Purification and Characterization of a Carboxylesterase from the Intestine of the Nematode *Caenorhabditis elegans*†

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ABSTRACT: The major intestinal esterase from the nematode *Caenorhabditis elegans* has been purified to essential homogeneity. Starting from whole worms, the overall purification is 9000-fold with a 10% recovery of activity. The esterase is a single polypeptide chain of M_r 60 000 and is stoichiometrically inhibited by organophosphates. Substrate preferences and inhibition patterns classify the enzyme as a carboxylesterase (EC 3.1.1.1), but the physiological function is unknown. The sequence of 13 amino acid residues at the esterase N-terminus has been determined. This partial sequence shows a surprisingly high degree of similarity to the N-terminal sequence of two carboxylesterases recently isolated from *Drosophila mojavensis* [Pen, J., van Beeumen, J., & Beintema, J. J. (1986) *Biochem. J. 238*, 691–699].

Carboxylesterases (EC 3.1.1.1) are a class of hydrolytic enzymes that have both broad substrate specificities and broad distributions in the tissues of most organisms (Myers, 1960; Krisch, 1971; Pearse, 1974; Peters, 1982; Luppa & Andra, 1983; Walker & Mackness, 1983). In only a few cases, however, has a physiological function been suggested (Gilbert & Richmond, 1982; Mentlein et al., 1985; Varki et al., 1986; Medda et al., 1986).

We have previously identified a nonspecific esterase activity that appears to be completely localized to the intestine of the small free-living nematode *Caenorhabditis elegans* (Edgar & McGhee, 1986). *C. elegans* is an excellent organism in which to study the biochemistry and genetics of development [e.g., Brenner (1974) and Sulston et al. (1983)], and the intestinal esterase should be an excellent biochemical marker with which to follow lineage-specific gene expression (Edgar & McGhee, 1986). Isoelectric focusing mutants in the gut esterase have been induced and mapped by classical genetics (McGhee & Cottrell, 1986). In this paper, the gut esterase is purified and characterized. The purpose of this is 2-fold: (i) to survey substrates and inhibitors that should help the design of genetic selection schemes and (ii) to obtain a partial amino acid sequence that can be used to clone the esterase gene.

The "nonspecific" esterases are usually regarded as rapidly evolving and have been commonly used to study population genetics and evolution. The current finding of a rather unexpected conservation between the N-terminal amino acid sequences of the gut esterase of *C. elegans* and two esterases from *Drosophila mojavensis* (Pen et al., 1986; Pen, 1986) suggests that esterases might actually be more strongly conserved than had previously been believed.

Experimental Procedures

Materials

Except where noted, all substrates and inhibitors were obtained from Sigma. The nematocides Aldicarb and Carbaryl were kindly provided by Bruce Milligan, Union Carbide of Canada. Phenyl-Sepharose, Superose, Sephacryl, and the fast-protein liquid chromatography (FPLC) system were all obtained from Pharmacia. [1,3-^{3}H]Diisopropyl fluorophosphate (DIFP) (5.5 Ci/mmol) was obtained from New England Nuclear.

Methods

(*a*) *Measurement of Enzyme Activity.* The large majority of the enzyme assays described in this paper were measured spectrophotometrically (Beckman DU-8B spectrophotometer; 1–10-cm path length cells; 23 ± 0.5 °C). The most common substrate was α-naphthyl acetate; the change in molar absorptivity ($\Delta\epsilon_{322}$) upon α-naphthyl acetate hydrolysis was estimated as 2350 M^{-1} cm^{-1} (pH 8). One unit of esterase activity was defined as the hydrolysis of 1 μmol of substrate/min.

Hydrolysis of acetylthiocholine and β-thionaphthyl acetate was followed according to the method of Ellman et al. (1961). Phosphatase activity was estimated by using the substrates α-naphthyl phosphate and *p*-nitrophenyl phosphate. Hydrolysis of [^{3}H]triolein (glycerol tri[9,10(n)-^{3}H]oleate) and cholesterol [1-^{14}C]oleate was kindly measured by Dr. D. Severson, Department of Pharmacology and Therapeutics, University of Calgary, by methods previously described (Ramirez et al, 1985). Protease activity was estimated by incubating purified esterase for 1–2 days at 25 °C with either (i) Azocoll (Calbiochem), followed by centrifuging and measuring the A_{520} of the supernatant or (ii) heat-denatured bovine serum albumin or trout histone H1 (kindly provided by Dr. Paul Wiersma), followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining, to detect any breakdown products. Protein concentrations were measured by a dye-binding assay (Read & Northcote, 1981) with bovine serum albumin as a standard.

(*b*) *N-Terminal sequencing* of the purified esterase was kindly done by Dr. D. McKay, Protein Sequencing Laboratory, University of Calgary, using an Applied Bio-Systems 470A Gas Phase sequenator, as described in more detail by McKay et al. (1985). The esterase was sequenced 4 times, with amounts of protein ranging from 0.25 to 2.5 nmol. Residues that were ambiguous in runs with a low amount of esterase were confirmed in runs with higher amounts. For sequencing and for amino acid analysis, the best recoveries of the modest amounts of esterase available were obtained by dialyzing the sample at room temperature against three changes of 0.15 M

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NaCl and 0.1% SDS and then against three changes of 0.02% SDS, as previously suggested (Hunkapillar & Hood, 1980). Spectrapore tubing (M_r cutoff 12000–14000) was well rinsed with water (but not boiled) before use and was shown to contribute neither to the sequence nor to the amino acid content.

