Biochemical Pharmacology, Vol. 42, No. 8, pp. 1577–1585, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITOR STUDIES OF PURIFIED HAEMOPOIETIC (MYELOID) CELL ESTERASES

EVIDENCE FOR THE EXISTENCE OF DISTINCT ENZYME SPECIES

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(*Received* 7 *May* 1991; *accepted* 18 *June* 1991)

Abstract—Human myeloid cells synthesize and express two major species of esterase, defined by isoelectric focusing (IEF). The first of these (MonEst) is specifically associated with haemopoietic cells of monocytic lineage, whereas the other species (ComEst) is common to all myeloid cells (granulocytes and monocytes) irrespective of lineage affiliation. Having recently purified these two species of human myeloid cell esterase, this present study extensively investigated the effects of 17 different inhibitors on their ability to hydrolyse the synthetic substrate alpha-naphthyl acetate (αNA). Significant inhibition of both ComEst and MonEst was exerted by 1% sodium dodecyl sulphate (SDS) and 1.0 mM diethyl pyrocarbonate (DEPC), but the patterns of inhibition for the two esterase species with the remaining compounds studied differed considerably; for example, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 5.0×10^{-3} M dichloroisocoumarin (DCIC) and 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) all inhibited MonEst but not ComEst. Mechanisms of inhibition were also examined and these studies established that SDS, PMSF, DCIC and TPCK irreversibly inactivated MonEst whilst the inhibition of ComEst by SDS was reversible. Analysis of inhibition kinetics further showed that (a) the reversible inhibition of both ComEst and MonEst by sodium fluoride (NaF) was noncompetitive (with K_i values of 1.28 and 0.01 mM, respectively, indicating a marked difference in sensitivity); (b) the inhibition of MonEst by PMSF was of 'mixed' noncompetitive–competitive type; and (c) that DEPC exerted noncompetitive inhibition with similar K_i values (0.05 mM) for both esterase species. These observations unequivocably demonstrate that ComEst and MonEst are unrelated enzyme species, with a common ability to hydrolyse αNA, and that these esterases show marked differences with respect to their active sites as adjudged by inhibitor sensitivities. These observations are particularly relevant to the histochemical analysis of these enzymes and to the elucidation of their *in vivo* functions.

Esterases represent a diverse spectrum of enzymes with a ubiquitous tissue distribution which, by definition, share certain features with regards to substrate specificity. Awareness of these substrate specificities, together with inhibitor characteristics, is particularly important in determining relationships between two apparently related enzyme species and, in addition, may be informative with regards to physiological function. Haemopoietic myeloid cells synthesize and express two main esterase isoenzyme groups which may be differentiated by isoelectric focusing (IEF) [1] or by patterns of substrate hydrolysis [2]. Myeloid cells of granulocytic and monocytic origin both express a series of cationic esterase isoenzymes within the pI range 6.3–7.9 (ComEst) and monocytic cells additionally express a closely related (pI range 5.5–6.2) series of isoenzymes (MonEst) which appear to be lineage-specific. Although little is known about the physiological function of these particular esterases, there is some evidence that esterases in general may be involved in the metabolism of xenobiotics of the ester or amide type, thus constituting part of the detoxification system of the body, and that MonEst in particular may function in pinocytosis, facilitation of diapedis and tissue migration, inflammatory processes and growth regulation [3]. Furthermore, it has been suggested that MonEst may have a role in tumour cell killing [4] and this is supported to some extent by observations that the incidence of certain lymphoid neoplasia may be increased in patients with MonEst deficiency [5].

We have recently purified these two histochemically important myeloid esterase species and although the possibility of their *in vivo* interconversion has been suggested [6], molecular [7] and substrate [2] studies of these purified fractions indicate that ComEst and MonEst are two distinct enzymes, representing monomeric acetylesterases and trimeric carboxylesterases, respectively. In order to substantiate these conclusions, and to further clarify the nature of these two myeloid esterase species, we analysed the effects of 17 different inhibitors on the activities of purified ComEst and MonEst fractions [7] by UV spectrophotometric assay, IEF and gel filtration chromatography.

