

# Do Damaged Proteins Accumulate in *Caenorhabditis elegans* L-Isoaspartate Methyltransferase (*pcm-1*) Deletion Mutants?<sup>1</sup>

Agnieszka Niewmierzycka and Steven Clarke<sup>2</sup>

Department of Chemistry and Biochemistry and the Molecular Biology Institute,  
University of California, Los Angeles, California 90095-1569

Received November 3, 1998, and in revised form January 11, 1999

The protein L-isoaspartate (D-aspartate) O-methyltransferase (E.C. 2.1.1.77) can initiate the conversion of isomerized and racemized aspartyl residues to their normal L-aspartyl forms and has therefore been hypothesized to function as a repair enzyme, responsible for helping to limit the accumulation of damaged proteins in aging organisms. In this study, the effect of a disruption in the *pcm-1* gene encoding the L-isoaspartyl methyltransferase was investigated in the nematode *Caenorhabditis elegans*. It was found that damaged proteins recognized by this enzyme accumulated to significant levels during long-term incubation of both *pcm-1*<sup>+</sup> and *pcm-1*<sup>-</sup> nematodes in a specialized larval stage called the dauer. The L-isoaspartyl methyltransferase-deficient mutants accumulated about twice the level of damaged proteins as the control nematodes during dauer aging. The mutants also accumulated higher levels of damage when both strains were incubated at 30°C for up to 3 days. However, when nonviable nematodes were removed in a Percoll separation, similar levels of damage were measured between the two strains following both dauer aging and 30°C incubation. Both strains were able to effectively eliminate damaged proteins recognized by the methyltransferase after recovery from dauer. Characterization of the methyl-accepting polypeptide substrates which accumulate in aged dauers revealed that although substrates of all molecular weights are present, the majority of substrates are peptides not precipitated by acetone. These results suggest that protein degradation, rather than repair, may be the major mechanism by which *C. elegans* eliminates damaged proteins containing L-isoaspartyl residues. © 1999 Academic Press

**Key Words:** methyltransferases; protein aging; protein repair.

Protein L-isoaspartate (D-aspartate) O-methyltransferase methylates the free carboxyl group of L-isoaspartyl and D-aspartyl residues on peptides and proteins to bring about their conversion to the L-aspartyl form (1). A major source of L-isoaspartyl and D-aspartyl residues is the spontaneous degradation of L-aspartate and L-asparagine residues of proteins over time (2). The isomerization of aspartyl residues causes the addition of a methylene group into the polypeptide backbone which can alter the conformation and compromise protein function (3–6). The protein L-isoaspartate O-methyltransferase has been shown to “repair” L-isoaspartyl residues *in vitro*, partially restoring the activity of damaged proteins such as calmodulin (7) and the bacterial phosphocarrier protein HPr (8). The L-isoaspartate O-methyltransferase is well conserved in a broad range of organisms including bacteria (9), plants (10), insects (11), nematodes (12), and mammals (13–15). Significantly, only the mammalian enzyme appears to be capable of recognizing D-aspartyl residues (16); the nonmammalian enzymes appear to recognize only L-isoaspartyl residues (9, 12).

The unique catalytic conversion which the L-isoaspartate O-methyltransferase carries out and its high level of conservation have led to the hypothesis that it may function to limit the accumulation of damaged proteins in aging organisms (17). Indeed, knockout mice lacking this methyltransferase have recently been shown to accumulate proteins with aspartyl and asparaginyl damage in all tissues, with the highest levels in the brain (18). The accumulation of damaged proteins is time-dependent and was found to be be-

<sup>1</sup> This work was supported by the National Institutes of Health Grant GM26020 (S.C.) and Medical Scientist Training Program GM08042 (A.N.).

<sup>2</sup> To whom correspondence should be addressed. Fax: 310-825-1968. E-mail: [clarke@ewald.mbi.ucla.edu](mailto:clarke@ewald.mbi.ucla.edu).

tween four- and eightfold higher in the null mutants than in wild-type mice at about 4 weeks of age (18). In addition, the L-isoaspartate methyltransferase-deficient mice showed a striking retardation of growth and most died of seizures at an average of 42 days after birth (18).

In contrast to the knockout mice, disruption of the L-isoaspartyl methyltransferase has had more subtle effects in other organisms. For example, a recent study has shown that a null mutant of the L-isoaspartyl methyltransferase in *Escherichia coli* survives normally during long-term growth in stationary phase (19), a stage which may be considered analogous to mammalian aging. The viability of the *E. coli* mutant was also normal when subjected to several different stresses such as heat shock and osmotic stress, although exposure to low concentrations of methanol and paraquat, high salt concentrations, or repeated heating at 42°C resulted in a reduction in viability of the mutants in stationary phase (19). In addition, the *E. coli* mutants showed a competitive disadvantage when grown in stationary phase with wild-type cells (19). However, no differences were observed in the levels of methyl-accepting substrates for the L-isoaspartyl methyltransferase between the mutant bacteria and wild-type control cells (20). The disparate effects of L-isoaspartyl methyltransferase deficiency on mammals and bacteria suggested that the role of this enzyme needed to be reevaluated.

In this study, the function of the L-isoaspartyl methyltransferase (*pcm-1*) in the nematode *Caenorhabditis elegans* was addressed. A recent construction of a null *C. elegans* mutant of this enzyme has provided a new system to study the function of the L-isoaspartyl methyltransferase (21). The L-isoaspartate methyltransferase-deficient mutants exhibit normal behavior and morphology, and a normal maximum lifespan of adult nematodes of about 20 days was measured (21). However, survival of the mutant nematodes was reduced 3.5-fold compared to the wild-type nematodes after 50 days in a specialized larval stage called dauer (21). The dauer larval stage is a developmentally arrested, non-aging stage which nematodes enter upon starvation or overcrowding (22). Dauer larvae can survive several months without feeding, a time period significantly longer than the maximum lifespan of adult nematodes (22). Interestingly, the activity of the L-isoaspartyl methyltransferase in wild-type nematodes in dauer was shown to increase twofold compared to wild-type adult nematodes (12). It was therefore hypothesized that the L-isoaspartyl methyltransferase may be important in maintaining the integrity of proteins during dauer, and its absence may be responsible for the reduced survival of mutant dauers. In addition, a 16% reduction in fitness was observed for the *pcm-1* deletion nematodes when the ability of these mutants to

compete reproductively against a wild-type population in mixed culture was measured (21). The purpose of the present study was to biochemically characterize L-isoaspartyl methyltransferase-deficient nematodes in order to further define the role of the L-isoaspartyl methyltransferase in an aging organism.

