *C. elegans* var. Bristol and its derivatives were used in this study. All mutant strains are descended from the wild-type strain N2 (BRENNER 1974). The markers used for various mapping experiments were *unc-29(e1072) I*, *sqt-1(scl) II*, *sqt-2(sc3) II*, *rol-1(e91) II*, *rol-6(e187) II*, *unc-4(e120) II*, *dpy-10(e128) II*, *fer-15(b26) II*, *unc-52(e669) II*, *let-257(mn235) II*, *let-259(mn210) II*, *unc-32(e189) III*, *dpy-13(e184) IV*, and *dpy-11(e224) V*. Genetic nomenclature follows HORVITZ *et al.* (1979). Mutant alleles isolated in our laboratory are given the prefix "j" followed by an isolation number and strains originating in our laboratory are prefixed by "PJ."

Stock cultures and small numbers of experimental animals were grown in 100-mm Petri dishes containing NG agar and lawns of *Escherichia coli* OP50 (BRENNER 1974). Growth was at 16° unless otherwise indicated. Large scale cultures were grown in liquid medium and the nematodes separated from excess *E. coli* as described by SARKIS *et al.* (1988).

Age-synchronous cultures were initiated by picking gravid hermaphrodites onto fresh plates to lay eggs, then removing the parental animals after 4 hr. The age of the animals is given from the middle of the egg-laying period. Because the amount of cathepsin D per animal is strongly dependent upon the age of the animal, we attempted during genetic experiments to do enzyme assays on animals of nearly the same age. In some genetic crosses, mated hermaphrodites were removed from the mating plates and allowed to lay eggs on fresh plates for 4–8 hr. When sufficient cross-progeny could not be obtained in this way, we simply tried to pick animals of about the same size and apparent age for assay.

Measurement of nematode growth rates: Age-synchronous populations of wild-type or mutant animals were grown at 25°. At intervals, 10–12 randomly chosen animals were videotaped, using a Panasonic video camera attached to a Wild dissecting microscope. During subsequent playback, lengths were measured directly from single-frame images on the video screen at a net magnification of $\times 105$ –210.

Isolation of mutants deficient in cathepsin D: Wild-type *C. elegans* (strain N2) were treated with 50 mM EMS for 4 hr according to BRENNER (1974). The worms were washed free of EMS and placed on plates with *E. coli* lawns for 1 hr to lay eggs. Ten mutagenized parental animals were picked onto each of 10 plates and incubated for 24 hr at 16°, then the parental animals were removed. The plates were incubated at 16° for 60 hr, then 5 F₁ progeny picked from the parental plates onto each of 200 plates. The F₁ were allowed to lay eggs for 24 hr at 16°, then removed from the plates. After 60 hr, approximately 48 F₂ were picked from each plate, one worm per well of a microtiter plate. Each well contained 40 μ l of 0.1 M acetate buffer, pH 5, with 0.1% NP-40. The lysate in each well was then assayed for *in vitro* inactivation of *E. coli* β -galactosidase as follows.

The worms were lysed by six cycles of freezing in liquid N₂ and thawing at about 10°, then the plates were allowed to stand at room temperature for 1 hr. Each well received 10 μ l of a solution containing 82 mM acetate buffer, pH 5, 50 mM 2-mercaptoethanol and 0.8 units/ml of *E. coli* β -galactosidase (Sigma Chemical grade VIII, specific activity 600–900 units/mg protein). The plates were incubated overnight at 37° to allow digestion of the β -galactosidase by proteases in the worm lysates. Each well then received 50 μ l of a substrate solution containing 100 mM Tris-HCl buffer, pH 8.25, 10 mM MgCl₂, 10 mM 2-mercaptoethanol

and 0.4 mM 4-methylumbelliferyl- β -D-galactoside such that the final pH of the reaction mixture was 7.5. The development of fluorescence due to hydrolysis of the substrate was monitored periodically under long-wave UV illumination for 4–8 hr. Wells showing strong purple-blue fluorescence, *i.e.*, those in which the β -galactosidase activity had survived incubation with the lysate, were considered to contain candidate protease-deficient mutants.

In some earlier experiments, we used *o*-nitrophenyl- β -D-galactoside as substrate. In these cases, the yellow color of the product was visualized after the addition of 50 μ l of 0.25 M NaHCO₃ to each well.

The progeny from 7 out of 200 F₂ plates showed positives in 2 or more microtiter wells, as well as some weaker positives. From each of these 7 plates, 30–40 sibling F₂ were picked onto Petri plates (one animal per plate), grown for 4 days at 16°, then 8 F₃ progeny from each plate reassayed for *in vitro* inactivation of β -galactosidase. One subclone, the only one which showed strong positives for all F₃ progeny, was maintained. Upon direct assay, this strain proved to have about 10% of wild-type cathepsin D levels per animal. This strain was named PJ01, the mutated gene named *cad-1*, and the mutant allele designated *jl*.

We assigned *cad-1* to a linkage group as follows: Wild type males were mated to *cad-1* mutant homozygotes (PJ01) and cathepsin D levels in the F₁ males compared to those in wild-type males. If *cad-1* were X-linked, the XO F₁ males would have been hemizygous for the *cad-1* mutation. From the fact that the two groups of males had identical enzyme levels, we inferred that *cad-1* was not X-linked.