MATERIALS AND METHODS

Purification of monocyte-specific (MonEst) and 'common' myeloid (ComEst) esterase isoenzymes. Monocyte-specific (pI range 5.5–6.2) and common myeloid esterases (pI range 7.3–7.9) were purified by chromatographic procedures as described previously

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Table 1. Inhibition studies of purified monocyte-specific (MonEst) and 'common' myeloid (ComEst) esterase isoenzyme species

Inhibitor	Concentration (mM)	ComEst (pI 7.3–7.9)	MonEst (pI 5.5–6.2)
2-Nitro-4-carboxyphenyl *N*,*N*-diphenyl-carbamate (NCDC)	2.0×10^{-3}	81	90
Phenylmethylsulphonyl fluoride (PMSF)	0.2	84	20
3-4-Dichloroisocoumarin (DCIC)	5.0×10^{-3}	89	3
Neostigmine (methyl sulphate)	20	99	99
Sodium fluoride (NaF)	1.0	52	5
Sodium dodecyl sulphate (SDS)	1.0 (%)	4	5
Sodium taurocholate	1.0	86	85
Acetylcholine iodide	0.1	91	99
Eserine	0.1	93	99
p-Hydroxymercuribenzoic acid (HMBA)	1.0	96	100
Iodoacetamide	2.0	73	100
N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	0.1	92	26
N-α-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK)	0.1	100	84
Diethyl pyrocarbonate (DEPC)	1.0	16	11
1,10-Phenanthroline	0.1	94	113
Ethylenediaminetetraacetic acid (EDTA)	1.0	100	91
Urea	2.5×10^{3}	94	88

Esterase activity was examined by UV spectrophotometric assay, using 0.5 mM αNA as substrate, in the presence and absence of inhibitors at the final concentrations stated. Esterase isoenzyme fractions were pre-incubated with each of the inhibitors for a constant period of 5 min prior to assay. The results indicate the observed enzymic activity, where 100% represents the activity obtained in the absence of inhibitor.

[7]. Esterase fractions prepared in this way were stored at −20° in 50% glycerol prior to kinetic analysis, with no evidence for loss of enzyme activity.

Inhibitor studies of MonEst and ComEst myeloid esterase isoenzyme species. The nature of enzyme–substrate interactions and the possibility that ComEst and MonEst esterase species differed in their reaction mechanisms were evaluated by incorporating a range of enzyme inhibitors in the standardized alpha-naphthyl acetate (αNA) UV spectrophotometric assay [8]. For the inhibitor kinetic studies, 10μL aliquots of chromatographically purified ComEst and MonEst fractions were pre-incubated for 5 min at 30° in a semimicro glass cuvette with 1.0 mL of 10 mM morpholino-ethane sulphonic acid (MES) pH 6.3 containing inhibitor solutions at the final concentrations specified in Table 1. At this time, the enzyme reactions were initiated by the addition of (10 μL) 50 mM stock αNA substrate (in 2-methoxyethanol), to give a final working concentration of 0.5 mM. After further mixing, the rate of increase in absorption at 235 nm (A_{235}) was measured at 12-sec intervals over a period of 2.0 min (Pye Unicam 8800 UV/VIS spectrophotometer). Enzyme reaction rates, both in the presence and absence of inhibitor, were corrected for spontaneous substrate hydrolysis and the results expressed in percentage terms as:

$$\frac{\text{Reaction rate } (A_{235})/\text{min in the presence of inhibitor}}{\text{Reaction rate } (A_{235})/\text{min in the absence of inhibitor}}.$$

As part of the assay standardization, preliminary analyses were also undertaken to determine the relationships between pH and reaction rates. This was carried out by the addition of standard amounts of both esterase species to 10 mM buffers, containing 0.5 mM concentrations of αNA or butyrate, at 0.5 pH unit intervals, through the range 4.0–9.0 (lactic acid, pH 4.0 and 4.5; piperazine, pH 5.0; MES, pH 5.5–6.5; phosphate, pH 7.0 and 7.5; and Tris, pH 8.0–9.0). Reaction rates at an absorption wavelength of 235 nm were measured over 2-min periods at 30° and the results expressed as O.D. change/min.

The type of inhibitory action (reversible or irreversible) exerted by a number of compounds on the two esterase isoenzyme species was further investigated by gel filtration and/or IEF. This involved pre-incubation of the esterase fractions with higher concentrations of inhibitor followed by (a) passage through an FPLC Superose-12 gel filtration column (Pharmacia) and fluorimetric esterase assay of eluted components [9], or (b) IEF electrophoresis and subsequent histochemical αNA esterase [10, 11] staining of plates. Reversible inhibition resulted in restoration of enzyme activity following chromatographic or electrophoretic separation from the inhibitor; irreversible inhibition was indicated by an inability to restore activity.