## EXPERIMENTAL PROCEDURES

**Growth and harvesting of *C. elegans* nematodes.** The L-isoaspartyl methyltransferase-deficient *C. elegans* strain used in this study was obtained by Tc1 transposon mutagenesis (*pcm-1*; XA201; Ref. (21)). Mutagenesis was carried out in the strain MT3126 (23), and the XA201 strain, which lacked exons 2–5 in the *pcm* gene, was isolated. This strain was outcrossed with the male-producing strain CB1489 (24) eight times and then selfed, utilizing CB1489 males and XA201 hermaphrodites in order to eliminate the MT3126 parent strain mutator background. The strain CB1489 was used as the wild-type control strain in this study. Liquid cultures of nematodes were grown by inoculating S medium containing *E. coli* OP50 cells and 50 units/liter nystatin (Sigma) with nematodes from saturated NGM plates as described (25). In several experiments nematodes were maintained on NGM or peptone rich plates seeded with OP50 *E. coli* (25, 26). After the nematodes were collected, they were typically cleared of bacteria by M9 washes and sedimentation at 4°C.

Sucrose floatation was performed as described (12, 25). Briefly, nematodes were resuspended in 0.1 M NaCl, an equal volume of ice-cold 60% (v/v) sucrose was added, and the suspension was centrifuged at 1500g for 5 min at 4°C. The surface layer of nematodes was collected and washed with 0.1 M NaCl solution. Percoll separation was performed as described (27) to separate live and dead dauers. Briefly, for each sample, 10 ml of ice-cold 40% (v/v) Percoll in M9 buffer was placed in a 15-ml Falcon tube. A thin layer of a worm suspension (<2 mm) was layered on the surface of the Percoll and centrifuged in a clinical centrifuge at 1500g at 4°C for 10 min. By visual inspection, the pellet typically contained >90% live worms, while >90% dead worms remained at the top of the Percoll.

**Preparation of *C. elegans* cytosolic extracts.** Nematodes were harvested as described above and the worm pellet was resuspended in an equal volume of homogenizing buffer (50 mM Tris-HCl pH 8, 10 mM sodium ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N'*-tetracetic acid, 2 mM sodium EDTA, 0.25 M sucrose, 0.5 mM dithiothreitol, 40  $\mu$ g/ml each of leupeptin and pepstatin, 200  $\mu$ M phenylmethylsulfonyl fluoride). Between 20 and 100  $\mu$ l of the nematode suspension was then placed in a mortar containing liquid nitrogen. Immediately after the liquid nitrogen evaporated, the frozen suspension was homogenized by grinding with a pestle. The frozen lysate was collected, allowed to thaw, and cellular debris was pelleted in microfuge tubes at 16,000g (using an Eppendorf Model 5415C Microcentrifuge) for 10 min at 4°C. Protein concentrations were determined using a Coomassie Plus Protein Assay Reagent (Pierce) against a standard of bovine serum albumin.

**Quantification of methyl-accepting proteins in cytosolic extracts.** The number of methyl-accepting residues in nematode cytosolic proteins was determined by using the human or nematode L-isoaspartate O-methyltransferase purified as described (hPCM,<sup>3</sup> Ref. (28); cPCM, Ref. (12)). The reaction mixture generally consisted of 8.9  $\mu$ M S-adenosyl-L-[methyl-<sup>14</sup>C]methionine (Amersham, 54 mCi/mmol), 12 to 66  $\mu$ g of cytosolic protein, human L-isoaspartate methyltrans-

<sup>3</sup> Abbreviations used: AdoMet, S-adenosyl-L-methionine; [<sup>14</sup>C]AdoMet, S-adenosyl-L-[methyl-<sup>14</sup>C]methionine; hPCM, recombinant human L-isoaspartate (D-aspartate) O-methyltransferase; cPCM, recombinant *C. elegans* protein L-isoaspartate (D-aspartate) O-methyltransferase.

ferase (1  $\mu$ l of a preparation with an activity of 1.8 pmol methyl groups transferred/min/ $\mu$ l) or *C. elegans* L-isoaspartate methyltransferase (4  $\mu$ l of a preparation with an activity of 0.65 pmol methyl groups transferred/min/ $\mu$ l), and 0.2 M sodium citrate, pH 6.0, to a final volume of 50  $\mu$ l. Reactions were incubated at 37°C (hPCM) or 22°C (cPCM) for 30, 60, or 90 min. The number of methyl groups transferred from [<sup>14</sup>C]AdoMet to L-isoaspartyl (or for the human enzyme, L-isoaspartyl and D-aspartyl) sites were measured using a vapor diffusion assay (29). In this assay, 50  $\mu$ l of 0.2 M NaOH and 1% (w/v) sodium dodecyl sulfate were added to each reaction and a 70- $\mu$ l aliquot was spotted on a 1.5  $\times$  2.7-cm piece of thick filter paper (Bio-Rad 165-090) which was inserted into a 1.5-ml microfuge tube. The opened microfuge tube containing the filter paper was placed upright in a 20-ml scintillation vial containing 6 ml of Safety-Solve counting fluor (RPI) in such a way that the filter paper did not come in contact with the scintillation fluid during the assay, but that the volatile radioactivity can diffuse from the filter paper to reach the scintillation fluid. The scintillation vial was then capped and allowed to equilibrate at room temperature for 2 h to allow the [<sup>14</sup>C]methanol produced by methyl ester hydrolysis to diffuse into the fluor. The microfuge tube containing the filter paper was then removed and the radioactivity in the fluor was counted. Each sample was assayed in duplicate or triplicate and an enzyme-only blank was subtracted as the background, representing an average of 98 pmol methyl groups transferred per milligram of cellular protein (for hPCM) or 26 pmol/mg (for cPCM). In order to monitor the nematode strains to confirm that the L-isoaspartyl methyltransferase activity is absent in the *pcm-1* mutant, assays were carried out as above using 12–66  $\mu$ g cytosolic protein from each strain as the enzyme source and 0.22 mM of the peptide Val-Tyr-Pro-L-IsoAsp-His-Ala (prepared as described in Ref. (15)) as the methyl acceptor.

**Dauer recovery experiments.** Nematodes in the following dauer recovery experiments were aged at 25°C on NGM plates and dauer entry was induced by food depletion. Dauers from 28-day-old nematode cultures were isolated, washed with M9 buffer, and transferred to the unseeded side of a 9-cm semiseeded plate. Dauers were allowed to crawl to the seeded side of the plate for 10 h and live nematodes were collected. The nematodes were washed three times by cold sedimentation in M9 buffer and then stored at –80°C. Dauers from 35-day-old nematode cultures to be used in recovery assays were washed in M9 buffer and then incubated for 16 h in 100 ml S medium seeded with OP50 *E. coli* prepared as described (25). The nematodes were then collected, washed in M9 buffer, and then transferred to the unseeded side of five semiseeded plates prepared with 25  $\mu$ M fluorodeoxyuridine (FudR) to maintain a synchronous population (30). Nematodes were allowed to crawl toward food for 24 or 72 h and were then collected from the seeded side of the plates, washed three times by cold sedimentation in M9 buffer, and stored at –80°C.