(*c*) *Worm Culture.* The wild-type strain, N2, of *C. elegans* was used for all isolation procedures and was maintained essentially as described by Brenner (1974). For large-scale cultures, worms were grown on egg plates, by a modification of a procedure originally suggested by Drs. R. Rosenbluth and D. L. Baillie, Simon Fraser University; see also Yarbrough and Hecht (1984). Incubation was for 6 days at 20 °C or until the number of healthy adult worms reached a maximum; at this point, roughly half of the mass of worms derives from larval forms. Worms were harvested by washing twice in phosphate-buffered saline (PBS) (0.795 g/L Na_2HPO_4, 0.144 g/L KH_2PO_4, 9 g/L NaCl; 4000*g* for 5 min at 4 °C), followed by two (or more) cycles of flotation in 35% (w/v) sucrose (700*g* for 5 min at 4 °C) and washing in PBS. After two final washes in PBS, the packed worm pellet was weighed, mixed with an equal volume of PBS, frozen as small (roughly 1 g) pellets by dropping into liquid nitrogen, and finally stored at −80 °C. We estimate the final purity of worms to be 80–90% by weight.

Enzyme Isolation Procedures

(*a*) *Crude Extract.* A typical esterase purification started with 100–200 g (wet weight) of worms. For each gram of packed worms, 5–8 mL of buffer (200 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 20 mM ethylenediaminetetraacetic acid (EDTA), 12 mM 2-mercaptoethanol, 10% (v/v) glycerol, pH 9.0) was added, and the worm pellets were allowed to thaw by stirring at room temperature. The suspension was cooled in ice and passed once (or twice if necessary) through a cooled motorized Stansted cell disruptor (Model TC/5 612W, obtained from Energy Service Co., Washington, D.C.); complete worm destruction was monitored by dark-field microscopy. The crude lysate was centrifuged (10000*g* for 30 min at 4 °C), and the supernatant was filtered through several layers of buffer-rinsed Miracloth (Calbiochem). We estimate that this crude extract contains at least 90% of the total α-naphthyl acetate hydrolyzing activity in *C. elegans*.

The crude supernatant was clarified by dropwise addition of 1 M $CaCl_2$ to a final concentration of 50 mM, incubation for 30 min at 0–4 °C, centrifugation (10000*g* for 30 min), and filtration through several layers of Miracloth. The calcium chloride supernatant could be further clarified by titration (in ice) to pH 5.5 by addition of 2.5 M acetic acid–2.5 M sodium acetate, centrifugation (10000*g* for 30 min), filtration of the supernatant through Miracloth, and immediate readjustment of the pH to 8.0 with 2.5 M Tris base.

(*b*) *Ammonium Sulfate Fractionation.* To the supernatant from the previous step was added solid ammonium sulfate to a final concentration of 1.5 M [218 g/L (Wood, 1976)] followed by centrifugation (10000*g* for 30 min) and Miracloth filtration. To the 1.5 M ammonium sulfate supernatant solid ammonium sulfate was added to a final concentration of 3.2 M [293 g/L of 1.5 M supernatant (Wood, 1976)] over a period of 60–120 min, at 4 °C. After centrifugation (10000*g* for 60 min), the pellets were suspended with the help of a Dounce homogenizer (A pestle; 5 strokes) in a minimum volume of cold 20 mM Tris-HCl, pH 8.0 ($^1/_{20}$–$^1/_{40}$ of the original crude extract volume), dialyzed overnight against 20–40 volumes of 20 mM Tris-HCl and 1 M ammonium sulfate, pH 8.0, and centrifuged (30000*g* for 15 min), and the supernatant was filtered through Miracloth.

Table I: Summary of Typical Purification of *C. elegans* Esterase

fraction	total vol (mL)	total protein (mg)	total enzyme units[a]	sp act. (units/mg)	purification (*x*-fold)
crude extract	1570	7360	354	0.048	≡1.0
calcium supernatant	1560	3390	260	0.077	1.6
pH 5.5 supernatant	1710	2860	318	0.11	2.3
1.5 M AS supernatant[b]	1812	1960	302	0.15	3.2
3.2 M AS pellet[b]	112	818	164	0.20	4.2
phenyl-Sepharose	100	63	101	1.6	33.2
Amicon concn	3.3	44	77	1.8	36.5
Superose	6	12	44	3.7	76.1
Mono-Q (pH 8)	2	0.46	32	70	1500
Mono-Q (pH 6)	2	0.12	38	332	6900

[a] Enzyme units defined as micromoles of α-naphthyl acetate hydrolyzed per minute, determined as described under Experimental Procedures. [b] AS, ammonium sulfate.

(*c*) *Column Chromatography.* See Figure 1 for details.

(*d*) *Storage.* In earlier preparations, the peak from the second Mono-Q column was neutralized by adding $^1/_{10}$ volume of 1 M Tris-HCl (pH 8.0) and then extensively dialyzed against 20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.2 mM dithiothreitol, and 50% glycerol. In later preparations, the Mono-Q peak was dialyzed directly into 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.2 mM dithiothreitol, 0.2 mM EDTA, and 50% glycerol. When stored at −20 °C, the enzyme loses activity at a rate of 10–20% per year.