Next we mated homozygous *cad-1* males (PJ01) to a series of strains, each homozygous for a visible mutation on one of the autosomal linkage groups. F₁ cross-progeny were picked, and from each cross about 30 F₂ animals homozygous for the visible marker were picked. F₃ progeny of each of these individuals were then assayed for cathepsin D levels. In the case of linkage groups I and III–V, the *cad-1* marker segregated approximately 1:2:1 (wild-type: heterozygotes: *cad-1* homozygotes) among the F₂, indicating that *cad-1* was segregating independently of the visible markers used. In the case of *dpy-10 II*, on the other hand, there was a large excess of *cad-1*⁺/*cad-1*⁺ animals among the homozygous Dpy F₂ (Table 2), indicating that *cad-1* was linked to *dpy-10* on LGII.

In the course of subsequent mapping experiments, we observed that *unc-52* homozygotes (any of five different alleles) were themselves markedly deficient (about 10% of wild-type) in cathepsin D. In contrast to the intermediate enzyme levels in *cad-1*⁺ heterozygotes (Table 1), *unc-52*/⁺ heterozygotes had normal enzyme levels. This effect on cathepsin D levels was apparently not specific to *unc-52* mutants, since other "uncoordinated" mutants showed degrees of cathepsin D deficiency which were more or less commensurate with their deficits in movement. For example, paralyzed animals homozygous for *unc-54(e190)* had about 15% of normal cathepsin D level, whereas animals with milder behavioral phenotypes (*unc-27(e155)* or *unc-101(m1)*) had 50–60% of normal level and some "uncoordinated" mutants (*unc-32(e189)*, *unc-4(e120)* and *unc-29(e1072)*) had essentially normal cathepsin D levels. An *unc-45(e286)* mutant, whose degree of behavioral deficit depends upon growth temperature (EPSTEIN and THOMSON 1974), had 20% of normal cathepsin D level when grown at nonpermissive temperature (25°) and 70% when grown at the permissive temperature (16°).

We will report elsewhere on this phenomenon, which probably involves physiological depression of cathepsin D levels by reduced food intake. For the present purpose of

mapping *cad-1* with respect to *unc-52*, we avoided problems by mapping in *cis* (F_1 *cad-1 unc-52/+ +*) and scoring for *cad-1* amongst the *unc-52⁺/unc-52⁺* F_2 (see Table 2).

After the *cad-1* gene had been assigned to linkage group II, PJ01 males were crossed with homozygous *unc-52 II* hermaphrodites, the non-Unc F_1 cross-progeny picked, and a *cad-1 unc-52* double homozygote chosen from among the Unc F_2 by assaying F_3 progeny for extreme deficiency of cathepsin D. The selection of the double mutant homozygotes was aided by the fact that the animals were smaller than *unc-52* homozygotes which were not also homozygous for *cad-1*. The *cad-1* mutation was then separated from *unc-52* by crossing the double mutant homozygote to wild-type males, choosing a non-Unc, cathepsin D-deficient (*unc-52 cad-1/+ cad-1*) F_2 and propagating to obtain a *cad-1* homozygote (strain PJ02) which no longer segregated Unc (paralyzed) progeny. Thus, strain PJ02 [*cad-1(j1) II*] has been outcrossed twice and in each outcross has experienced a recombination event between *cad-1* and *unc-52*. Strain PJ02 was used for all subsequent experiments.

Two new mutant alleles were then isolated by treating wild-type (N2) males with EMS, mating to *cad-1 unc-52* hermaphrodites and screening the non-Unc F_1 for cathepsin D deficiency, using the microtiter plate assay for inactivation of β -galactosidase. Mutant candidates were recovered from the F_2 progeny (from eggs laid by the F_1 before assay) and obtained in homozygous form by choosing animals which segregated no paralyzed progeny. Only one representative of each original mating plate was retained. Cathepsin D deficiency in these new isolates was then confirmed by direct enzyme assay.

Isolation of chromosomal deficiencies: Chromosomal deficiencies (see Figure 3) uncovering the *unc-52* marker on the right end of LGII were isolated in three different ways:

First, wild-type (N2) males were irradiated with 1500 rad of γ -radiation from a ^{137}Cs source, then mated to hermaphrodites of genotype *mnC1 dpy-10 unc-52 II*. *mnC1* is a presumed chromosomal rearrangement which acts as a dominant suppressor of recombination (HERMAN 1978) between *dpy-10* and *unc-52* (normally about 24 cM apart). The F_1 were screened visually for animals which were Unc (*unc-52* homozygotes are paralyzed) but not Dpy, and two individuals were found. Both of these segregated Unc and Dpy Unc progeny in approximately 2:1 ratio. Successive propagation of Unc individuals identified none which failed to segregate Dpy Unc progeny, suggesting that each of the two new strains carried a mutant chromosome deficient for *unc-52*, but not *dpy-10*.

The mutant chromosome from the first isolate proved to carry deficiency *jDf1*, which failed to complement all tested markers on the right half of LGII, including *unc-4*, *fer-15*, *rol-1*, *let-257*, *unc-52*, *let-259* and *cad-1*, but not *rol-6*. This places the left endpoint of *jDf1* right of *rol-6* and left of *unc-4*. The second isolate carried deficiency *jDf2*, which proved to uncover *unc-52*, *cad-1* and *let-259*, but not *ace-3*, *let-257* or any marker farther left on LGII. (Complementation tests with lethals were performed according to SIGURDSON, SPANIER and HERMAN (1984). The complementation test with *ace-3* was done by J. B. RAND). This deficiency was later stored as a strain of genotype *unc-4 let-257/unc-4 jDf2*, in which form the deficiency is stably maintained.