## RESULTS

**Levels of cytosolic methyl-accepting substrates in 5-day-old *pcm-1* and wild-type nematodes from liquid cultures.** The *C. elegans* disruption mutants of *pcm-1* which were constructed recently (21) were used in this study to determine the effect of L-isoaspartate methyltransferase deficiency on the levels of damaged proteins in the nematode. It was confirmed at the beginning of the study and at various times throughout that L-isoaspartate methyltransferase activity was completely absent in *pcm-1* mutants (0.010  $\pm$  0.014 pmol methyl groups transferred/min/mg) in comparison to the control strain (0.94  $\pm$  0.17 pmol methyl groups transferred/min/mg).

TABLE I

Methyl-Accepting Substrate Levels in Cytosolic Proteins of 5-Day-Old Wild-Type (wt) and L-Isoaspartate Methyltransferase-Deficient (*pcm-1*) Nematodes<sup>a</sup>

Substrate source <sup>b</sup>	$\mu$ g of protein	pmol methyl groups/mg protein <sup>c</sup>
wt	33	93 $\pm$ 16
<i>pcm-1</i>	33	70 $\pm$ 1
wt	66	82 $\pm$ 1
<i>pcm-1</i>	66	92 $\pm$ 16

<sup>a</sup> Liquid cultures of control (wt) and L-isoaspartate methyltransferase-deficient (*pcm-1*) nematodes were incubated for 5 days at 20°C with shaking at 200 rpm (25). Nematodes were pelleted at 4°C at 3000g, washed with 0.1 M NaCl solution, and cleaned by sucrose floatation as described under Experimental Procedures. Nematodes were rotated at 25°C for 30 min to allow the digestion of any remaining bacteria present in the gut. Worms were spun down and stored at –80°C before cytosol preparation as described under Experimental Procedures.

<sup>b</sup> Methyl-accepting substrate levels in cytosolic extracts of control (wt) and L-isoaspartate methyltransferase deficient (*pcm-1*) dauer nematodes were assayed in duplicate for 30 min at 37°C using recombinant human L-isoaspartate methyltransferase as described under Experimental Procedures.

<sup>c</sup>  $\pm$  Standard deviation.

As a first step to characterizing the effect of an L-isoaspartyl methyltransferase deficiency in *C. elegans*, the levels of damaged proteins in cytosolic extracts of L-isoaspartate methyltransferase deficient nematodes were assessed compared to a wild-type control strain. Although the effect of a deficiency in this enzyme was expected to be most apparent after long-term incubation of the nematodes, when proteins had enough time to accumulate damage, an initial measurement was made in fresh worms. Cytosols from 5-day-old cultures of *pcm-1* mutants and wild-type nematodes were tested for methyl-accepting substrates of this enzyme using the recombinant human L-isoaspartate methyltransferase as an analytical probe as described under Experimental Procedures. As expected for fresh worms, there was no significant difference in the levels of methyl-accepting substrates in cytosolic extracts from *pcm-1* and wild-type nematodes (Table I). While wild-type nematode extracts contained an average of 88 pmol methyl-accepting sites/mg extract protein, methyltransferase-deficient *pcm-1* extracts contained an average of 81 pmol/mg (Table I).

**Levels of cytosolic methyl-accepting substrates in *pcm-1* and wild-type dauers aged in liquid cultures.** It was reasoned that 5 days may be an insufficient amount of time to accumulate enough spontaneously damaged proteins for a difference to be detected between the *pcm-1* and wild-type strains. Also, since the entire life cycle of *C. elegans* is 3 days (22), new larvae

are present in large numbers in a 5-day mixed nematode culture, and protein synthesis is active. Any differences in levels of damaged proteins between the L-isoaspartate methyltransferase deficient nematodes and the control strain could be masked by new protein synthesis. In order to synchronize the worms and to allow damaged proteins to accumulate for extended periods of time, nematodes were induced to enter a specialized larval stage called the dauer. This nonaging, developmentally arrested stage allows the worms to survive for much longer periods of time than adult nematodes. In addition, in contrast to adult nematodes, dauers deficient in L-isoaspartate methyltransferase have impaired survival compared to wild-type dauers (21).

Nematodes were induced to enter dauer by allowing saturated liquid cultures of the *pcm-1* and control strains to become depleted of food (2 weeks from the time of inoculation; dauers are first present after about 1 week). Dauers were isolated, placed in M9 buffer and incubated with shaking for an additional 29 days at 20°C. At days 0 and 29 after placement in M9, dauers were collected and their cytosols were assayed for methyl-accepting substrates. After 43 days in culture, there was a significant accumulation of methyl-accepting substrates in cytosolic extracts from both strains (Table II, experiment 1). While fresh dauers prepared from a 2-week liquid culture showed levels of damaged proteins of 55 pmol/mg for wild-type and 134 pmol/mg for *pcm-1*, the levels of damage increased 6.9- and 5.0-fold, respectively, for the two strains aged for a total of 43 days (Table II, experiment 1). In addition, methyl-accepting substrate levels in L-isoaspartyl methyltransferase-deficient dauers were about 2-fold higher than in the control strain after both 14 and 43 days in culture. These results show that both nematode strains accumulate damaged proteins with time and that the L-isoaspartyl methyltransferase may slow down accumulation of damaged proteins in the aging wild-type nematode.