Results and Discussion

Esterase Purification. In crude extracts of *C. elegans*, five to seven nonspecific esterase activities can be distinguished by starch gel electrophoresis (Butler et al., 1981) or by isoelectric focusing (McGhee & Cottrell, 1986), especially when sensitive fluorescent substrates are used. However, when α-naphthyl acetate is used as the substrate, one of these activities predominates (Edgar & McGhee, 1986; McGhee & Cottrell, 1986) and comprises 60–90% of the total α-naphthyl acetate hydrolyzing activity in the worm. This major esterase has the lowest isoelectric point of all the worm esterases (McGhee & Cottrell, 1986) and can be detected in isolated guts (data not shown). As shown in detail by Edgar and McGhee (1986), essentially all of the histochemically detectable α-naphthyl acetate hydrolyzing activity in both adult and embryonic worms is localized to the intestine. The following purification procedure was developed by following total α-naphthyl acetate hydrolyzing activity and by verifying at each step that it was indeed the lowest p*I* esterase that was being purified.

The major esterase activity does not correspond to any of the esterases present in the usual food source, *Escherichia coli*, and can be detected both in embryonic (i.e., nonfeeding) worms (Edgar & McGhee, 1986; McGhee et al., 1986) and in worms grown axenically (data not shown). Moreover, isoelectric focusing mutants in the major esterase map to chromosome V of the worm (McGhee & Cottrell, 1986), definitively ruling out any worries about undefined bacterial contaminants.

Table I summarizes a typical esterase isolation procedure, beginning with 176 g of frozen packed worms. As described in detail under Experimental Procedures, purification involves

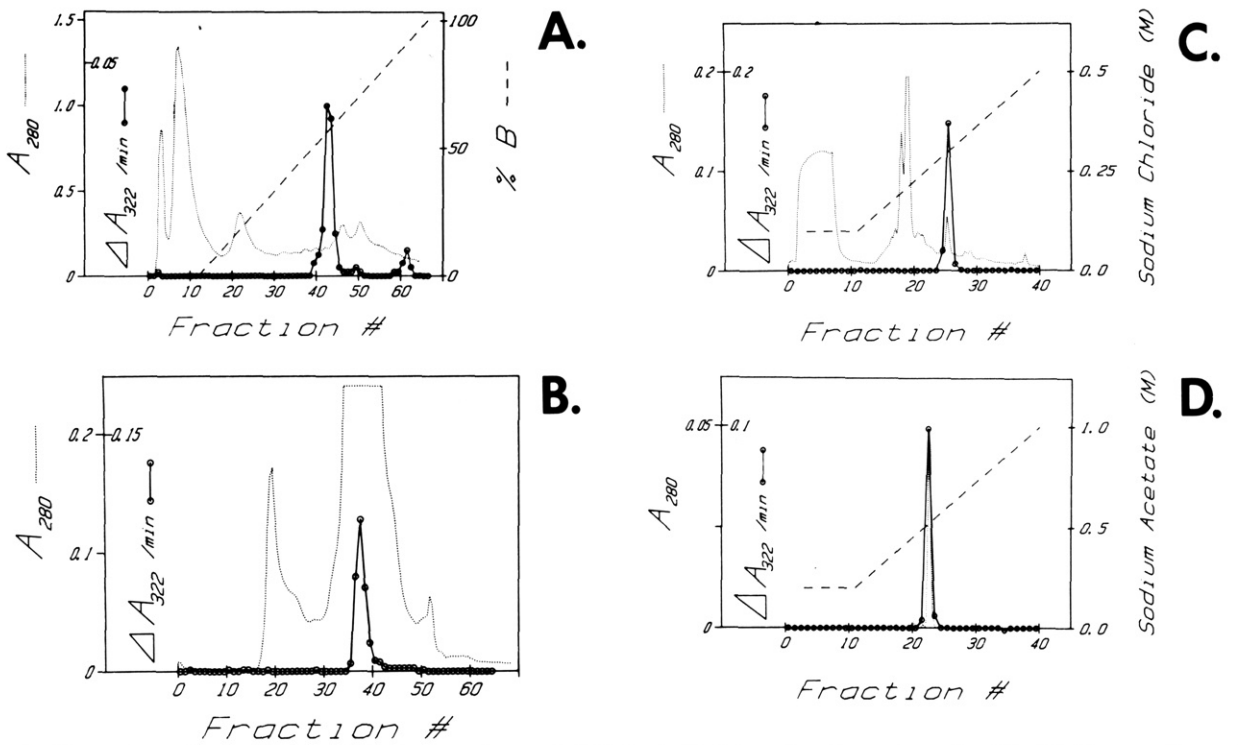

FIGURE 1: Chromatographic purification of *C. elegans* esterase. (a) Hydrophobic interaction chromatography. The 3.2 M ammonium sulfate pellet was dialyzed against buffer A (20 mM Tris-HCl, pH 8.0, and 1 M ammonium sulfate) and applied to a 2.6 × 35 cm phenyl-Sepharose column at 4 °C. The column was washed with 300 mL of buffer A and eluted with a linear gradient of buffer B [20 mM Tris-HCl, pH 8.0, and 50% (v/v) ethylene glycol]. Flow rate = 1.5 mL/min; fraction size = 25 mL. Esterase activity was measured on 20 μL of each fraction, with α-naphthyl acetate as substrate. (b) Gel filtration of *C. elegans* esterase. The esterase peak from the phenyl-Sepharose column was concentrated to 4 mL by pressure filtration (Amicon YM-10), applied to a 1.6 × 60 cm column of Superose, and eluted with 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 10% (v/v) glycerol. Flow rate = 0.15 mL/min; fraction size = 2 mL; 10 μL of each fraction was assayed for α-naphthyl acetate hydrolysis. (c) Anion exchange (first Mono-Q Column). The Superose peak (6 mL) was applied to a Pharmacia Mono-Q anion-exchange column, washed with 5 mL of 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 10% glycerol, and eluted by raising the NaCl concentration. Flow rate = 1 mL/min; fraction size = 1 mL; 5 μL of each fraction was assayed for α-naphthyl acetate hydrolysis. (d) Second Mono-Q column. The esterase peak from the previous Mono-Q column was diluted, reapplied to the Mono-Q column, washed with 5 mL of 20 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris), pH 6.0, 0.1 M sodium acetate, and 10% glycerol, and eluted with increasing sodium acetate concentrations. Flow rate = 1 mL/min; fraction size = 1 mL; 5 μL of each fraction was assayed for α-naphthyl acetate hydrolysis.