Second, we mutagenized *unc-4/mnC1 dpy-10 unc-52* animals with HCHO (MOERMAN and BAILLIE 1981) and selected paralyzed non-Dpy progeny. The single resulting isolate evidently contained a mutation which failed to complement *unc-52*, but the chromosome carrying this *unc-52* mutation

complemented all other LGII markers tested. We classify this as a deficiency (*jDf4*) on two grounds: (1) repeated outcrossing to wild-type males failed to detect any viable segregant which was homozygous for the new *unc-52* mutation and (2) heterozygotes of genotype *jDf4/jDf2* are inviable, as shown by the following experiment. Hermaphrodites of genotype (*mnC1 dpy-10 unc-52/jDf2*) were crossed with wild-type males and the F_1 males crossed to *dpy-10 unc-4/unc-4 jDf4* hermaphrodites. Individual paralyzed (Unc-52) cross-progeny were propagated. All of these segregated both paralyzed and paralyzed Dpy progeny, and were thus of genotype *mnC1 dpy-10 unc-52/unc-4 jDf4*. The absence of paralyzed animals which segregated only paralyzed non-Dpy progeny shows that *jDf2/jDf4* animals are inviable, presumably because the overlapping deficiencies both uncover at least one essential gene.

Third, we screened for spontaneous recombinants in a background containing the *mnC1* crossover suppressor. Plates of *unc-4/mnC1 dpy-10 unc-52* animals were screened visually for animals which were paralyzed but not Dpy. Approximately 15 independent isolates were obtained, five of which (carrying *jDf5*, *jDf6*, *jDf7*, *jDf8* and *jDf9*) have been characterized further. All of these deficiencies were homozygous inviable (*cf.* HERMAN 1978) and genetically indistinguishable from *jDf2*, in that they uncovered *unc-52*, *let-259* and *cad-1*, but not other LGII markers. Some were coupled to *unc-4(e120)* in the original isolates.

Deficiency *jDf2* was used to map the lethal marker *let-259* as follows. Males of genotype *unc-4 let-259/unc-52* were constructed and immediately crossed with paralyzed hermaphrodites of genotype *jDf2/mnC1 dpy-10 unc-52*. Non-Unc F_1 cross progeny were picked and propagated individually, then scored for the segregation of the marked chromosomes in the F_2 . Because *jDf2* uncovers both *unc-52* and *let-259*, the wild-type F_1 mostly (110 of 115) segregated only wild-type and Dpy Unc progeny and thus represented nonrecombinants of inferred genotype *unc-4 let-259/mnC1 dpy-10 unc-52* or recombinants of genotype *let-259/mnC1 dpy-10 unc-52*. We also found five individuals which, as a result of recombination between *let-259* and *unc-52*, inherited a chromosome bearing neither *let-259* nor *unc-52* from the male parent. These recombinants were of three classes: (1) those segregating wild type and substantial numbers of Unc-4 progeny (2 of 115), of presumed genotype *unc-4 + +/jDf2*; (2) those segregating only wild-type progeny (2 of 115), of presumed genotype *+ + +/jDf2*; and (3) those segregating wild type, paralyzed Dpy and substantial numbers of Unc-4 progeny (1 of 115), of presumed genotype *unc-4 + +/mnC1 dpy-10 unc-52*.

These results indicate that the distance between *unc-52* and *let-259* (as measured in the male parent) is about 2 cM.

Assay of cathepsin D: Worms to be assayed (usually ten per tube) were hand-picked into 20 μl of lysing buffer (0.1 M acetate, pH 5, containing 0.2% NP-40) and lysed by six cycles of freezing in liquid N_2 and thawing at about 10° . Reactions (0.2 ml) contained 0.1 M sodium formate buffer, pH 4, FITC-hemoglobin (prepared according to RINDERKNECHT 1962) at a final concentration of 2.5 mg/ml, and 50 nM leupeptin (synthetic hemisulfate, Sigma Chemical Co.). Reactions were initiated by the addition of FITC-hemoglobin, then incubated for 1–5 hr at 37° .

Reactions were stopped by the addition of an equal volume of 10% (w/v) Cl_3CCOOH and the precipitated proteins removed by centrifugation at 10° for 5 min in a Fisher microcentrifuge (approximately $12,000 \times g$). A sample (0.1 ml) of the supernatant was withdrawn, diluted with 0.9 ml of 0.2 M NaOH, and the fluorescence measured

in a Farrand Mark I spectrophotofluorimeter at an excitation wavelength of 490 nm and an emission wavelength of 515 nm. Control reactions without extract, or with extract added after the Cl_3CCOOH , were included in each experiment and the average fluorescence of four such blanks subtracted from each experimental value.

One unit of enzyme activity is defined as that amount which produces a net fluorescence increase of 0.001 in 1 hr under these conditions. This corresponds to the release of 0.64 fmol of peptide-bound FITC per hour. We have determined that digestion of FITC-hemoglobin under these conditions is totally inhibited by 0.1 mM pepstatin; thus, the assay is specific for the pepstatin-sensitive aspartyl protease, cathepsin D (L. A. JACOBSON, in preparation).