In order to confirm that the *pcm-1* nematodes accumulate a greater number of damaged proteins compared to the control strain as observed in the previous experiment, a second similar study was undertaken. After allowing nematodes of both strains to deplete liquid cultures of food and enter dauer (2 weeks), dauers were placed in three flasks of M9 for each strain and incubated with shaking for 17 days. The incubation temperature was raised to 26°C in this experiment in an attempt to speed up the accumulation of L-isoaspartyl residues. Dauers were collected on days 0 and 17 after placement in M9 buffer, and cytosols were assayed for levels of methyl-accepting substrates. In order to assure that all available methyl-accepting substrates were detected in the assay of damaged substrates, a low amount of protein (12 µg) and long

TABLE II  
Methyl-Accepting Substrate Levels in Cytosolic Extracts of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Dauer Nematodes<sup>a</sup>

Substrate source <sup>b</sup>	Age of culture (days)	pmol methyl groups/mg protein <sup>c</sup>
Experiment 1 (aging at 20°C)		
wt	14	55 ± 15
<i>pcm-1</i>	14	134 ± 6
wt	43	381 ± 62
<i>pcm-1</i>	43	678 ± 74
Experiment 2 (aging at 26°C)		
wt	14	280 ± 47
<i>pcm-1</i>	14	338 ± 136
wt	31	943 ± 211
<i>pcm-1</i>	31	1717 ± 77

<sup>a</sup> Control (wt) and methyltransferase-deficient (*pcm-1*) dauers were prepared by incubating nematodes at 20°C in S medium with OP50 *E. coli* for 14 days with shaking at 200 rpm. Upon entering the dauer stage, the nematodes were cooled on ice, spun down at 3000g, and washed with M9 buffer. Nondauers were eliminated by incubation in 30 ml of 1% sodium dodecyl sulfate at 25°C for 30 min. Dauers were isolated using sucrose floatation as described under Experimental Procedures and washed with M9 buffer. About 100 mg of each strain of nematodes was saved at -80°C as the 14-day time point. For experiment 1, the remaining dauers were placed in 2 × 250-ml flasks containing 100 ml of M9 buffer each and were shaken in a water bath at 20°C for 29 additional days. Aged dauers from both strains were collected, pelleted at 3000g, and saved immediately at -80°C for later analysis. For experiment 2, dauers were placed in M9 buffer (3 × 125-ml fluted flasks per strain containing 50 ml M9 buffer), and aged at 26°C for 17 days with shaking at 200 rpm. On Days 0 and 17 of incubation in M9 buffer, dauers were collected, washed with M9 buffer, and saved at -80°C. The results in experiment 2 are from Kagan *et al.* (21).

<sup>b</sup> Methyl-accepting substrate levels in cytosolic extracts of control (wt) and L-isoaspartate methyltransferase-deficient (*pcm-1*) dauer nematodes were assayed at 37°C for 30 min (experiment 1) or 90 min (experiment 2) using recombinant human L-isoaspartate methyltransferase as described under Experimental Procedures. In experiment 1, the values and the standard deviations shown are based on an average of two separate assays. Different amounts of cytosolic proteins were used in the first assay: 14 days, 216 µg (wt) and 193 µg (*pcm-1*); and 43 days, 69 µg (wt) and 56 µg (*pcm-1*). In the second assay, 12.4 µg cytosolic protein was used for all samples. In experiment 2, assays were performed in hexuplicate using 12 µg cytosolic protein.

<sup>c</sup> ±Standard deviation.

incubation times (90 min) were used (Table II, experiment 2).

As seen in the previous experiment, the level of damaged proteins in dauers aged for a total of 31 days increased significantly in comparison to 14-day-old dauers, with a 3.4-fold increase for the wild-type strain and a 5.1 increase for the *pcm-1* strain. In addition, there was again an almost 2-fold increase in the level of

methyl-accepting substrates in the aged *pcm-1* strain versus the wild-type strain ( $1717 \pm 77$  pmol/mg vs  $943 \pm 211$  pmol/mg, respectively; Table II, experiment 2). Interestingly, the levels of damaged substrates in this experiment was about 2.5-fold higher than in the previous experiment, presumably due to the increased temperature used in this experiment for culturing the dauer worms.

The high level of damaged cytosolic proteins which accumulate in the wild-type strain with aging was surprising because the methyltransferase is expected to be fully active in wild-type nematodes and should be able to maintain the level of damage to a minimum. It was reasoned the 16,000*g* centrifugation of cytosolic extracts during the preparation of nematode cytosols may not have eliminated plasma membranes, organelles, and extracellular material. A fraction of the proteins of these components are normally inaccessible to the cytosolic methyltransferase, and the presence of these components could contribute a high background of methylation during *in vitro* assays. However, when cytosolic extracts from wild-type dauers (day 14 or day 31 dauers from above) were centrifuged at 100,000*g* for 1 h, the levels of methyl-accepting substrates remained the same as seen with centrifugation at a 16,000*g* (332 pmol/mg versus 281 pmol/mg for 14-day cytosolic extracts and 921 pmol/mg versus 943 pmol/mg for aged cytosolic extracts centrifuged at 100,000*g* and 16,000*g*, respectively). Therefore, centrifugation of cytosolic extracts at 16,000*g* yields mainly cytosolic methyl acceptors, and the high level of substrates measured in wild-type nematodes appear to be cytosolic.

*Levels of cytosolic methyl-accepting substrates in pcm-1 and wild-type dauers aged on NGM plates and enriched for live worms by Percoll centrifugation.* In the above experiments, it was observed that in the later time point of dauer aging, a significant number of worms appeared dead in the aliquots collected for methyltransferase assays. It was suspected that these dead worms might contribute to the unusually high background methylation observed in wild-type aged nematodes. The following experiments were carried out in which aliquots of aged dauers were enriched for live dauers by centrifugation through a Percoll gradient (27). For these experiments, dauers were aged on NGM plates instead of liquid culture to reduce the chance of contamination during long-term aging.

Nematodes of both strains (*pcm-1* and control) were placed on *E. coli* seeded agar plates and allowed to saturate each plate and enter dauer after food depletion. The plates were incubated at 25°C for a total of 20 or 34 days from the time of inoculation to harvesting. Centrifugation of aged dauers through a Percoll gradient resulted in a greater than 90% separation of live and dead worms. The levels of methyl-accepting sub-

TABLE III

Methyl-Accepting Substrate Levels in Cytosolic Extracts of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Aged Dauer Nematodes Fractionated by Density to Live and Dead Worms<sup>a</sup>

Substrate source <sup>b</sup>	Percoll separation fraction	Age of culture (days)	pmol methyl groups/mg protein <sup>c</sup>
wt	Live	20	327 ± 12
<i>pcm-1</i>	Live	20	364 ± 20
wt	Live	34	1444 ± 251
<i>pcm-1</i>	Live	34	1383 ± 131
wt	Dead	34	2775 ± 307
<i>pcm-1</i>	Dead	34	3470 ± 84

<sup>a</sup> Nematodes were inoculated on 5 × 5-cm NGM plates seeded with OP50 *E. coli* and were grown at 25°C for 20 or 34 days. Dauers were collected and centrifuged through a Percoll gradient to separate live and dead dauers as described under Experimental Procedures. Data for live 34-day worms are from Kagan *et al.* (21).

<sup>b</sup> Methyl-accepting substrate levels in cytosolic extracts of control (wt) and L-isoaspartate methyltransferase deficient (*pcm-1*) dauer nematodes were assayed in duplicate for 90 min at 37°C using recombinant human L-isoaspartate methyltransferase and 12 μg (6-day dauers) or 25 μg (20-day dauers) cytosolic protein as described under Experimental Procedures.