several initial steps necessary to clarify the lipid-rich crude extract, followed by ammonium sulfate fractionation, hydrophobic interaction chromatography, gel filtration, and high-pressure anion exchange chromatography (Figure 1). In 13 preparations, the average overall purification was 8800-fold (SD ±3600) and the mean recovery of total esterase activity was 10% (SD ±5%). This corresponds to roughly 1–5 million molecules of esterase per gut cell.

The purity and identity of the final esterase preparation are demonstrated in Figure 2. SDS–polyacrylamide gel electrophoresis reveals only one band detected by silver staining (Figure 2a); i.e., the preparation is at least 95% pure by weight. The position of the protein band corresponds exactly to the position of a band observed when the enzyme is preincubated with [^{3}H]DIFP and subjected to SDS–polyacrylamide gel electrophoresis, and the incorporated radioactivity is detected by fluorography (Figure 2b). When the enzyme is subjected to polyacrylamide gel electrophoresis under nondenaturing conditions, again only one band is detected by silver staining (Figure 2c), the position of which corresponds precisely to the position of a band detected by esterase activity (Figure 2d). We thus conclude that the major gut esterase of *C. elegans* has been purified to essential homogeneity (>95%).

Physical Properties of the Esterase. Analysis of several SDS–polyacrylamide gels (such as that shown in Figure 2a) indicates that the denatured molecular weight of the esterase is 60 000 ± 2000. From gel filtration chromatography (Figure 3) the native molecular weight is also estimated to be 60 000 ± 2000. We thus conclude that the *C. elegans* gut esterase is a single polypeptide chain. The genetic behavior of esterase mutants is completely consistent with this conclusion (McGhee & Cottrell, 1986).

The isoelectric point of the purified esterase (measured at 20 °C relative to known standards) is 4.5 ± 0.1 (data not shown). Figure 4 shows that the esterase is most stable between pH 6 and pH 7.5 and becomes particularly unstable at lower pH. The presence of 10% glycerol increases the enzyme stability by 3–4-fold (data not shown).

Kinetic Properties. (*a*) *General.* Overall, the kinetic behavior of the purified esterase appears straightforward, with none of the complications such as substrate activation or inhibition sometimes seen with other carboxylesterases [see, e.g., Barker and Jencks (1969), Main and Rush (1980), and Farb and Jencks (1980)]. The rate of hydrolysis of α-naphthyl acetate by the purified esterase is not significantly altered by the presence of 10 mM concentrations of dithiothreitol, $MgCl_2$, $MnCl_2$, $CaCl_2$, EDTA, or ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Figure 5a shows that the pH optimum is close to 8.0. Figure 5b shows that the addition of most simple alcohols (with the possible exception of ethanol) leads to a mild stimulation (at most 2-fold) of esterase activity. This same extent of stimulation by simple alcohols has been reported for other esterases (Junge & Heymann, 1979; Farb & Jencks, 1980) and has been interpreted as a direct reaction of the alcohol with the acetyl-enzyme intermediate to generate the corresponding aliphatic ester. Addition of various salts (up to 1 M concentrations of KCl, CsCl, sodium acetate, ammonium sulfate, NaCl, NaBr,

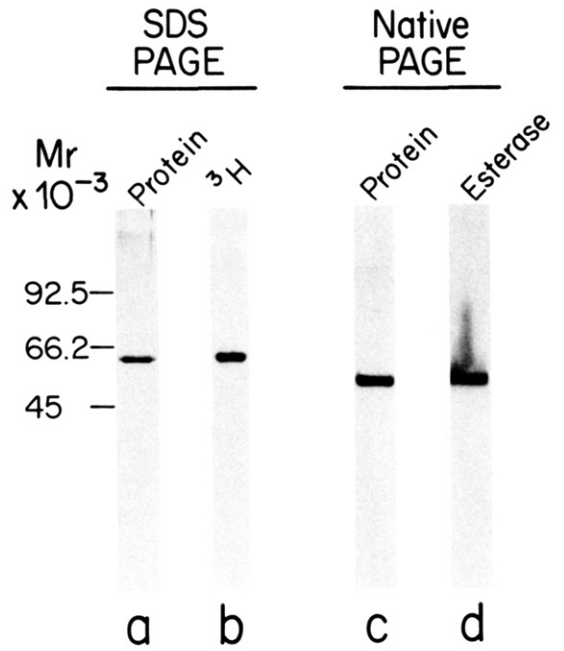

FIGURE 2: Purity and identity of *C. elegans* esterase. Lanes a and b: 10% SDS–polyacrylamide gel, according to Laemmli (1970). (a) Final gut esterase preparation (0.25 μg) detected by silver staining; migration distances of standards (phosphorylase *b* = 92 500 daltons; bovine serum albumin = 66 200 daltons; ovalbumin = 45 000 daltons) are indicated on the left. (b) Final gut esterase (0.25 μg) incubated for 1 h with excess [^{3}H]DIFP and electrophoresed on a parallel gel lane; incorporated radioactivity was detected by fluorography. No band was detected if the esterase was heat-denatured or incubated with excess E600 prior to addition of DIFP (data not shown). Lanes c and d: 7% nondenaturing polyacrylamide gel (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, 10% glycerol). Lane c, 0.2 μg of final esterase preparation detected by silver staining. Lane d, 0.1 μg of esterase electrophoresed on a parallel lane and esterase activity detected with the substrate α-naphthyl acetate and freshly diazotized pararosaniline as previously described (McGhee & Cottrell, 1986).

