Purification and characterization of carboxylesterases from rat lung

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Carboxylesterase (EC 3.1.1.1) has played an important part in our understanding of the toxicokinetic behaviour of the organophosphorus cholinesterase inhibitors. Carboxylesterases are a heterogeneous group of enzymes that can be separated on the basis of their isoelectric points and by their substrate-specificity. We have purified the isoenzyme (pI 5.8) present in greatest activity in rat lung to near homogeneity. The enzyme was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration, chromatofocusing, separation on anion- and cation-exchangers and hydrophobic-interaction chromatography. The purified enzyme has a molecular mass of approx. 180 kDa with subunits of 60 kDa. The substrate and inhibitor specificities of the enzyme have been characterized. Edman degradation revealed the first 19 amino acid residues of the enzyme. The *N*-terminus was found to be tyrosine. Inhibition of the enzyme by organophosphorus compounds differed greatly from that of cholinesterases, despite the partial analogy at the *N*-terminal region.

INTRODUCTION

Carboxylesterases (EC 3.1.1.1) have been shown to play an important part in the detoxification of organophosphorus compounds (Sterri & Fonnum, 1984; Fonnum *et al.*, 1985). They are a heterogeneous group of enzymes that differ in isoelectric point and in substrate-specificity. Most investigations have been conducted with the esterases of rat liver. In the last years, six carboxylesterases have been isolated from rat liver microsomal fractions (Mentlein *et al.*, 1980; Robbi & Beaufay, 1983). However, all the esterases examined probably exist in microheterogeneous forms.

The isoelectric points and molecular masses of the liver microsomal carboxylesterases have been determined, but only short fragments of the active-site and the *N*- and *C*-terminal amino acid sequences are known (Heymann *et al.*, 1970; Mentlein *et al.*, 1984). The isoelectric points and the substrate specificities of the carboxylesterases from other tissues have in part been determined, but very little knowledge exists with regard to their purification.

In the present investigation we wanted to characterize carboxylesterases from rat lung, which is one of the main tissues where they can be found. We report on the purification and characterization of the most abundant isoenzyme from this tissue.

MATERIALS AND METHODS

Chemicals

4-Nitrophenyl butyrate, 4-nitrophenyl acetate, α -naphthyl acetate and bis-(4-nitrophenyl) phosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A. Butanilcaine (*N*-butyl-aminoacetyl-2-chloro-6-methylanilide) was from Hoechst A.G., Frankfurt, Germany. Sephadex G-25 (medium grade), Ultrogel AcA34, Polybuffer exchanger 94, Polybuffer 74, Mono Q HR 5/5, Mono S HR 5/5 and alkyl-Superose HR 5/5 were from Pharmacia LKB Biotechnology Division, Uppsala, Sweden. Paraoxon (diethyl 4-nitrophenyl phosphate) was from Koch-Light Laboratories, Colnbrook, Berks., U.K. Di-isopropyl

phosphorofluoridate was from Du Pont NEN Research Products, Dreieich, Germany. Soman (pinacolyl methylphosphonofluoridate), sarin (isopropyl methylphosphonofluoridate), cyclosarin (cyclohexyl methylphosphonofluoridate) and tabun (*O*-ethyl *NN*-dimethylphosphoramidocyanidate) were synthesized in this laboratory by the procedures of Bryant *et al.* (1960) and Holmstedt (1951), and the purity was found to be greater than 95% by n.m.r. spectroscopy. All other chemicals were of analytical-grade quality.

Animals

Male Wistar rats (200–300 g weight) were purchased from the National Institute of Public Health, Oslo, Norway.

Purification procedures

Lungs were collected from male Wistar rats (200–300 g), which were macroscopically free from disease. A 40–70 g batch of the tissue was rinsed with cold 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl and homogenized in 4 vol. of the same buffer with a Polytron instrument for 20 s. The homogenate was filtered through glass-wool to remove fat, and heated at 55 °C for 15 min to remove unstable proteins, followed by centrifugation at 9000 *g* for 20 min at 4 °C. The supernatant was further centrifuged at 100 000 *g* for 60 min. The high-speed supernatant was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The 35–70%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction was resuspended in a small volume of 50 mM-Tris/HCl buffer, pH 8.0.