<sup>c</sup> ±Standard deviation.

strates in cytosolic extracts from live aged dauers increased significantly with the age of the dauers (Table III) in a way similar to that seen for unfractionated worms (Table II). However, in contrast to unfractionated preparations of dauers, aged *pcm-1* and control dauers enriched for live worms did not differ in the levels of methyl-accepting substrates (Table III). Surprisingly, when Percoll fractions containing mostly dead worms were assayed, the levels of methyl-accepting substrates were the highest seen in any experiment and were 2 to 2.5 times higher than in similarly aged live worms (Table III). In addition, dead *pcm-1* worms showed a level of damaged proteins higher than that seen for wild-type dead worms (Table III). This result suggests that the presence of dead worms may have contributed to the increased levels of damaged proteins measured in previous experiments for *pcm-1* mutant cytosolic extracts.

*The effect of dauer recovery on the levels of cytosolic methyl-accepting substrates in pcm-1 and wild-type aged nematodes.* One possibility for the failure to detect any differences in substrate levels between the live fraction of the L-isoaspartyl methyltransferase-deficient nematodes and the control strain was that during dauer, the L-isoaspartyl methyltransferase could be inactive due to such factors as low levels of ATP (31), the biosynthetic precursor of the methyl group donor AdoMet. It is possible that the absence of the methyltransferase in the mutant would only have a

noticeable effect during recovery of the nematodes from dauer. Experiments were therefore carried out in which aged dauers of both strains were allowed to recover from dauer, and methyl-accepting substrate levels were measured.

Commitment to recovery from dauer stage is known to occur within 1 h after the animal is placed in a fresh environment with food (22). The nematode begins to feed after 2–3 h and resumes development after an additional 8–10 h (22). In these experiments, the dauers were recovered for 10 h, 40 h, and 3 days. For experiments where recovery was carried out for 40 h or 3 days, fluorodeoxyuridine was included in the culture to prevent the nematodes from laying eggs (30). Live dauers were recovered by allowing the dauers to crawl to a source of food on a semiseeded agar plate as described under Experimental Procedures. Only the live fraction of each population was assayed for methyl-accepting substrates.

After recovery for 10 h, the dauers still showed high levels of methyl-accepting substrates, comparable to levels seen before recovery (Table IV versus Table III). This result indicates that after 10 h, the nematodes have not yet begun to repair or replace damaged proteins. There was no difference in the level of methyl-accepting substrates between the *pcm-1* and control strains at this stage (Table IV). After 40 h and 3 days of recovery, the levels of methyl-accepting substrates dropped significantly for both strains compared to pre-recovery levels to about 18% after 40 h and to about 9% after 3 days (Table IV). However, there was again no difference in substrate levels between the strains. The ability of both strains to clear damaged proteins after recovery from dauer indicates that the L-isoaspartyl methyltransferase is not essential for this function in the nematode.

All the experiments until this point were performed using the human L-isoaspartyl methyltransferase to measure methyl-accepting substrates in nematode cytosolic extracts. However, it is unknown whether the human and the worm enzymes have the same substrate specificities, and a high level of activity of the human enzyme on substrates which are not recognized by the worm enzyme could obscure any potential differences between the *pcm-1* mutant and the control strain. Another dauer recovery experiment was therefore performed in which methyl-accepting substrates were assayed using the purified *C. elegans* L-isoaspartyl methyltransferase (12). Dauers aged for 28 days and enriched for live nematodes using Percoll were assayed before and after a 26-h exposure to food. After 26 h of recovery from dauer, the number of damaged sites for both strains dropped to about 85% (Table V). However, using the nematode L-isoaspartyl methyltransferase to assay cytosolic extracts did not reveal any differences between the *pcm-1* mutant and the

TABLE IV  
Methyl-Accepting Substrate Levels (pmol Methyl Groups Transferred per mg Cytosolic Protein<sup>a</sup>) in Cytosolic Extracts of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Live Dauer Nematodes after Recovery from Dauer<sup>b</sup>

	Age of culture (days):	28	35	35
	Recovery time (hours):	10	40	88
wt		3183 ± 301	548 ± 38	315 ± 7
<i>pcm-1</i>		3416 ± 461	667 ± 155	306

<sup>a</sup> ± Standard deviation. Insufficient cytosolic protein was obtained to perform a duplicate assay for the final *pcm-1* data point (35 days and 88 h recovery), so no standard deviation is available.

<sup>b</sup> Dauers aged on NGM plates for either 28 or 35 days were recovered from dauer by transferring the dauers to food for the indicated number of hours as described under Experimental Procedures. Substrate levels in control (wt) and methyltransferase-deficient (*pcm-1*) dauer nematodes were assayed in duplicate for 90 min at 37°C using recombinant human L-isoaspartate methyltransferase and 12 µg cytosolic protein as described under Experimental Procedures.

control strain in either prerecovery or postrecovery samples (Table V). Surprisingly, the values obtained using the purified nematode enzyme were significantly lower than those measured using the human enzyme, suggesting that the human methyltransferase may recognize additional substrates compared to the nematode enzyme.

*Effect of heat exposure on methyl-accepting substrate levels of wild-type and pcm-1 nematodes.* In the experiments above, an attempt was made to induce accumulation of damaged proteins by allowing nematodes to age during the dauer stage. Although damaged proteins accumulated to high levels with aging, a difference in methyl-accepting substrates was not observed between the L-isoaspartyl-deficient nematodes and the control strain. It was reasoned that exposure of nematodes to heat could induce faster formation of L-isoaspartyl residues than is possible during dauer aging and may reveal a difference in substrate levels between the two strains. Since the optimal growth temperature for *C. elegans* is 20°C (26), nematodes were incubated at 30 and 37°C in the following experiments.

Nematodes of both strains (*pcm-1* and control) grown on agar plates were first exposed to 37°C for 19 h. This resulted in the death of all nematodes. In contrast, incubation of nematodes at 30°C caused them to become sluggish, but most survived a 19-h incubation at this temperature. However, when the heat-exposed nematodes were assayed for methyl-accepting sub-

TABLE V

Methyl-Accepting Substrate Levels in Cytosolic Extracts of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Nematodes before and after Recovery from Dauer Measured with the *C. elegans* L-Isoaspartate Methyltransferase<sup>a</sup>

Substrate source <sup>b</sup>	pmol methyl groups/mg protein <sup>c</sup>
wt—prerecovery	58 ± 2
<i>pcm-1</i> —prerecovery	60 ± 5
wt—postrecovery	49 ± 6
<i>pcm-1</i> —postrecovery	50 ± 6

<sup>a</sup> Control (wt) and methyltransferase-deficient (*pcm-1*) nematodes were incubated on NGM plates at 25°C for 28 days. Aged dauers were collected, washed, and enriched for live dauers by centrifugation through a 40% Percoll gradient. Aged dauers from another pool were recovered from dauer by adding fresh food to each plate and incubating at 25°C for 16 h. Live recovered worms were separated by allowing washed worms to crawl towards food on half-seeded plates for 10 h.