Analytical methods for proteins: Procedures for preparation of extracts and subsequent SDS gel electrophoresis or analytical slab gel isoelectric focusing were as given by SARKIS *et al.* (1988). Protein concentrations in solutions of pure cathepsin D were determined with fluorescamine (UDENFRIEND *et al.* 1972) using pepsin as standard.

For immunodetection of cathepsin D after separation by electrophoresis or isoelectric focusing, we used a polyclonal rabbit antibody against pure nematode cathepsin D, with detection by the binding of goat anti-rabbit IgG, labeled with ^{125}I . Bands on autoradiograms were quantitated by densitometric scanning in a transverse direction (*i.e.*, perpendicular to the direction of electrophoresis) with a slit width slightly greater than the width of the bands. Thus, the integrated intensity measured the total area of each band. Control experiments indicated that this integrated intensity was proportional to the amount of protein applied to each lane. Details of the enzyme purification, antibody production and immunodetection methods will be given elsewhere (L. A. JACOBSON *et al.*, in preparation).

RESULTS

Isolation of mutants deficient in cathepsin D: To simplify the isolation of protease-deficient mutants, we sought to score the proteolytic inactivation of an "indicator" enzyme. *E. coli* β -galactosidase proved to be a suitable indicator. There is little or no neutral β -galactosidase activity in *C. elegans* (BOLANOWSKI, JACOBSON and RUSSELL 1983) and reconstruction experiments with crude worm extracts or lysates of individual wild-type animals showed that *in vitro* inactivation of *E. coli* β -galactosidase was totally inhibited by pepstatin. The latter observation suggested that β -galactosidase inactivation required the activity of cathepsin D.

We found that β -galactosidase was irreversibly denatured by prolonged incubation at pH 4, the normal optimum for cathepsin D activity. β -Galactosidase was also inactivated at pH 5, but could be reactivated upon titration to pH 7.5 provided that a reducing agent (2-mercaptoethanol) was present throughout, and that Mg^{2+} was added upon renaturation.

These considerations led us to design the mutant identification protocol described in MATERIALS AND METHODS. Control experiments showed that lysates of single wild-type worms completely inactivated the β -galactosidase under these conditions, whereas in

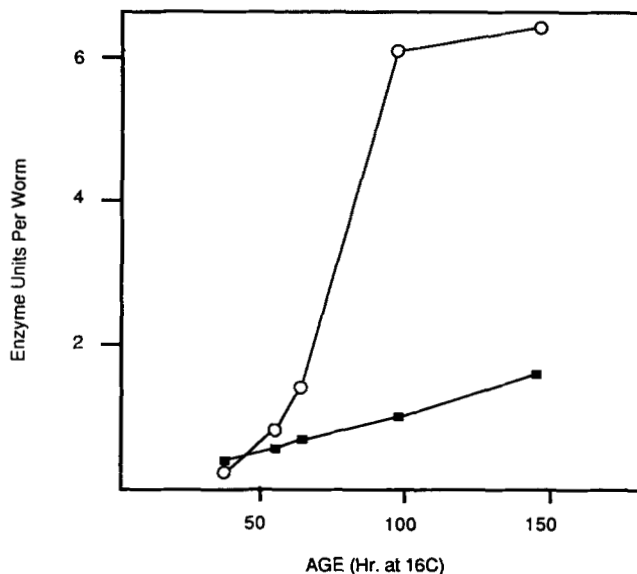


FIGURE 1.—Age-dependence of cathepsin D activity. (○) Wild-type strain N2; (■) mutant strain PJ02 [*cad-1(j1) III*].

the presence of pepstatin, survival of β -galactosidase was nearly as good as in the absence of worm lysate.

Our first isolation attempt, using EMS as mutagen, led to the identification of a single mutant. The mutant allele was named *j1* in a gene we designate as *cad-1*. This isolate was outcrossed twice (see MATERIALS AND METHODS) before being used for subsequent experiments.

The *cad-1(j1)* homozygote proved upon enzyme assay to be deficient in cathepsin D activity *in vitro*. As shown in Figure 1, the level of cathepsin D in each worm increases as the animal grows to adulthood. The mutant strain had 10–20% of the wild-type cathepsin D level at adulthood, indicating that its deficiency of cathepsin D was not simply the result of delayed development. In fact, the growth rate and developmental schedule of the mutant appears to be nearly the same as that of the wild type (Figure 2).

Morphologically, the *cad-1* mutant was unexceptional after outcrossing to remove extraneous mutations. Its principal visible abnormality is a "clear" or "bubbled" appearance of the intestinal cells upon examination with a dissecting microscope or with high magnification Nomarski differential interference contrast optics. In the electron microscope, the intestinal cells appear more highly vacuolated than those of wild-type animals. The rate of uptake of fluorescent-labeled proteins and dextrans into the lysosomes of the intestinal cells (CLOKEY and JACOBSON 1986) appeared normal.

We then used a strain containing the first *cad-1* mutation to screen for additional mutations in the same complementation group (see MATERIALS AND METHODS). These screens led to the isolation of the independent *j12* and *j14* mutations. As shown in

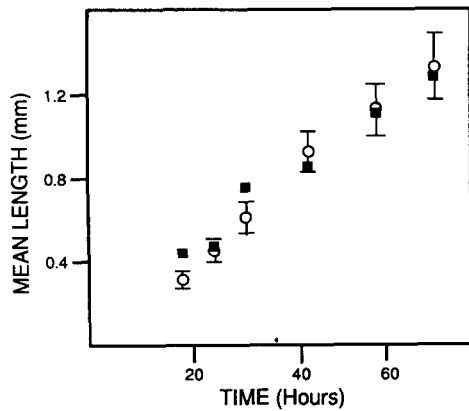


FIGURE 2.—Growth rates of wild-type strain N2 (○) and mutant strain PJ02 (■) at 25°. Details of procedure are given in MATERIALS AND METHODS. Standard deviations (error bars) for both data sets were of comparable magnitude; error bars for PJ02 have been omitted for clarity.