Gel filtration. The 35–70%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to an Ultrogel AcA34 gel-filtration column (60 cm $\times$ 2.5 cm) at 4 °C. The column was equilibrated and eluted with 50 mM-Tris/HCl buffer, pH 8.0. The flow rate was 0.35 ml/min, and 4 ml fractions were collected. Fractions containing 80–90% of the total activity were combined and concentrated to 5–6 ml by means of an Amicon ultrafiltration cell, with a YM-10 membrane.

Chromatofocusing. The preparation was chromatofocused at 4 °C as described in the chromatofocusing kit instructions (Pharmacia LKB). The sample buffer was exchanged with the

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starting buffer 25 mM-imidazole/HCl buffer, pH 7.4, on a Pharmacia PD-10 column. The elution buffer was an 8-fold dilution of Polybuffer 74, adjusted to pH 4.0 with 1.0 M-HCl. A column (0.9 cm × 28 cm) was packed with PBE 94 and equilibrated with the starting buffer. The sample was applied after running through 5 ml of the elution buffer. In all, 200 ml of elution buffer was followed by 2 column vol. of 1 M-NaCl solution. The elution rate was 0.40 ml/min, and 2 ml fractions were collected. Fractions containing 80 % of the total activity were combined and concentrated to 6 ml with an Amicon ultrafiltration cell.

The chromatofocusing procedure was repeated, but with other buffers. The same column (PBE 94) was equilibrated with the starting buffer 25 mM-histidine/HCl buffer, pH 6.2, and the elution buffer was a 10-fold dilution of Polybuffer 74/HCl, pH 5.0. Fractions with the highest activity (fraction A) were combined and concentrated to 2.5 ml with an Amicon ultrafiltration cell.

Anion-exchange chromatography (f.p.l.c.) on Mono Q HR 5/5. The buffer of fraction A (pI 5.8) was exchanged with the starting buffer 25 mM-Bis/tris/HCl buffer, pH 7.0. The material was applied to a Mono Q HR 5/5 column, which was eluted (1.0 ml/min) with 6 ml of the starting buffer followed by 20 ml of a linear gradient of 0–0.5 M-NaCl in the starting buffer.

Cation-exchange chromatography (f.p.l.c.) on Mono S HR 5/5. The buffer of the combined fractions was exchanged with the next starting buffer 25 mM-Mes/NaOH buffer, pH 5.1. The preparation was applied to a Mono S HR 5/5 column, which was eluted (1.0 ml/min) with 6 ml of the starting buffer followed by 20 ml of a linear gradient of 0–0.5 M-NaCl in the starting buffer.

Hydrophobic interaction (f.p.l.c.) on alkyl-Superose HR 5/5. The buffer of the collected fractions was exchanged with 50 mM-sodium phosphate buffer, pH 7.0, containing 1.5 M-(NH₄)₂SO₄. The preparation was applied to alkyl-Superose HR 5/5 equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 2.0 M-(NH₄)₂SO₄. The column was eluted (0.5 ml/min) with a linear gradient of 2.0–0 M-(NH₄)₂SO₄ in the starting buffer. Fractions with the highest activity were combined, and the buffer was exchanged with 25 mM-ammonium bicarbonate buffer, pH 8.0. The protein solution was concentrated by drying under vacuum.

Enzyme assay methods

Carboxylesterase activity was measured spectrophotometrically with 4-nitrophenyl butyrate as substrate (Sterri *et al.*, 1985). The assay mixture at 30 °C consisted of 0.1 M-sodium phosphate buffer, pH 7.8, 2 mM-4-nitrophenyl butyrate and tissue in a total volume of 3.02 ml. The absorbance of 4-nitrophenol was monitored at 400 nm with a Beckman DU-50 spectrophotometer. The molar absorption coefficient for 4-nitrophenol at pH 7.8, used in this investigation, was 17000 M⁻¹·cm⁻¹.