<sup>b</sup> Substrate levels in cytosolic extracts of nematodes were assayed for 60 min at 22°C in triplicates (prerecovery) or duplicates (postrecovery) with recombinant *C. elegans* L-isoaspartyl methyltransferase and 33 μg (prerecovery) or 11 μg (postrecovery) of cytosolic protein as described under Experimental Procedures.

<sup>c</sup> ±Standard deviation.

strates, there was no difference in substrate levels between the two strains (Table VI). In another experiment, dauers aged for 26 days and exposed to 30°C for 3 days showed a higher level of methyl-accepting substrates for the *pcm-1* mutant only when unfractionated preparations of nematodes were tested (Table VII). However, in fractions enriched for live nematodes, there was no difference in the levels of methyl-accepting substrates between the *pcm-1* and control strains (Table VII). Interestingly, preliminary experiments suggested that L-isoaspartyl methyltransferase-deficient nematodes incubated at 30°C for up to 7 days die at a faster rate than the control strain (A. Niewmierzycka and S. Clarke, unpublished), suggesting that short-term incubation of nematodes at 30°C may have the same phenotypic consequence as long-term aging of nematodes in dauer. It appears that the absence of L-isoaspartyl methyltransferases can lead to phenotypic effects (such as reduced survival in dauer or at 30°C) in the absence of an observable increase in total levels of methyl-accepting substrates.

The majority of L-isoaspartate methyltransferase methyl-accepting substrates are found in acetone-soluble fractions of cytosolic extracts. Previous experiments characterizing methyl-accepting substrates of the L-isoaspartyl methyltransferase in aged dauers have shown that the majority of these substrates are polypeptides lower than 20 kDa (21). This finding prompted the question whether small cellular peptides

TABLE VI

Methyl-Accepting Substrate Levels in Cytosolic Extracts of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Nematodes Incubated at 30°C for 19 h

Substrate source <sup>a</sup>	MTase source	pmol methyl groups/mg protein <sup>b</sup>
wt	Nematode	34 ± 2
<i>pcm-1</i>	Nematode	24 ± 1
wt	Human	176 ± 3
<i>pcm-1</i>	Human	142 ± 1

<sup>a</sup> Five-day-old control (wt) and L-isoaspartate methyltransferase-deficient (*pcm-1*) nematodes were incubated on *E. coli* seeded plates at 30°C for 19 h. Methyl-accepting substrate levels in 66 μg cytosolic protein from dauer nematodes of each strain were assayed in duplicates for 60 min at 37°C (using the recombinant human L-isoaspartate methyltransferase) or at 22°C (using the *C. elegans* L-isoaspartyl methyltransferase) as described under Experimental Procedures. MTase, methyltransferase.

<sup>b</sup> ±Standard deviation.

may also act as substrates for this enzyme. When total cytosolic extracts from both *pcm-1* and control aged dauer nematodes were fractionated using acetone to separate peptides from proteins, the majority of methyl-accepting species were found in the acetone-soluble fractions of both strains (Table VIII). This result indicated that peptides may account for the majority of methyl acceptors in aged dauers and could be

TABLE VII

Methyl-Accepting Substrate Levels in Cytosolic Extracts of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Aged Dauer Nematodes after 3 Days of Incubation at 30°C

Substrate source <sup>a</sup>	MTase source	pmol methyl groups/mg protein <sup>b</sup>
wt—total	Human	2564 ± 460
<i>pcm-1</i> —total	Human	3183 ± 62
wt—total	Nematode	234 ± 52
<i>pcm-1</i> —total	Nematode	284 ± 36
wt—live	Nematode	72 ± 16
<i>pcm-1</i> —live	Nematode	94
wt—dead	Nematode	451 ± 12
<i>pcm-1</i> —dead	Nematode	374 ± 51

<sup>a</sup> Nematodes were induced into dauer by starvation and aged on NGM plates at 25°C for 28 days. The plates were then placed at 30°C for 3 days. Cytosols were prepared from either total washed worms (total) or washed worms separated on 40% Percoll (live and dead worms). 28 μg of cytosolic protein was assayed in duplicate for 60 min at 37°C (using the recombinant human L-isoaspartate methyltransferase) or 22°C (using the *C. elegans* L-isoaspartyl methyltransferase) as described under Experimental Procedures. MTase, methyltransferase.

<sup>b</sup> ±Standard deviation. Insufficient cytosolic protein was available to perform a duplicate assay for *pcm-1* (live) so no standard deviation is available.

TABLE VIII

Fractionation of Wild-Type and L-Isoaspartate Methyltransferase-Deficient Nematode Cytosols Containing Methyl-Accepting Substrates by Acetone Precipitation<sup>a</sup>

Substrate source <sup>b</sup>	Insoluble (pmol/mg <sup>c</sup> )	Soluble (pmol/mg <sup>c</sup> )
wt	184 ± 0	412 ± 15
<i>pcm-1</i>	334 ± 27	1161 ± 184

<sup>a</sup> Thirty-one-day aged dauers from control (wt) and methyltransferase-deficient (*pcm-1*) strains were same as those described in Table II. 25 µg cytosolic protein from each strain was combined with an equal volume of -20°C acetone (9.3 µl for the control strain and 14.8 µl for the *pcm-1* strain). Samples were spun down at 4°C at 16,000g for 10 min. Supernatants were saved. Pellets were redissolved in 10 µl water and 10 µl of -20°C acetone was added. Samples were spun down and supernatants collected as above. Supernatants for each strain were pooled. Pellets and supernatants were dried in a vacuum desiccator at 25°C for 16 h.

<sup>b</sup> Methyl-accepting substrate levels of acetone soluble and insoluble fractions from control (wt) and L-isoaspartate methyltransferase-deficient (*pcm-1*) aged dauer nematode cytosolic extracts were assayed in duplicate for 90 min at 37°C using recombinant human L-isoaspartate methyltransferase as described under Experimental Procedures.

<sup>c</sup> pmols of methyl groups transferred/mg total cellular extract ± standard deviation.

derived from further proteolysis of damaged proteins. Interestingly, the L-isoaspartyl-deficient aged dauers accumulated 2.5-fold higher levels of acetone soluble methyl-accepting species than control dauers (Table VIII). This suggested that the *pcm-1* mutant may be less able to clear L-isoaspartyl-containing peptides in the absence of the methyltransferase, and the accumulation of these damaged peptides could account for its diminished survival during dauer.