Inhibition kinetics of ComEst and MonEst isoenzyme species. Two inhibitors that are commonly incorporated in histochemical staining procedures for the demonstration of cellular esterases are sodium fluoride (NaF) and phenylmethylsulphonyl fluoride (PMSF). Together with another agent, diethyl pyrocarbonate (DEPC), these were further investigated in kinetic assays to establish whether or not they exerted differential types of inhibition on ComEst and MonEst species. By measuring the effect of increasing NaF and PMSF concentrations on the rate of αNA substrate hydrolysis, the inhibition constants (K_i or K_I) for both myeloid esterase species could be determined and, moreover,

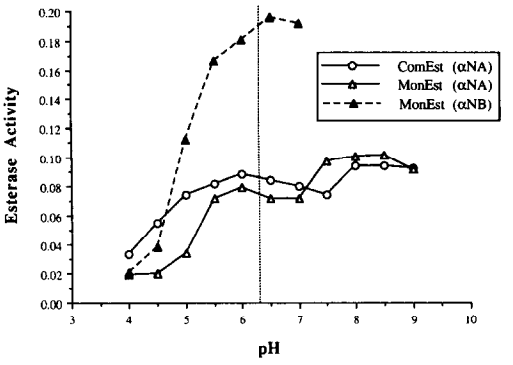

Fig. 1. Effect of pH on ComEst and MonEst reaction rates. Enzyme activities, at 30°, of ComEst and MonEst with 0.5 mM alpha-naphthyl acetate (αNA) and alpha-naphthyl butyrate (αNB) substrates were determined at 0.5 pH intervals in the range of 4.0 and 9.0. Note that ComEst did not cause significant hydrolysis of αNB and that because of a high degree of spontaneous αNB hydrolysis above pH 7.0, the reaction rates for MonEst with this substrate were not evaluable. The pH at which inhibition kinetics were carried out in this study is shown by the vertical dotted line.

the mechanisms of enzyme inhibition could be further clarified.

Inhibitor kinetics were determined by pre-incubating 10 μL of ComEst (or MonEst) fraction with 1.0 mL of 10 mM MES, pH 6.3, containing known concentrations of NaF, PMSF or DEPC in a semimicro quartz cuvette for 5 min at 30°. At this time, 4, 8, 12 or 16 μL of stock 25 mM αNA solution (in 2-methoxyethanol) was added to the cuvette contents (final substrate concentrations, 0.1–0.4 mM) and the rate of increase in absorption at 235 nm measured at 12-sec intervals over a 2 min period. At each inhibitor concentration studied, a Lineweaver–Burk plot of 1/initial reaction rate ($1/v$) against 1/substrate concentration ($1/[s]$) was constructed and the substrate–inhibitor V_{max} estimated. For each inhibitor, a secondary plot of the reciprocal substrate–inhibitor V_{max} ($1/V'_{max}$) values against inhibitor concentration was made and, from this, the inhibition constant (K_i or K_I) determined. In this present study, the NaF inhibitor concentrations examined ranged from 0 to 1.0 mM; PMSF concentrations (prepared immediately prior to assay from stock PMSF solutions in 2-isopropranol) ranged from 0 to 0.2 mM; and DEPC concentrations from 0 to 0.1 mM.

RESULTS

Inhibitor studies

The inhibitory effects on ComEst and MonEst esterase species of a wide range of enzyme inhibitors were examined in this study. The substrate used throughout these analyses was αNA and the inhibitors, their concentrations, and effects are summarized in Table 1. Based upon arbitrary definitions of significant and partial inhibition as <50% and 50–80% of control (no inhibitor) activity, respectively, only two compounds (1% SDS; and 1.0 mM DEPC) exerted significant inhibitory effects on both ComEst and MonEst. PMSF (0.2 mM), 3, 4-dichloroisocoumarin (DCIC, 5.0×10^{-3} mM), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 0.1 mM) inhibited MonEst, but not ComEst; NaF (1.0 mM) was shown to significantly inhibit MonEst, with partial inhibition of ComEst; and iodoacetamide (2.0 mM) induced partial inhibition of ComEst, but not MonEst. All the other inhibitors examined failed to cause any significant inhibitory effects on either of the esterase isoenzyme species.

Initial studies to examine relationships between pH and substrate reaction rates of ComEst and MonEst in the absence of inhibitor, showed (Fig. 1) that both esterase species hydrolysed αNA with only marginal differences through the pH range 5.5–7.0 and that the observed increases in reaction rates above this pH were insignificant. Hydrolysis of α-naphthyl butyrate by the MonEst species (ComEst caused no detectable hydrolysis of this substrate) showed similar reaction rates in the pH range 6.0–7.0 but, because of marked spontaneous substrate breakdown above pH 7.0, further analysis was not possible.