Table II: Substrate Preference of *C. elegans* Esterase

substrate	relative hydrolysis rate[a]
α-naphthyl acetate	≡1.0
α-naphthyl propionate	0.51
β-naphthyl acetate	1.02
β-naphthyl propionate	0.46
p-nitrophenyl acetate	1.00
p-nitrophenyl propionate	0.56
p-nitrophenyl butyrate	2.3
methylumbelliferyl acetate	0.95
phenyl acetate	0.99
naphthyl-AS-MX acetate[b]	<0.01
Br-Cl-indoxyl acetate	0.09
ethyl *m*-aminobenzoate (tricaine)	<0.01
salicylic acid acetate (aspirin)	<0.01
acetylthiocholine	<0.01
β-thionaphthyl acetate	0.43

[a] Measured in 0.1 M Tris-HCl, pH 8.0, and expressed relative to hydrolysis rate measured with α-naphthyl acetate (0.018 unit). Substrates present at 0.05–0.5 mM depending on solubility; (2-fold changes in substrate concentration did not significantly change the measured hydrolysis rate). All rates measured by absorbance changes at peak of the difference spectra. [b] 3-Acetoxy-2-naphthoic acid 2,4-dimethylanilide.

and NaI) has little significant effect on esterase activity. The exception is NaF, which strongly inhibits enzyme activity; 50% inhibition is achieved at approximately 5 mM NaF. Inhibition by this concentration of fluoride ion is common in carboxylesterases (Krisch, 1971).

(*b*) *K_m and V_{max}.* Eadie–Hofstee plots, using the substrate α-naphthyl acetate, were linear (data not shown). Our best

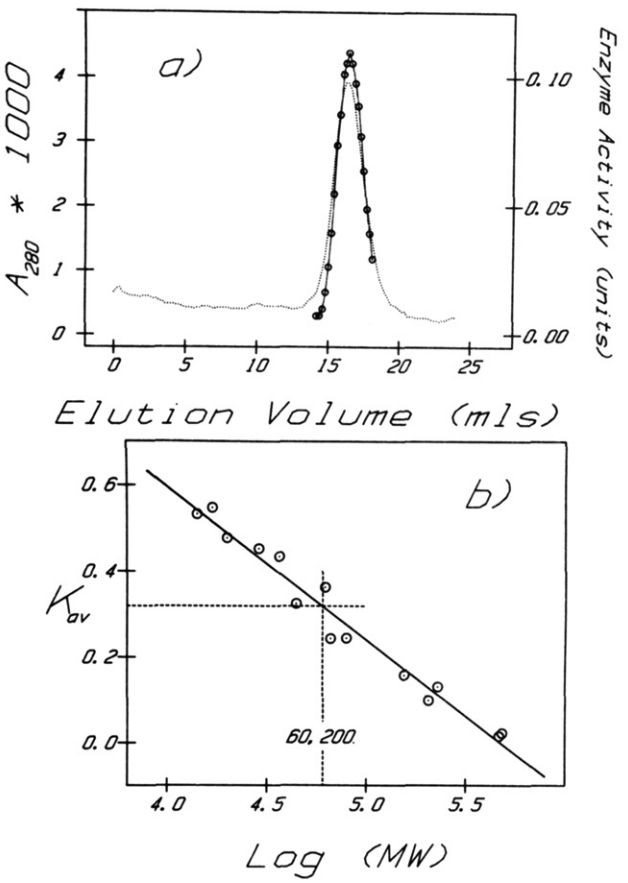

FIGURE 3: Native molecular weight of *C. elegans* esterase. (a) Six micrograms of purified esterase was chromatographed on a 1 × 36 cm column of Sephacryl S-200 and eluted with 20 mM Tris-HCl, pH 8.0, and 0.1 M NaCl at a flow rate of 0.2 mL/min. Protein concentration (A_{280}) and α-naphthyl acetate hydrolyzing activity were determined as indicated. (b) Calibration curve of the Sephacryl column used in (a). Proteins (and their respective $M_r \times 10^{-3}$) used as standards were urease (483), ferritin (467), catalase (230), β-amylase (206), aldolase (156), alcohol dehydrogenase (79.6), bovine serum albumin (66.3), bovine hemoglobin (62.1), ovalbumin (44.5), β-lactoglobulin (36.7), carbonic anhydrase (29.0), soybean trypsin inhibitor (20.1), myoglobin (17.0), and α-lactalbumin (14.2). Interpolation using the least-squares regression line estimates the native molecular weight of the esterase to be 60 000 ± 2000. (The uncertainty in esterase molecular weight was estimated from variations in esterase elution position in repeated runs, as well as from uncertainties in published values of the standard molecular weights.)

estimate of the K_m is 8 ± 2 μM and of the k_{cat} is 500 molecules of α-naphthyl acetate hydrolyzed per molecule of enzyme per second (0.1 M Tris-HCl, pH 8; 23 °C).