TABLE 1

Complementation tests and gene dosage for *cad-1* alleles

Strain or construction	Genotype at <i>cad-1</i>	Cathepsin D ^a	
		Enzyme units/M ₁	Percent wild type
Wild type	+/+	6.2 ± 1.7	100
<i>cad-1</i>	<i>j1/j1</i>	0.7 ± 0.4	12
<i>cad-1;unc-32</i>	<i>j1/j1</i>	0.8 ± 0.3	13
<i>cad-1</i>	<i>j12/j12</i>	0.5 ± 0.1	8
<i>cad-1</i>	<i>j14/j14</i>	0.7 ± 0.2	11
wt ♂ × <i>cad-1;unc-32</i>	<i>j1/+</i>	3.7 ± 0.4	59
<i>cad-1</i> ♂ × <i>unc-32</i>	<i>j12/+</i>	3.8 ± 0.7	62
<i>cad-1</i> ♂ × <i>unc-32</i>	<i>j14/+</i>	3.9 ± 0.4	63
<i>cad-1(j12)</i> ♂ × <i>cad-1(j1);unc-32</i>	<i>j12/j1</i>	1.3 ± 0.1	21
<i>cad-1(j14)</i> ♂ × <i>cad-1(j1);unc-32</i>	<i>j14/j1</i>	0.9 ± 0.2	15
<i>dpy-10 unc-4/jDf1</i>	<i>jDf1/+</i>	3.5 ± 0.2	57
<i>unc-4 let-257/unc-4 jDf2</i>	<i>jDf2/+</i>	3.4 ± 0.6	54
<i>unc-4 let-257/unc-4 jDf7</i>	<i>jDf7/+</i>	3.6 ± 0.4	58

^a Data show means and standard deviations of quadruplicate or triplicate (deficiency heterozygotes only) determinations on ten-worm samples.

Table 1, these new presumptive alleles failed to complement the original *j1* allele by direct assay of cathepsin D activity in heteroallelic strains.

The *j12* allele, like the *j1* allele, is closely linked to *unc-52*, since 8/8 *unc-52*⁺/*unc-52*⁺ segregants from *j12/unc-52* animals had a deficiency of cathepsin D comparable to that of *j12* homozygotes. Furthermore, 10 of 10 *unc-4/unc-4* recombinants in the F₂ progeny of *j12/unc-4 unc-52* animals were deficient in cathepsin D, confirming that *j12* lies on the right side of LGII. We therefore think it likely that the *j1*, *j12* and *j14* mutations are allelic.

Dominance and gene dosage effects: The cathepsin D level in *j1/+* heterozygotes was midway between that of mutant and wild-type homozygotes (Table 1). Similar results were obtained with the *j12*

and *j14* alleles (Table 1). In strains carrying chromosomal deficiencies (see below) which uncovered the *cad-1* locus, and which were thus hemizygous for *cad-1*⁺, cathepsin D levels were about one-half of that found in wild-type animals (Table 1).

The gene dosage effects observed are consistent with the hypothesis that *cad-1* is a structural gene for cathepsin D (also see below). Furthermore, the ability to distinguish heterozygotes from mutant homozygotes or wild types by enzyme assay greatly simplified the genetic mapping of the *cad-1* mutation.

Mapping the *cad-1* mutation: After assigning the *cad-1* mutation to LGII (see MATERIALS AND METHODS), we chose four visible markers on LGII for a series of two-point crosses. The data (Table 2) indicate that *cad-1* lies on the right end of LGII, about 1 cM from *unc-52*.

The paucity of markers with visible phenotypes at the right extremity of LGII, compelled us to use lethal markers for the ordering of genes. We first mapped *let-259* with respect to *unc-52* (see MATERIALS AND METHODS). The results indicate a recombinational distance of about 2 cM between *let-259* and *unc-52*. Because the *unc-4* marker is so far (21 cM) from *unc-52*, however, the gene order was not established unambiguously in this experiment.

We then constructed a heterozygote of genotype *unc-4 let-259/cad-1 unc-52* and picked viable Unc-4 progeny. These animals carried at least one chromosome which had sustained a recombination event between *unc-4* and *let-259*. Of 305 Unc-4 animals whose progeny we examined, 303 segregated both Unc-4 and Unc-52 (paralyzed) progeny, and thus had sustained no second recombination event. Two Unc-4 animals segregated only Unc-4 and no paralyzed progeny, and had thus sustained a second recombination event between *let-259* and *unc-52*.

If *let-259* lay right of *unc-52*, recombinants of the latter type could have arisen by a single crossover between *unc-52* and *let-259*. The predicted incidence of these animals would be 2 of 23 or about 9%. If *let-259* lay left of *unc-52*, the predicted incidence of these double crossovers would be 2% (since we forced a crossover between *unc-4* and *let-259* in all animals examined). The data are clearly more consistent with the latter alternative, implying the gene order *unc-4-let-259-unc-52* (Figure 3). It seems likely that the observed incidence of double crossovers was reduced by interference.