Table 2. Mechanisms of esterase inhibition*

Inhibitor†	ComEst species	MonEst species	Analytical methods
1% SDS	Reversible	Irreversible	Gel filtration
20 mM NaF	Reversible	Reversible	Gel filtration and IEF
5.0 mM PMSF	Resistant	Irreversible	IEF
5.0 mM DEPC	Reversible	Reversible	IEF
5.0 mM TPCK	Resistant	Irreversible	IEF

* Purified monocyte-specific (MonEst) and 'common' myeloid (ComEst) esterase isoenzyme fractions incubated with various inhibitors and then analysed following gel filtration chromatography or isoelectric focusing (IEF) to assess whether inhibition was reversible or irreversible.

† Inhibitors: SDS, sodium dodecyl sulphate; NaF, sodium fluoride; PMSF, phenylmethylsulphonyl fluoride; DMPC, diethyl pyrocarbonate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

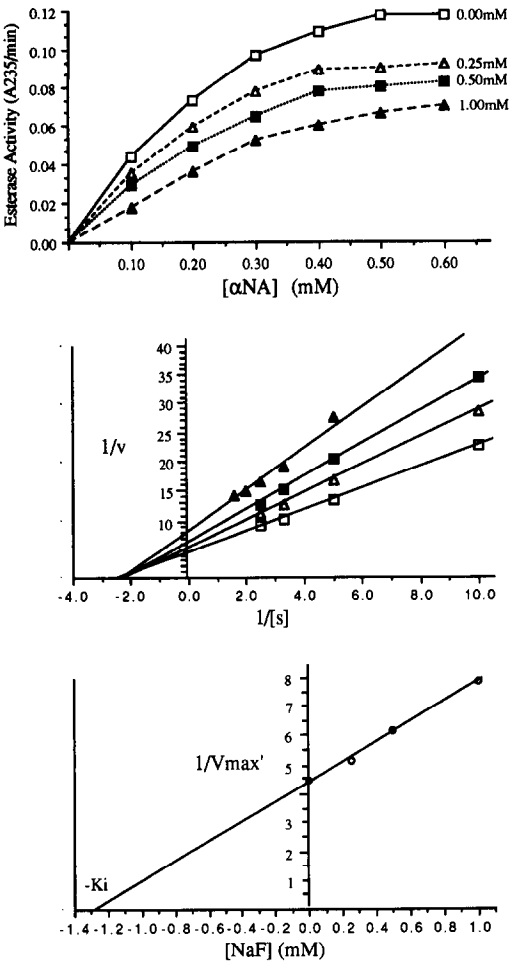

Fig. 2. Effect of sodium fluoride (NaF) on the observed rate of alpha-naphthyl acetate (αNA) hydrolysis by the purified ComEst species. The rate of αNA hydrolysis was determined by UV spectrophotometry [8] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A_{235}/min with each data point representing a mean of duplicate determinations. The upper diagram shows the ComEst rates of αNA hydrolysis with a range (0.1–0.6 mM) of substrate concentrations in the presence of increasing NaF (0–1.0 mM). The Lineweaver–Burk plot (centre diagram) indicates that NaF exerts noncompetitive inhibition on ComEst; a secondary plot of altered $1/V_{max}$ ($1/V'_{max}$) values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (K_i) for inhibition of ComEst by NaF.