(*c*) *Substrate Preference.* The hydrolysis rate of a number of common esterase substrates was measured, and the results are collected in Table II. It can be seen that different histochemical substrates are hydrolyzed at vastly different rates, and such preferences must obviously be considered in designing genetic selection or screening schemes.

Table II also shows that hydrolysis of acetylthiocholine is essentially undetectable although β-thionaphthyl acetate is cleaved readily; thus it is unlikely that the enzyme has any acetylcholinesterase activity. In addition, none of the genetic loci of the three known acetylcholinesterases in *C. elegans* correspond to the map position of the gut esterase, and an *ace-1 ace-2* mutant (either strain NW129 or NW131, kindly provided by Dr. J. G. Culotti), which lacks >95% of wild-type acetylcholinesterase activity, still exhibits the gut esterase at roughly normal levels (data not shown).

The final esterase preparation shows negligible hydrolysis of cholesterol oleate or triolein (i.e., <0.01% of the rate with

Table III: Inhibition Properties of *C. elegans* Esterase

inhibitor	concn (M)	relative V_{max}[a]	inhibitor	concn (M)	relative V_{max}[a]
control		≡1.0	Eserine	10^{-5}	0.05
diisopropyl fluorophosphate (DIFP)	10^{-6}	0.00		10^{-6}	0.13
diethyl *p*-nitrophenyl phosphate (E600)	10^{-6}	0.00	Trichlorfon	10^{-5}	0.00
tris(*p*-nitrophenyl) phosphate	10^{-6}	0.04		10^{-6}	0.08
bis(*p*-nitrophenyl) phosphate	10^{-4}	0.01	*p*-(chloromercuri)benzenesulfonic acid (PCMBSA)	10^{-3}	1.08
	10^{-5}	0.04	*p*-(chloromercuri)benzoic acid (PCMB)	10^{-3}	1.20
	10^{-6}	0.62	*N*-ethylmaleimide	10^{-3}	1.15
phenylmethanesulfonyl fluoride	10^{-3}	0.17	acetazolamide	10^{-3}	1.05
	10^{-4}	0.32	carbachol	10^{-3}	1.03
	10^{-5}	0.81	levamisole	10^{-3}	0.95
	10^{-6}	1.04	neostigmine	10^{-3}	0.67
Aldicarb	10^{-5}	0.11		10^{-6}	0.97
	10^{-6}	0.51	sodium deoxycholate	10^{-3}	0.10
Carbaryl	10^{-5}	0.03	sodium taurocholate	10^{-3}	0.18
	10^{-6}	0.08	Triton X-100	10^{-3}	0.78

[a] Purified esterase incubated at 23 °C with 10^{-6}–10^{-3} M inhibitors, and surviving esterase activity assayed by the hydrolysis of 1 mM α-naphthyl acetate in 0.1 M Tris-HCl, pH 8.0, containing the same concentration of inhibitor. Identical results were obtained with 30- and 60-min incubations with inhibitor, and average esterase activity is shown in the table. Esterase concentration during preincubation = (3–6) × 10^{-8} M.

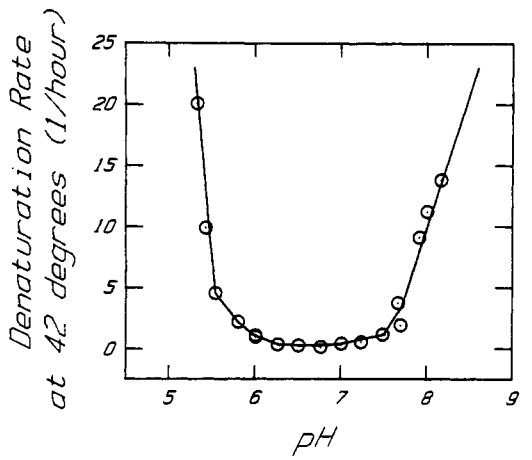

FIGURE 4: pH–stability curve of *C. elegans* esterase. Purified esterase was incubated at 42 °C in various buffers (acetate, phosphate, and Tris-HCl), all at 20 mM concentration, containing 0.1 M NaCl and 0.1 mg/mL of bovine serum albumin. At six intervals between 0 and 45 min, aliquots were removed, chilled, and centrifuged briefly; surviving esterase activity was determined in 0.1 M Tris-HCl with 1 mM α-naphthyl acetate as substrate. Denaturation rate constants were determined from the slopes of the (linear) first-order decay curves and plotted as a function of incubation pH.

α-naphthyl acetate). The esterase also shows no detectable phosphatase or protease activity.

(*d*) *Inhibitors.* One of the principal classifications of esterases is by their inhibition properties (Myers, 1960; Krisch, 1971; Pearse, 1974; Peters, 1982; Luppa & Andra, 1983; Walker & MacKness, 1983). A survey of different inhibitors should also allow more decisive interpretation of histochemical staining patterns and of the physiological role of the esterase. In addition, inhibitors could possibly be used in genetic selection schemes. A number of esterase inhibitors were investigated, and the results are collected in Table III.