The substrate-specificity of rat lung carboxylesterase was also examined with 4-nitrophenyl acetate and butanilcaine. 4-Nitrophenyl acetate was used under the same absorption coefficient. When butanilcaine was used, the assay mixture consisted of 0.06–5.00 mM substrate and 50 mM-Tris/HCl buffer, pH 8.6, as described by Heymann *et al.* (1981). The increase in absorbance was monitored at 285 nm, and a molar absorption coefficient of 2080 M⁻¹·cm⁻¹ was used.

Measurement of inhibition

The purified enzyme was incubated with different organophosphates in 0.1 M-sodium phosphate buffer, pH 7.8, at 30 °C. After various times (5–20 min) the substrate, 4-nitrophenyl

butyrate, was added and the residual activity determined as described above.

The bimolecular rate constants were calculated from the equation:

$$k_a = [\ln(100/x)]/(t \cdot c_i)$$

where c is the concentration of inhibitor, x is the percentage of esterase activity remaining and t is the time of preincubation of enzyme and inhibitor before the addition of substrate (Aldridge & Reiner, 1972).

SDS/PAGE

SDS/PAGE was carried out as described by Laemmli (1970), on the Phast Gel System from Pharmacia. A polyacrylamide-gradient gel, Phast Gel Gradient 8–25, was used with Phast Gel SDS buffer strips.

Determination of molecular mass

Molecular mass was determined by the gel-sieving method of Johnson (1979). The PAGE was run with a Mini Protean II cell from Bio-Rad Laboratories. Different concentrations of acrylamide (4, 7 and 10 %) were used. The standard proteins used were the high-molecular-mass standards of Bio-Rad Laboratories. The protein was located in the gels by using Coomassie Brilliant Blue or the substrate α -naphthyl acetate. The gels were run and stained by the method of Øien & Stenersen (1984).

Amino acid sequence analyses

Sequence analysis was carried out on an Applied Biosystems 477A Protein Sequencer. The amino acid phenylthiohydantoin derivatives were determined on-line with the aid of an Applied Biosystems 120 phenylthiohydantoin analyser, as outlined by Cornwell *et al.* (1988).

Other analytical methods

Protein was determined as described by Lowry *et al.* (1951), with BSA as standard.

RESULTS

In rat lung the highest carboxylesterase activity (more than 80 % of total activity) was found in the 100000 *g* supernatant, with 4-nitrophenyl butyrate as substrate. The yield of solubilized

Fig. 1. Chromatofocusing II of rat lung preparation

The eluted fractions were analysed for carboxylesterase activity (●), pH (⊗) and A_{280} (○). The column was eluted with 1 M-NaCl after the fraction indicated by an arrow. The thick arrows indicate the three isoelectric points (pI). The hatched peak contained the fraction with the highest hydrolytic activity on 4-nitrophenyl butyrate, and the fraction (A) was used in the next purification step.

Fig. 2. SDS/PAGE of purified carboxylesterase (pI 5.8)

The electrophoresed gel was stained with Coomassie Brilliant Blue R-250. Lane A, 4.5 μ g of protein. Lane B, molecular-mass markers (phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme in order of decreasing molecular mass).

enzyme did not increase after use of the glycoside detergent saponin (final concn. 1%, w/w) to extract the microsomal activity. The rest of the activity or the microsomal activity was distributed in various subcellular fractions (present in the 600 *g*, 6500 *g*, 12000 *g* and 100000 *g* pellets).

Carboxylesterase was separated into different isoenzymes according to their isoelectric points (pI) by chromatofocusing. Fig. 1 show three different isoenzymes, pI 5.8, pI 5.5 and pI 5.3. The isoenzyme present in greatest activity, pI 5.8, was purified by f.p.l.c. to a single band on SDS/PAGE with a monomer mass of 60 kDa (Fig. 2). The molecular mass was determined to be 180 kDa by the gel-sieving method. Because the protein appeared to be essentially homogeneous, we have to assume a trimeric structure of identical subunits. In the purification experiment reported in Table 1, the yield of the enzyme was 1.0 mg of

protein/70 g of tissue. The low enzyme recovery was in part due to the fact that only one of the isoenzymes was selectively purified.