To test the segregation of *cad-1* in those chromosomes which had recombination events between *unc-52* and *let-259*, progeny homozygous for the recombinant chromosome were obtained by picking individuals and scoring for increased brood size and the absence of larval lethality (homozygous *let-259* segregants). These Unc-4 animals were then assayed for cathepsin D deficiency. We found one recombinant

TABLE 2
Mapping of *cad-1* by two-point crosses

Marker ^a	F ₂ Selected		Observed No. of			Distance (cM)
	Phenotype	Genotype	<i>cad</i> ⁺ / <i>cad</i> ⁺	<i>cad</i> ⁺ / <i>cad</i>	<i>cad</i> / <i>cad</i>	
<i>dpy-10(e128)</i>	Dumpy	<i>dpy/dpy</i>	23	15	2	24
<i>unc-4(e120)</i>	Uncoordinated	<i>unc/unc</i>	21	12	2	23
<i>sqt-2(sc02)</i>	Roller	<i>sqt/+</i>	6	28	16	33
<i>sqt-1(sc01)</i>	Roller	<i>sqt/+</i>	10	42	8	18
<i>unc-52(e669)</i> ^b	Non-Unc ^c	<i>unc</i> ⁺ / <i>unc</i> ⁺	114	2	0	0.9

^a Hermaphrodites homozygous for each of these LGII markers were crossed with homozygous *cad-1(j1)* mutant males. Cross progeny from Dumpy or Uncoordinated parents were recognized as non-Dumpy or non-Unc. Cross progeny from Squat parents were recognized as rollers. These F₁ were picked and animals showing the indicated phenotype in the F₂ were separately propagated so that the *cad-1* genotype could be scored by assaying the F₃ for cathepsin D. Appropriate wild-type, *cad-1/cad-1* and *cad-1/+* animals were included in each experiment as controls.

^b This cross was performed in *cis*, as wild-type males × *cad-1 unc-52* hermaphrodites, such that the F₁ was *cad-1 unc-52/+ +*.

^c F₃ chosen for assay were progeny of F₂ which segregated no uncoordinated progeny in the F₃; thus the F₂ genotype was *unc-52*⁺/*unc-52*⁺.

which had normal cathepsin D levels, and one which had cathepsin D deficiency comparable to that of *j1* homozygotes. This indicates that *cad-1* probably lies between *let-259* and *unc-52*.

In an attempt to confirm this gene order, we isolated a series of chromosomal deficiencies (see MATERIALS AND METHODS), all of which were selected for their failure to complement *unc-52*. Of the eight independent deficiencies, six proved to uncover *cad-1* and *let-259* as well as *unc-52* (Figure 3), but did not uncover *let-257*. These data are consistent with the placement of *cad-1* between *let-257* and *unc-52*, but do not further resolve the order of *let-259* and *cad-1*.

The genetic map in Figure 3 summarizes our present knowledge of the genes at the right end of LGII.

Properties of the mutant cathepsin D: The reduced levels of cathepsin D in the *cad-1* mutants could, in principle, represent a reduction in the amount of enzyme present, a reduction in the catalytic activity of the enzyme, or both. We have explored this question in several ways.

First, we purified cathepsin D from both wild-type and mutant (PJ02) animals by affinity chromatography on immobilized pepstatin (L. A. JACOBSON, in preparation). The two preparations showed identical band patterns on SDS gel electrophoresis, including essentially identical distribution among the multiple cathepsin D bands [32–44 kilodaltons (kDa)] characteristic of the wild-type enzyme. The catalytic activity of the mutant enzyme, however, was about tenfold lower (on a per-mass basis) than that of the wild-type enzyme (Figure 4). These data show that each molecule of mutant enzyme has less catalytic capacity than each molecule of wild-type enzyme, suggesting that the mutation has produced a structural alteration in cathepsin D.

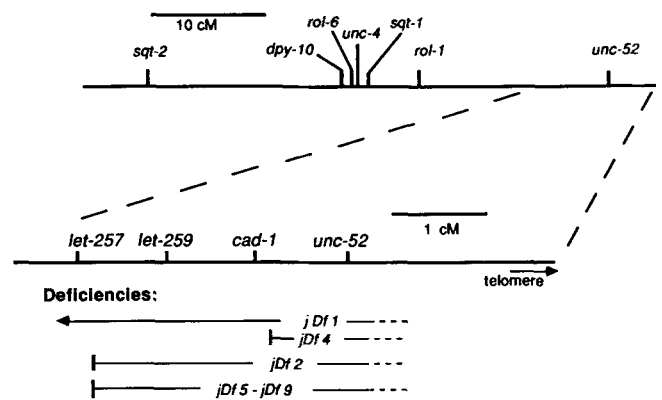


FIGURE 3.—Genetic map of linkage group II of *C. elegans*.

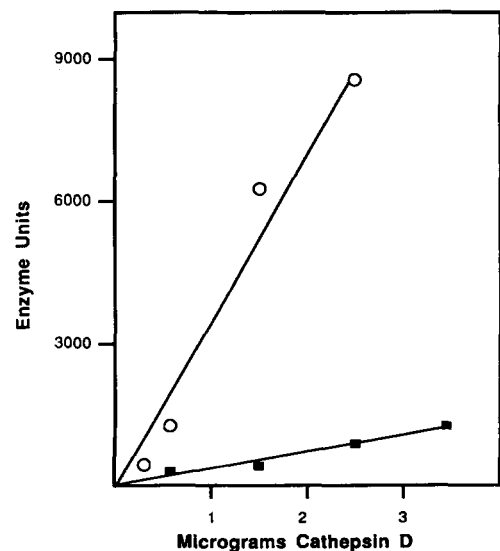


FIGURE 4.—Activity of cathepsin D purified from strain N2 (○) or from mutant strain PJ02 (■). Procedures for activity assays and protein determinations are given in MATERIALS AND METHODS.