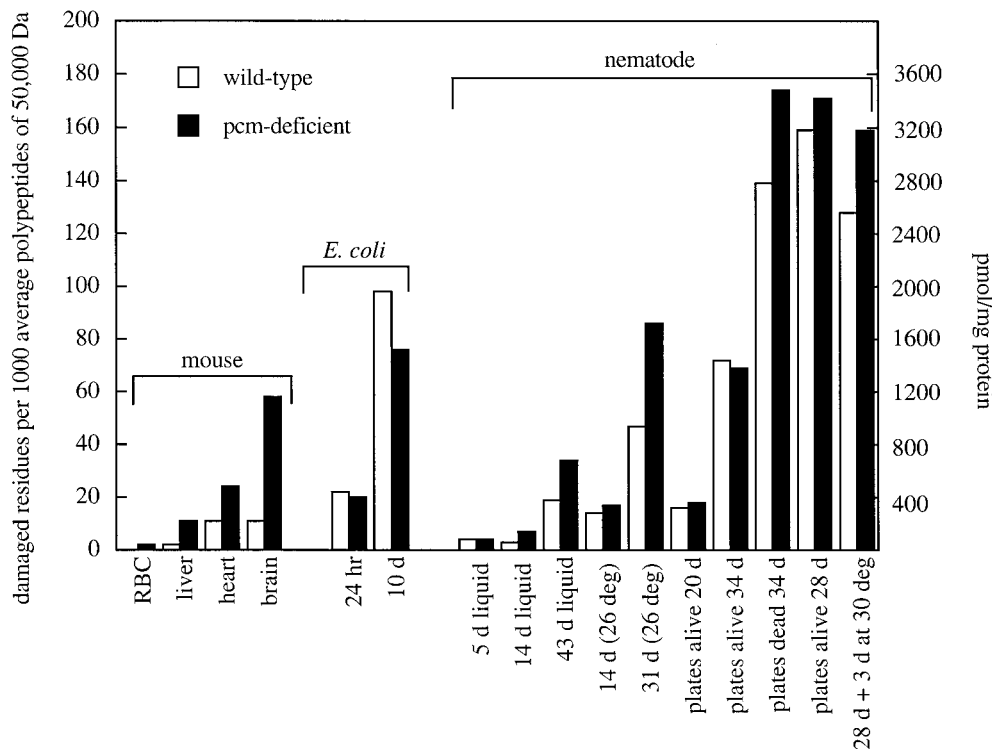
## DISCUSSION

In this study, the role of the L-isoaspartyl methyltransferase in the nematode *C. elegans* was investigated. According to the "repair hypothesis," this enzyme is responsible for limiting the accumulation of damaged proteins which have become isomerized and racemized at aspartyl and asparaginyl residues by converting these abnormal residues back to L-aspartyl residues. Although a number of *in vitro* studies have yielded support for a repair function for L-isoaspartyl methyltransferase, the first *in vivo* evidence of this function has come recently from knockout mice (18). Mice deficient in the L-isoaspartate (D-aspartate) O-methyltransferase accumulate damaged proteins in all tissues at levels 4- to 8-fold higher than the control mice (18). In contrast, this study has shown that the L-isoaspartyl methyltransferase in *C. elegans* is apparently not effective at maintaining a low level of dam-

aged proteins in either the wild-type nematodes, or in mutants deficient in this enzyme. High levels of L-isoaspartyl damage accumulated in the nematode during a specialized metabolically inactive larval stage called dauer. Dauers of both strains aged for about 1 month accumulate methyl-accepting substrates to levels up to 40-fold higher than those found in 5-day-old nematodes. Interestingly, these levels are almost 3-fold higher than the highest levels seen in the brains of 1-month-old knockout mice and about 2-fold higher than levels seen in *E. coli* aged for 10 days (Fig. 1). In fact, the amount of damage measured in cytosolic extracts from aged dauers is at the highest level observed thus far for any organism.

The dauer stage of nematodes represents a metabolically dormant state in which the systems designed for clearing L-isoaspartyl damaged proteins appear to be inactive. Although the measured L-isoaspartyl methyltransferase activity is twofold higher in the dauer stage than in adult nematodes (12), insufficient ATP may be made during this larval stage to produce an adequate amount of S-adenosylmethionine needed for methylation (31). In addition, alternate mechanisms for clearing L-isoaspartyl damage, such as proteolysis, may also be less active during dauer. However, this study has shown that recovery from dauer results in the elimination of the majority of methyl-accepting substrates from the nematodes, indicating the L-isoaspartyl damage can eventually be eliminated by both mutant and control strains. The nematode may rely more heavily on proteolysis of damaged proteins rather than repair during dauer, accumulating damaged peptides and partially proteolyzed proteins until they can be cleared completely after dauer recovery. In fact, in support of this hypothesis, the majority of methyl-accepting species which accumulate in aged dauers were shown to be low-molecular-weight proteins and acetone-soluble peptides (Ref. (21) and this study). This result may also suggest that the methyltransferase-mediated repair may be directed at partially proteolyzed peptides where the presence of L-isoaspartyl residues may impede their further digestion.

Surprisingly, this study did not demonstrate a large difference in the levels of damaged proteins, or in the nature of the methyl-accepting substrates, between the L-isoaspartyl methyltransferase-deficient nematodes and the control strain. Although a twofold increase in substrate levels was observed in the mutants in several trials, enrichment of the samples for live nematodes eliminated any differences between the strains. The higher substrate levels of mutants in unfractionated nematode samples may simply reflect the decreased viability which has been observed for *pcm*-deficient dauers (21). In fact, cytosolic extracts prepared from dead nematodes contained some of the highest levels of substrates observed in this study. However, it is diffi-



**FIG. 1.** Comparison of the level of damaged polypeptides in mice, *E. coli*, and *C. elegans* recognized by the protein L-isoaspartate (D-aspartate) O-methyltransferase. The average number of methylatable residues per 1000 polypeptides of 50,000 Da was estimated for cytosolic extracts from mice, *E. coli*, and nematodes. The recombinant human L-isoaspartyl methyltransferase (28) was used to quantitate the number of damaged sites in the mouse and nematode studies shown, and the *Thermatoga maritima* L-isoaspartyl methyltransferase (16) was used for the *E. coli* studies. Extracts from L-isoaspartyl methyltransferase-deficient organisms are compared to those obtained for wild-type cytosolic extracts. Levels of damage in cellular extracts of various tissues from 30- to 40-day-old mice were presented by Kim *et al.* (18). Levels of damage in *E. coli* extracts aged for 24 h or 10 days in LB broth are those presented by Visick *et al.* (20). The nematode data are presented in the same order as in this study. RBC, red blood cells; d, days.

cult to judge whether the presence of unusually high levels of damaged proteins resulted in the death of a greater percentage of mutant nematodes or whether the mutants died at a higher rate, accumulating damage after death due to a shutdown of metabolic activity.