Substrate-specificity

The results on the substrate-specificity of rat lung carboxylesterase (pI 5.8) are given in Table 2. The activities were determined at various substrate concentrations, and maximal velocity, *V*, and the Michaelis constant, *K_m*, were determined graphically from Lineweaver–Burk plots.

4-Nitrophenyl acetate and 4-nitrophenyl butyrate are examples of esters with an aromatic alcohol group. Both substrates are rapidly hydrolysed by rat lung carboxylesterase, with the highest *V* and *K_m* for 4-nitrophenyl acetate. Substrate inhibition was observed at high concentrations (> 1.6 mM) for both substrates.

Butanilcaine, a representative of the aromatic amides, is hydrolysed at a much lower rate than the esters. This is typical for carboxylesterases.

Inhibition by organophosphorus compounds

The rates of inactivation of different esterases, including carboxylesterase (pI 5.8), by organophosphorus compounds are shown in Table 3. The bimolecular rate constant of inhibition was determined from a number of experiments with different concentrations and incubation times. The inactivation reaction followed first-order kinetics, since the decrease in log(activity) was linear with time for several concentrations of inhibitors. The inactivation reaction follows first-order kinetics when $c_i/[E_0] \geq 10$ (Aldridge & Reiner, 1972), where $[E_0]$ and c_i are initial concentrations of enzyme and inhibitor. In our experiments the maximal value of $[E_0]$, estimated by assuming pure enzyme, varied from 0.3 to 1.6 nM. Whereas acetylcholinesterase showed wide variations in specificity towards inhibitors, the carboxylesterases gave inhibitor rate constants in a narrow range. Bis-(4-nitrophenyl) phosphate is a very poor inhibitor of

Table 1. Purification of carboxylesterase (pI 5.8) from rat lung

For experimental details see the text.

Fraction	Volume (ml)	Protein (mg)	Specific activity (μ mol/h per mg)	Purification (fold)	Recovery (%)
I Protein extract	350	8000	17	1	100
II 35–70 % -satd.-(NH ₄) ₂ SO ₄ fraction	30	960	60	4	42
III Gel filtration on Ultragel AcA34	7.3	201	168	10	25
IV Chromatofocusing I	6.0	38	297	18	8
V Chromatofocusing II	1.2	11	920	54	7
VI Anion-exchange chromatography on Mono Q HR 5/5 and cation-exchange chromatography on Mono S HR 5/5	2.0	1.7	3750	221	5
VII Hydrophobic-interaction chromatography on alkyl-Superose HR 5/5	0.3	1.0	5650	332	4

Table 2. Kinetics of the hydrolysis of esters and amides by carboxylesterase (pI 5.8)

The enzyme concentration ($[E_0]$) was 3.0 nM. Results are means $\pm$ S.E.M. ($n = 4$).

Substrate	Range of substrate concentration (mM)	<i>K_m</i> (mM)	<i>V</i> (μ mol/min per mg)
4-Nitrophenyl acetate	0.5–0.08	0.06 ± 0.01	107 ± 4
4-Nitrophenyl butyrate	0.6–0.05	0.02 ± 0.005	64 ± 3
Butanilcaine	5.0–0.06	0.43 ± 0.01	15 ± 2

Table 3. Bimolecular rate constants for the reaction of rat lung carboxylesterase (pI 5.8), horse liver carboxylesterase and bovine erythrocyte acetylcholinesterase with organophosphorus compounds

Results for rat lung carboxylesterase are means $\pm$ S.E.M. ($n = 6-9$). Values for horse liver carboxylesterase and bovine erythrocyte acetylcholinesterase are taken from Ooms (1961).