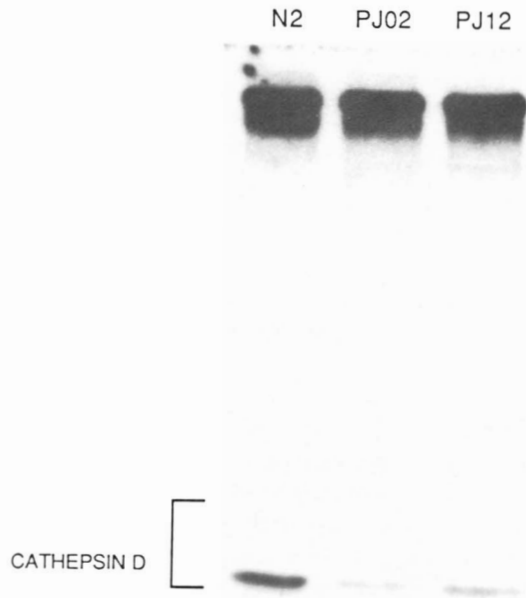


FIGURE 5.—Immunoblot analysis of cathepsin D content of wild-type strain N2 and the *cad-1* mutant strains PJ02 (*j1*) and PJ12 (*j12*). Equal amounts (15 μ g) of crude extract protein were loaded into wells of a 12.5% acrylamide SDS slab gel. After electrophoresis and transfer to nitrocellulose membrane, cathepsin D was detected with polyclonal rabbit anti-cathepsin D and 125 I-labeled goat anti-rabbit IgG, followed by autoradiography. The identity of the cross-reacting material at the top of the gel (>205 kDa) is unknown, but it is evidently not cathepsin D (see text).

Second, we made crude lysates of mutant and wild-type animals, subjected these to SDS gel electrophoresis and estimated the amount of cathepsin D present in each lysate by immunodetection with polyclonal anti-cathepsin D antibody. The results (Figure 5) show that strains homozygous for either the *j1* or *j12* alleles contain a slightly reduced amount of cathepsin D antigen. The autoradiograms from four independent experiments similar to that in Figure 5 were quantitated densitometrically. If the integrated intensity of the cathepsin D band from wild-type animals is assigned a relative value of 100, the corresponding integrated intensity (mean \pm SD) for *j1* homozygotes was 52 ± 10 and for *j12* homozygotes it was 47 ± 20 . This two-fold decrement in cathepsin D antigen content is too small to account for the 8 to 12-fold lower activity in the mutant animals (*cf.* Table 1).

There was no indication of any change in the M_r of cathepsin D in the mutant homozygotes. Note that the unidentified cross-reacting material at >205 kDa did not appear in affinity-purified cathepsin D, and fractions enriched in this band were devoid of cathepsin D activity. It is not an aggregated form of cathepsin D, since its migration was not affected by the inclusion of 8 M urea or nonionic detergents in

the SDS gels. We believe this material was detected because of fortuitous immunological cross-reaction.

Third, we measured the activity of cathepsin D in crude extracts of *cad-1* mutant and wild-type worms, and in mixtures of these extracts in various proportions. In all cases, the activities of the mutant and wild-type extracts were strictly additive, eliminating the remote possibility that the mutant had lower cathepsin D activity because of the presence of an unusual inhibitor.

Despite the altered catalytic activity of the mutant product of the *j1* allele, we have detected no other change in physicochemical properties. The temperature-activity profiles of the *j1* mutant and wild-type enzymes are identical ($Q_{10} = 1.96$ in the range 16° to 45°), as are their pH optima and thermal inactivation rates ($t_{1/2} = 40$ min at 50°). Furthermore, we observed no alteration in isoelectric point of cathepsin D produced in strains homozygous for any of the mutant alleles, under conditions where even a single charge difference should have been detectable.

DISCUSSION

The number of genes for cathepsin D: The affinity-purified cathepsin D from *C. elegans* shows 5–6 distinct bands on SDS gel electrophoresis and fractions enriched in each of these bands show equivalent enzymatic activity (L. A. JACOBSON, unpublished observations). We must therefore consider the possibility that “cathepsin D” represents more than one gene product. For both mutant and wild-type cathepsin D purified by affinity chromatography, about 80–90% of the protein and the activity reside in two bands at 34 kDa and 36.5 kDa. Amino acid sequence data (L. A. JACOBSON, unpublished observations) show that these two bands are identical for at least the 12 amino acids at the N terminus, and are thus probably products of the same gene. Lectin-binding data indicate that these are glycoproteins which differ in the attached glycosides (L. A. JACOBSON, unpublished data); glycoside variations may be sufficient to account for the migration differences on SDS gels. We do not yet know if the C-terminal amino acid sequences are different.

The minor bands in cathepsin D preparations (about 32 kDa, 38 kDa and 44 kDa) have not yet been purified free of the 34-kDa and 36.5-kDa major components. Fractions enriched in these minor forms have specific activities no lower than those of the purified major forms, so the minor bands are unlikely to be inactive contaminants. It is at present impossible to determine if these represent variant processed forms of the major components or distinct proteins.