Low concentrations of organophosphates completely inhibit the *C. elegans* esterase. As shown above (Figure 2), [^{3}H]-diisopropyl phosphate can be covalently incorporated into the purified enzyme. Furthermore, within the accuracy of determining the low concentrations of available protein (±20%), inhibition is complete at one molecule of E600 (diethyl *p*-nitrophenyl phosphate) per 60 000 daltons of esterase (data not shown). We have shown previously (Edgar & McGhee, 1986) that treatment of sectioned worms with 10^{-5} M E600 abolishes the great majority of gut esterase staining. Organophosphates can be used to show that the physiological role

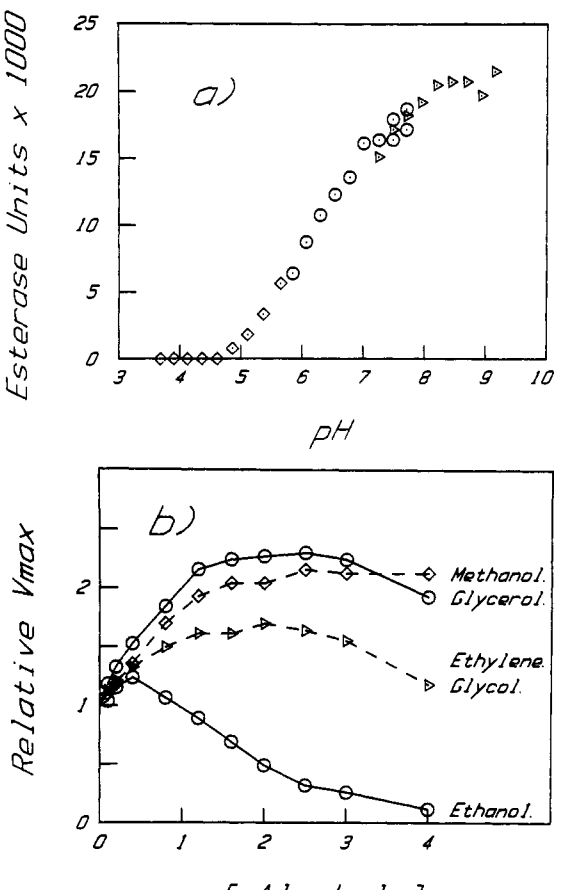

FIGURE 5: Survey of kinetic variables influencing *C. elegans* esterase. (a) pH–activity curve. Purified esterase was assayed at 23 °C, with 0.9 mM α-naphthyl acetate as substrate, in 0.1 M NaCl and 20 mM of one of the following buffers: Sodium acetate (◇); sodium phosphate (○); Tris-HCl (▷). Rates were corrected for substrate hydrolysis and for pH-dependent changes in the product absorbance. Esterase activity was difficult to measure at a pH much greater than 9 because of high background hydrolysis rates and because of esterase denaturation. (b) Effect of alcohols. Purified esterase was assayed at 23 °C, with 0.9 mM α-naphthyl acetate as substrate, in 0.1 M Tris-HCl, pH 8.0, and various alcohols at the indicated molarities. Rates were corrected for substrate-only hydrolysis, and it was verified that the absorbance of α-naphthol did not change significantly with alcohol addition. The average apparent pH change in the most concentrated alcohol solutions was −0.17.

of the major gut esterase is probably not essential. Apart from being uncoordinated (undoubtedly due to loss of acetyl-

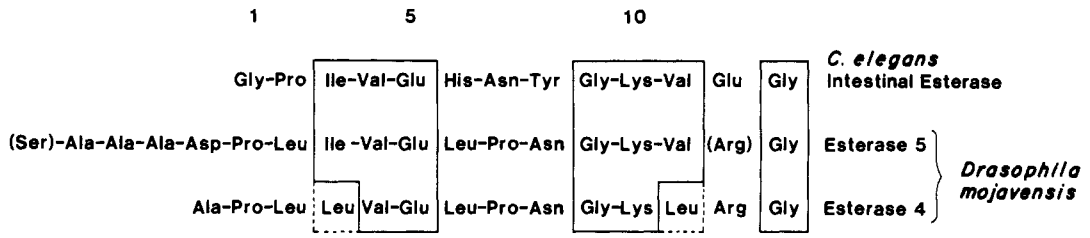

FIGURE 6: N-Terminal amino acid sequence of *C. elegans* intestinal esterase (top line), aligned to show similarity with the N-terminal sequence of two esterases isolated from *D. mojavensis* (Pen et al., 1986; Pen, 1986). Regions of similarity are indicated by solid boxes. Two conservative amino acid substitutions in esterase 4 are indicated by broken lines.

Table IV: Amino Acid Composition of *C. elegans* Esterase

amino acid	mol %[a]	amino acid	mol %[a]
Asx	12.1 (0.6)	Met	2.3 (0.3)
Thr	3.7 (0.7)	Ile	5.9 (0.3)
Ser	6.9 (0.6)	Leu	6.8 (0.2)
Glx	12.1 (0.6)	Tyr	4.5 (0.6)
Pro	6.9 (0.3)	Phe	5.2 (0.2)
Gly	8.3 (0.6)	His	2.0 (0.2)
Ala	6.3 (0.4)	Lys	6.3 (0.5)
Val	6.8 (0.3)	Arg	4.1 (0.1)

[a] Mean of three determinations; standard deviations in parentheses.

cholinesterases), worms appear to grow and reproduce quite normally in the presence of 5×10^{-6} M E600; under these conditions, the major gut esterase activity can no longer be detected in crude extracts (J. C. Birchall and J. D. McGhee, unpublished results).

Table III shows that the *C. elegans* esterase is also inhibited by other well-known serine esterase inhibitors [tris(*p*-nitrophenyl) phosphate, bis(*p*-nitrophenyl) phosphate, and phenylmethanesulfonyl fluoride] but with varying effectiveness. The esterase is sensitive to low concentrations of carbamates (Aldicarb, Carbaryl, and Eserine) and is also sensitive to the phosphonate Trichlorfon. No inhibition is seen with sulfhydryl modifying reagents, such as *N*-ethylmaleimide, *p*-(chloromercuri)benzoate and *p*-(chloromercuri)benzenesulfonic acid. The esterase is not inhibited by high concentrations of acetazolamide, suggesting it is not a carbonic anhydrase. The esterase also shows no inhibition by levamisole; this drug causes hypercontraction of nematode muscles (Lewis et al., 1980) and has turned out to be useful to bring about extrusion of guts in cut worms, thereby allowing rapid esterase staining in individual animals (unpublished results). Bile salts activate mammalian lipases (Krisch, 1971) but clearly inhibit the *C. elegans* gut esterase.