Organophosphorus compound					k_a ($M^{-1} \cdot \text{min}^{-1}$)		
	General formula	X	R	R'	Carboxylesterase from:		Acetylcholinesterase from bovine erythrocytes
					Rat lung	Horse liver	
Bis-(4-nitrophenyl) phosphate		$\text{OC}_6\text{H}_4\text{NO}_2$	$\text{OC}_6\text{H}_4\text{NO}_2$	O^-	$(1.8 \pm 0.3) \times 10^5$		
Paraoxon		$\text{OC}_6\text{H}_4\text{NO}_2$	OC_2H_5	OC_2H_5	$(1.4 \pm 0.2) \times 10^7$	7.9×10^6	4.5×10^5
Soman		F	CH_3	$\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_2$	$(7.0 \pm 0.6) \times 10^6$	2.3×10^5	6.0×10^7
Sarin		F	CH_3	$\text{OCH}(\text{CH}_3)_2$	$(6.0 \pm 0.2) \times 10^5$	1.7×10^5	1.4×10^7
Cyclosarin		F	CH_3	OC_6H_{11}	$(2.2 \pm 0.4) \times 10^6$	5.3×10^5	3.3×10^8
Di-isopropyl phosphorofluoridate		F	$\text{OCH}(\text{CH}_3)_2$	$\text{OCH}(\text{CH}_3)_2$	$(2.8 \pm 0.3) \times 10^6$	3.2×10^5	4.6×10^4
Tabun		CN	OC_2H_5	$\text{N}(\text{CH}_3)_2$	$(3.6 \pm 0.4) \times 10^6$	7.0×10^5	3.6×10^6

Fig. 3. Sequence comparison of the N-terminal regions of (1) rat lung carboxylesterase (pI 5.8), (2) 60 kDa rabbit liver microsomal esterase, (3) *Torpedo* acetylcholinesterase and (4) human butyrylcholinesterase

The ringed amino acids indicate identical sequences between (1), (2), (3) and (4). Arrows indicate functionally related amino acids and codons that differ by a single nucleotide base. The 60 kDa rabbit liver microsomal esterase sequence was taken from Korza & Orzols (1988), the acetylcholinesterase sequence from MacPhee-Quigley *et al.* (1985) and the butyrylcholinesterase sequence from Lockridge *et al.* (1987).

acetylcholinesterase (the bimolecular rate constant is not shown). The data for rat lung are strikingly higher than those previously published for horse liver (Ooms, 1961). It was also shown that physostigmine, at a concentration of 1 mM, did not inhibit carboxylesterase (pI 5.8).

N-Terminal amino acid sequence

The N-terminal sequence of the rat lung carboxylesterase shows partial analogy with the N-terminal sequence of *Torpedo* acetylcholinesterase and slightly lower analogy with human serum butyrylcholinesterase. The lung carboxylesterase sequence shows a high degree of similarity to a recently published microsomal esterase from rabbit liver (Fig. 3). Of the esterases compared, only butyrylcholinesterase has a glycosylation site within the 19 residues of the N-terminal sequence.

DISCUSSION

The carboxylesterase activity of rat lung (measured with the substrate 4-nitrophenyl butyrate) was mainly found in the cytosolic fraction, and there was no advantage in extracting the small amount of microsomal activity. Also, malathion

carboxylesterase from lung has been shown to be localized predominantly in the 100 000 g supernatant, the cytosolic fraction (Ali & Imamura, 1985). In contrast, malathion carboxylesterase activity in liver was found to be mainly localized (75%) in the microsomal fraction (Talcott, 1979). These results show that the subcellular pattern of carboxylesterases in the lung is different from that in liver. The most likely explanation is that the enzyme is localized almost exclusively in the soluble fraction of the lung, but autolysis, which readily solubilizes the membrane-bound carboxylesterase, cannot be excluded.