***cad-1* is probably a structural gene:** Two lines of evidence indicate that *cad-1* is a structural gene for cathepsin D. First, heterozygotes (*cad-1/+*) have in-

intermediate enzyme levels and *cad-1*⁺ hemizygotes contain about half the normal level of cathepsin D. This proportionality between gene dosage and enzyme level is expected for a structural gene, and has been observed for other presumed structural genes in *C. elegans* (JOHNSON *et al.* 1981; RAND and RUSSELL 1984).

Second, purified cathepsin D from a strain homozygous for the *j1* mutant allele had about 10% of the catalytic activity, on a mass basis, of the corresponding pure enzyme from a wild-type strain. Alteration of the catalytic properties of an enzyme is most consistent with a mutation in the structural gene.

This conclusion is somewhat complicated by the observation (Figure 5) that both the *j1* and *j12* mutations reduce the amount of cathepsin D antigen present in mutant homozygotes by about twofold. The fact that mutant heterozygotes contain intermediate enzyme levels (Table 1) suggests that the mutations are unlikely to affect a *trans*-acting regulatory element, and we have seen no evidence from SDS gels that the *j1*, *j12* or *j14* mutations affect the post-translational processing of cathepsin D. We think it most probable that the mutations have rendered the mutant cathepsin D proteins somewhat less stable *in vivo*, and that this accounts for the twofold decreased amount of antigen present.

In order to have reduced the catalytic activity of cathepsin D by about tenfold, the *cad-1* mutation must lie in the gene which codes for the two predominant forms of cathepsin D. In the affinity purification of cathepsin D from the *cad-1* mutant, however, the 34-kDa and 36.5-kDa major bands were not resolved from the minor bands, so the formal possibility remains that the *cad-1(j1)* mutation has rendered the major forms completely inactive (yet still able to bind to pepstatin), with 10% residual activity deriving from the minor bands as products of another gene or genes. We cannot rigorously exclude this possibility.

None of the three mutations we have isolated produces any change in the physical properties of cathepsin D. In particular, we have observed no change in isoelectric focusing behavior under conditions where mutational alteration of even a single charged amino acid should have been readily detected. The further observation that the molecular weight distribution of cathepsin D forms is unaltered in the mutants indicates that post-translational proteolytic processing and glycosylation are also unaffected by the mutations. In the absence of such evidence for direct physical effects of mutation in the *cad-1* gene on cathepsin D, we believe that correspondence between the nucleotide sequence of the *cad-1* gene and amino acid sequence of the major forms of cathepsin D will provide the only conclusive proof that *cad-1* is a structural gene.

Is cathepsin D a dispensable enzyme? So far as we know, this is the first report of mutations affecting a lysosomal protease in any metazoan. Although there are many well-known cases of genetic deficiency in lysosomal glycosidases, deficiencies in lysosomal proteases had not been observed, leading to speculation that such mutations might have lethal consequences (HOLTZMANN 1976). It may be significant in this context that all *cad-1* mutants so far isolated are "leaky," with 3–10% residual cathepsin D activity relative to the wild-type. We do not yet know whether true null alleles of *cad-1* would be lethal.

Despite the fact that cathepsin D is required for >95% of the *in vitro* proteolysis by crude extracts of *C. elegans*, it is evident that the *cad-1* mutant strains survive, without noticeable impairment, with <10% of the normal amount of enzyme activity. We have also observed that double mutants, homozygous for the *j1* allele of *cad-1* and either *unc-52(e669) II* or *daf-4(e1364) III*, may have as little as 1–5% of normal cathepsin D levels, presumably because the *unc-52* or *daf-4* mutations impose a net nutritional deficit and consequent underproduction (or underaccumulation) of cathepsin D protein (J. HAWDON and L. A. JACOBSON, unpublished observations). These double mutants have no further morphological, developmental or behavioral abnormalities beyond those normally associated with *unc-52* or *daf-4* mutant homozygotes (with the single exception of the somewhat reduced size of *cad-1 unc-52* double mutant homozygotes). These observations suggest that cathepsin D has no unique or indispensable physiological role in *C. elegans*.

Similar questions have arisen in evaluating the effects of mutations which affect protease activities in other organisms. In *Saccharomyces cerevisiae*, for example, mutants deficient in vacuolar acid proteases A and B also show little obvious abnormality (MECHLER and WOLF 1981; WOLF and EHMANN 1979) except under very special physiological conditions (WOLF and EHMANN 1979). It has been suggested, therefore, that the many kinds of proteases found in eukaryotic cells and tissues have at least partially redundant functions in protein degradation *in vivo*, such that even a drastic decrement in activity of any single protease has little net effect on the overall rate of protein degradation. In *C. elegans*, SARKIS *et al.* (1988) have described at least four other proteases, but it is not yet clear to what extent these enzymes may share the ability to attack particular target proteins.

It should be borne in mind, however, that the choice of any one protein as substrate for *in vitro* proteolysis may present a deceptive picture of the proteolytic process *in vivo*, where a mixture of proteases acts in concert (and/or sequentially) upon a mixture of protein substrates. We believe that a mutational analysis of several proteases, in conjunc-

tion with direct measurement of proteolysis rates *in vivo*, will be required to assess the physiological role of each of the proteases.

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