Overall, the inhibition pattern revealed in Table III indicates that the *C. elegans* enzyme should be classified as a B esterase or carboxylesterase. However, the many problems and limitations of esterase classification have often been noted (Myers, 1960; Krisch, 1971; Pearse, 1974; Peters, 1982; Luppa & Andra, 1983; Walker & MacKness, 1983).

Amino Acid Sequence and Composition. Figure 6 shows the sequence of 13 amino acid residues at the esterase N-terminus, determined as described under Experimental Procedures. The N-terminal sequences from two esterases recently isolated from *D. mojavensis* (Pen et al., 1986; Pen, 1986) are also shown, aligned to give maximum similarity. Out of the 13 positions, 5 amino acids in the worm sequence are identical with those in esterase 4 and 7 are identical with those in esterase 5 of *D. mojavensis*. The impression that this degree of similarity is much greater than would be expected by chance is confirmed by a similar comparison with several unrelated proteins (data not shown). The amino acid composition of the *C. elegans* esterase is shown in Table IV and is clearly similar to the composition of the two esterases from *D. mojavensis* (Pen et al., 1986; Pen, 1986). Considering the limited sequence data available and considering that DNA sequences should be available in the future, attempts at a more rigorous assessment of esterase homologies seem premature.

In summary, the major intestinal esterase of the nematode *C. elegans* has been purified and characterized. The esterase can be classified as a carboxylesterase or B esterase (EC 3.1.1.1) and behaves as if it has a serine residue in the active site. Substrates and inhibitors were surveyed as a background for genetic selection schemes, but the physiological function of the esterase remains unknown. The N-terminal amino acid sequence obtained should be adequate to allow gene cloning via an oligonucleotide probe. Both the sequence and the composition reveal similarities to two carboxylesterases recently isolated from *D. mojavensis* (Pen et al., 1986; Pen, 1986). This raises intriguing questions about esterase evolution. Nonspecific esterases, primarily because of their large number of electrophoretic variants, are customarily regarded as highly variable enzymes, and yet there appears to be sequence conservation between nematodes and flies, maintained over the better part of a billion years. As clones of esterase genes become available, we can expect much more insight into esterase evolution. Perhaps we can also expect to find out what esterases actually do.

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Steady-State and Stopped-Flow Kinetic Measurements of the Primary Deuterium Isotope Effect in the Reaction Catalyzed by *p*-Cresol Methylhydroxylase†

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ABSTRACT: Steady-state kinetic studies for the reaction of the flavocytochrome *p*-cresol methylhydroxylase with the reducing substrates (S) *p*-cresol, 4-ethylphenol, and their corresponding α-deuteriated analogues are presented. The results from these experiments and those from studies involving various reoxidizing substrates support the proposed apparent ping-pong mechanism. With phenazine methosulfate (PMS) as the reoxidant for studies at pH 7.6 and 6 or 25 °C, the isotope effects on k_{cat} are lower than the intrinsic isotope effect. The values for $^{D}(k_{cat}/K_S)$ are equal to the intrinsic effect for *p*-cresol at 25 °C and for 4-ethylphenol at both 6 and 25 °C. However, the value for this steady-state parameter at 6 °C for *p*-cresol is lower than the intrinsic effect. The values for $^{D}(k_{cat}/K_{PMS})$ are nearly equal to 1.0 under all conditions. In contrast, the steady-state kinetic analysis for the isolated flavoprotein subunit of *p*-cresol methylhydroxylase involving *p*-cresol and PMS as substrates indicates that a random-binding mechanism is operating. Additionally, several of the steady-state parameters yield values for the apparent intrinsic isotope effect for the flavoprotein. The results of stopped-flow kinetic studies are also reported. At pH 7.6 the intrinsic isotope effect ($^{D}k_2$) for the reduction of the enzyme by 4-ethylphenol is 4.8–5.0 at 25 °C and 4.0 at 6 °C. This technique yields a value for $^{D}k_2$ of 7.05 at 6 °C and pH 7.6 for *p*-cresol. The combined results from the stopped-flow and steady-state kinetic experiments at pH 7.6 and 6 °C for *p*-cresol also allow the calculation of several important kinetic parameters for this enzyme. These calculations are viewed with caution, since some discrepancies develop when a comparison of the data from the two kinetic methods is made for both *p*-cresol and 4-ethylphenol. Surprisingly, the stopped-flow method could not be used to measure an isotope effect for the isolated flavoprotein subunit.

Over the past few years a variety of properties of the flavocytochrome *p*-cresol methylhydroxylase from *Pseudomonas putida* have been investigated. The three-dimensional structure of this tetrameric enzyme, which is composed of two identical flavoprotein and two identical *c*-type cytochrome subunits, is known at 6-Å resolution (Shamala et al., 1986). Additionally, the primary structure of the cytochrome subunit is available (McIntire et al., 1986), and studies are under way to determine the amino acid sequence of the flavoprotein from the corresponding DNA sequence. It is also known that FAD is covalently bound to the flavoprotein through a tyrosyl ether

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