There are no previous reports on the purification of rat lung carboxylesterases, but the isoelectric points have previously been measured. The isoelectric points found here for the two peaks of carboxylesterase activity in lung, pI 5.8 and pI 5.5 (see Fig. 1), are close to those reported by Mentlein *et al.* (1987) (pI 6.0 and pI 5.6). The small differences observed could be due either to the fact that two different outbred strains of Wistar rats were used or to the fact that isoelectric focusing was used by Mentlein *et al.* (1987) whereas we used chromatofocusing. Experiments have shown that the pI may be slightly changed by repeated isoelectric focusing (Heymann & Junge, 1979) and also by chromatofocusing in ampholytes (Goepfinger *et al.*, 1978).

The isoenzyme (pI 5.8) was shown by SDS/PAGE to have a subunit mass of about 60 kDa, but a molecular mass of 180 kDa was obtained by the gel-sieving method. It is therefore reasonable to assume a trimeric structure and that each subunit has one active site. The lung isoenzyme therefore bears some resemblance to the rat liver carboxylesterase (pI 6.0), which is also a trimer (Mentlein *et al.*, 1984). A trimeric structure has also been reported for a pig liver carboxylesterase (Heymann & Junge, 1979).

The length of the alkyl and alkoxy groups seems to be important for the inhibitory properties of organophosphorus compounds. An analogous effect is found with substrates; the rate of hydrolysis depends on the number of carbon atoms in the acyl group (Aldridge & Reiner, 1972). Considering the two substrates 4-nitrophenyl acetate and 4-nitrophenyl butyrate (Table 2), lengthening the substrate by two carbon atoms nearly doubles the rate constant (V/K_m). The pattern for the inhibitors is more difficult to explain (Table 3). The rate constant for the carboxylesterase inactivation is increased when the alkoxy group is lengthened. Inhibition of acetylcholinesterase also increases with the length of the alkoxy group of the organophosphorus compound, but it differs from carboxylesterase in that two bulky alkoxy groups showed the lowest rate of inactivation (Table 3). A striking difference between the two enzymes is the fact that acetylcholinesterase is not inhibited by bis-(4-nitrophenyl) phosphate. The negative charge on the inhibitor probably prevents binding to the esteratic locus of the acetylcholinesterase active site because of the nearby anionic locus. Carboxylesterase (pI 5.8) differs further from cholinesterases in that it is not inhibited by physostigmine at a concentration of 1 mM. We therefore exclude any relationship to the cholinesterases, despite the sequence similarities at the *N*-terminal region.

In general, good inhibitors are found to be compounds that resemble the structure of substrates. An example is paraoxon, the best inhibitor of the organophosphorus compounds examined, the structure of which resembles that of 4-nitrophenyl butyrate.

Soman and sarin have rate constants of inactivation of carboxylesterase (pI 5.8) that approach the rate constants of inhibition of acetylcholinesterase (Table 3). These results explain how carboxylesterase could function as a scavenger in soman detoxification (Fonnum *et al.*, 1985).

The six rat liver carboxylesterase isoenzymes have different *N*-terminal amino acid residues, which are tyrosine and aspartic acid/asparagine (Mentlein *et al.*, 1984), but the structural analyses of these preparations have yet to be performed. The *N*-terminal residue of carboxylesterases from other species such as pig liver was shown to be glycine, but no sequence has been determined (Heymann & Iglesia, 1974). These results show that the isoenzymes separated from various animals and tissues with a subunit mass of approx. 60 kDa have differences in the *N*-terminus and can illustrate heterogeneity of the carboxylesterases.

The recently presented amino acid sequence of the 60 kDa glycoprotein esterase from rabbit liver microsomal fractions

(Korza & Ozols, 1988) shows a high degree of similarity to the reported *N*-terminal sequence of carboxylesterase (pI 5.8). The differences observed are in positions 1, 4 and 19 (Fig. 3), and we have to assume a close relationship between these two enzymes.

The present paper reports the first amino acid sequence of a rat carboxylesterase, and the data could give further information on the relationship between the different carboxylesterases, and on the understanding of physiological detoxification.

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