The effects due to a disruption of the *C. elegans* L-isoaspartyl methyltransferase are subtle compared to the significant growth retardation and fatal seizures observed in knockout mice. The reduction in viability seen during long-term growth in dauer and the reduced ability of these nematode mutants to compete in mixed culture with their wild-type counterparts are more similar to the phenotypes seen in *E. coli* deficient in the L-isoaspartate methyltransferase (19). It is possible that in these nonmammalian organisms, the repair function of the L-isoaspartate methyltransferase may be less crucial to the survival of the species and has not been as refined as it has in mammalian systems. In fact, the  $K_m$  values of the *E. coli* and *C. elegans* enzymes for various isoaspartyl-containing peptides are significantly higher than those measured for the human enzyme (12). For example,  $K_m$  values of the *C. elegans* enzyme are 17- to 67-fold higher than those of

the human enzyme for several L-isoaspartyl-containing peptides (12). It is therefore possible that the lower efficiency of the nonmammalian enzymes results in a slower repair reaction (15), and a deficiency of the methyltransferase is therefore less detrimental to these organisms. Another possibility is that in the nematode, another enzyme works in concert with the protein L-isoaspartyl methyltransferase, such as the L-isoaspartyl dipeptidase in *E. coli* (32), and a disruption of both of these enzymes would be necessary to observe a significant defect in the processing of L-isoaspartyl containing proteins.

Alternatively, it is possible that the L-isoaspartyl methyltransferase may play a completely different role in lower organisms, with the repair role having evolved as an additional function of this enzyme in higher organisms. In fact, only the mammalian enzyme acts on D-aspartate residues in addition to L-isoaspartate residues, demonstrating its expanded role for clearing damaged proteins (16). It remains to be seen whether a separate function can be demonstrated for the L-isoaspartate methyltransferase in lower organisms.

## REFERENCES

1. Lowenson, J. D., and Clarke, S. (1995) *in* Deamidation and Isoaspartate Formation in Peptides and Proteins (Aswad, D. W., Ed.), pp. 47–64, CRC Press, Boca Raton, FL.
2. Clarke, S. (1987) *Int. J. Pept. Protein Res.* **30**, 808–821.
3. Di Donato, A., Ciardiello, M. A., de Nigris, M., Piccoli, R., Mazzarella, L., and D'Alessio, G. (1993) *J. Biol. Chem.* **268**, 4745–4751.
4. Cacia, J., Keck, R., Presta, L. G., and Frenz, J. (1996) *Biochemistry* **35**, 1897–1903.
5. Catanzano, F., Graziano, G., Capasso, S., and Barone, G. (1997) *Protein Sci.* **6**, 1682–1693.
6. Noguchi, S., Miyawaki, K., and Satow, Y. (1998) *J. Mol. Biol.* **278**, 231–238.
7. Johnson, B. A., Langmack, E. L., and Aswad, D. W. (1987) *J. Biol. Chem.* **262**, 12283–12287.
8. Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B., and Clarke, S. (1994) *J. Biol. Chem.* **269**, 24586–24595.
9. Ichikawa, J., and Clarke, S. (1998) *Arch. Biochem. Biophys.* **358**, 222–231.
10. Mudgett, M. B., Lowenson, J. D., and Clarke, S. (1997) *Plant Physiol.* **115**, 1481–1489.
11. O'Connor, M. B., Galus, A., Hartenstine, M., Magee, M., Jackson, F. R., and O'Connor, C. M. (1997) *Insect Biochem. Mol. Biol.* **27**, 49–54.
12. Kagan, R. M., and Clarke, S. (1995) *Biochemistry* **34**, 10794–10806.
13. O'Connor, C. M., Aswad, D. W., and Clarke, S. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7757–7761.
14. Galletti, P., Ingrosso, D., Manna, C., Clemente, G., Zappia, V. (1995) *Biochem. J.* **306**, 313–325.
15. Lowenson, J. D., and Clarke, S. (1991) *J. Biol. Chem.* **266**, 19396–19406.
16. Lowenson, J. D., and Clarke, S. (1992) *J. Biol. Chem.* **267**, 5985–5995.
17. McFadden, P. N., and Clarke, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2460–2464.
18. Kim, E., Lowenson, J. D., MacLaren, D. C., Clarke, S., Young, S. G. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 6132–6137.
19. Visick, J. E., Cai, H., and Clarke, S. (1998a) *J. Bacteriol.* **180**, 2623–2629.
20. Visick, J. E., Ichikawa, J., and Clarke, S. (1998b) *FEMS Microbiol. Lett.* **167**, 19–25.
21. Kagan, R. M., Niewmierzycka, A., and Clarke, S. (1997) *Arch. Biochem. Biophys.* **348**, 320–328.
22. Riddle, D. L. (1988) *in* The Nematode *Caenorhabditis elegans* (Wood, W. B., Ed.), pp. 393–412, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Zwaal, R. R., Broeks, A., van Meurs, J., Groenen, J. T., and Plasterk, R. H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7431–7435.
24. Hodgkin, J., Horvitz, H. R., and Brenner, S. (1979) *Genetics* **91**, 67–94.
25. Sulston, J., and Hodgkin, J. (1988) *in* The Nematode *Caenorhabditis elegans* (Wood, W. B., Ed.), pp. 587–606, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. Lewis, J. A., and Fleming, J. T. (1995) *in* *Caenorhabditis elegans*: Modern Biological Analysis of an Organism (Epstein, H. F., and Shakes, D. C., Eds.), pp. 3–29, Academic Press, San Diego, CA.
27. Egilmez, N. J., and Shmookler Reis, R. J. (1994) *Mutat. Res.* **316**, 17–24.
28. MacLaren, D. C., and Clarke, S. (1995) *Protein Expr. Purif.* **6**, 99–108.
29. Gilbert, J. M., Fowler, A., Bleibaum, J., and Clarke, S. (1988) *Biochemistry* **27**, 5227–5233.
30. Mitchell, D. H., Stiles, J. W., Santelli, J., and Sanadi, D. R. (1979) *J. Gerontol.* **34**, 28–36.
31. Wadsworth, W. G., and Riddle, D. L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8435–8438.
32. Gary, J. D., and Clarke, S. (1995) *J. Biol. Chem.* **270**, 4076–4087.