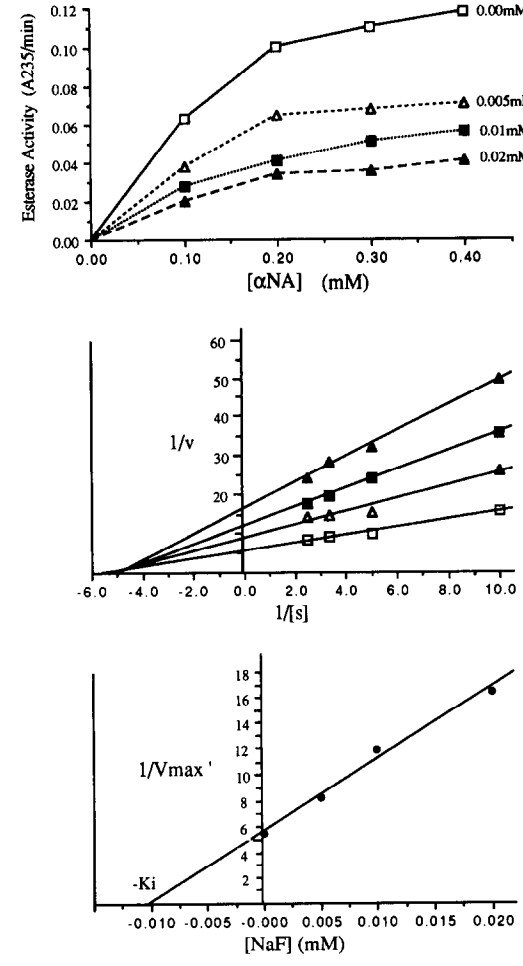

Fig. 3. Effect of sodium fluoride (NaF) on the observed rate of alpha-naphthyl acetate (αNA) hydrolysis by the purified MonEst species. The rate of αNA hydrolysis was determined by UV spectrophotometry [8] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A_{235}/min with each data point representing a mean of duplicate determinations. The upper diagram shows the MonEst rates of αNA hydrolysis with a range (0.1–0.4 mM) of substrate concentrations in the presence of increasing NaF (0–0.02 mM). The Lineweaver–Burk plot (centre diagram) indicates that NaF exerts noncompetitive inhibition on MonEst; a secondary plot of altered $1/V_{max}$ ($1/V'_{max}$) values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (K_i) for inhibition of MonEst by NaF.

Chromatographic and electrophoretic (IEF) studies which further investigated the mechanisms of inhibition revealed that higher concentrations of PMSF (5.0 mM) and DCIC (0.05 mM) irreversibly inhibited MonEst isoenzyme activity, with no significant inhibition of ComEst forms (Table 2). For comparison, 20 mM NaF inhibited both species of esterases but this inhibition was reversible in that enzyme activity was restored following electrophoretic or chromatographic fractionation. A further finding of note was that 1% SDS, which inhibited both ComEst and MonEst isoenzyme activities in the UV spectrophotometric assay, was shown to inhibit ComEst in a reversible fashion whereas, in distinct contrast, SDS treatment of MonEst isoenzymes caused irreversible inhibition. Finally, DEPC was shown to reversibly inhibit both ComEst and MonEst species, compared to TPCK which inhibited MonEst irreversibly with little significant effect on ComEst activity.

Inhibition kinetics

A series of kinetic studies were undertaken in

Table 3. NaF, PMSF and DEPC inhibitor kinetic studies of 'common' myeloid (ComEst) and monocyte-specific (MonEst) esterase isoenzyme species*

	ComEst species		MonEst species	
Inhibitor	Inhibition type	K_i (mM)	Inhibition type	K_i/K_I (mM)
NaF (0–1.0 mM)	Noncompetitive	1.28	Noncompetitive	0.01
PMSF (0–0.2 mM)	No inhibition	—	Mixed-inhibition†	
			(noncompetitive	0.01)
			(competitive	0.11)
DEPC (0–0.1 mM)	Noncompetitive	0.05	Noncompetitive	0.05

* Alpha-naphthyl acetate esterase activities were determined in the presence of varying concentrations of sodium fluoride (NaF) or phenylmethylsulphonyl fluoride (PMSF) by UV spectrophotometric assay. The type of inhibition was determined by Lineweaver–Burk plots and the K_i (or K_I for competitive inhibition) was estimated using secondary plots of $1/V'_{max}$ against inhibitor concentration.

† At concentrations exceeding 0.5 mM, PMSF was shown to cause irreversible inhibition of MonEst; K_i and K_I values for the noncompetitive and competitive components, respectively, are given separately.

order to further characterize the mechanisms of inhibitory action on ComEst and MonEst isoenzyme species by NaF, PMSF and DEPC. The type of inhibition (competitive, uncompetitive, noncompetitive, or mixed) exerted by these three compounds, as well as their inhibition constants, was assessed by constructing secondary plots of altered V_{max} values against inhibitor concentration.

The Lineweaver–Burk plots of $1/v$ against $1/[s]$ for the inhibition of MonEst and ComEst isoenzymes by NaF clearly showed that both esterase species were inhibited in a noncompetitive manner (Figs 2 and 3). Furthermore, the calculated K_i values for NaF indicated that MonEst isoenzymes were approximately 130 times more sensitive to inhibition than ComEst (Table 3). Similarly, the reversible inhibition of esterase species by DEPC was shown to be noncompetitive for both ComEst and MonEst (Figs 4 and 5) with similar K_i values of 0.05 mM (Table 3). For comparison, PMSF which was shown by electrophoretic analyses to irreversibly inhibit MonEst isoenzymes at high concentrations (5.0 mM), appeared to show 'mixed competitive–noncompetitive' inhibition at lower concentrations (Fig. 6). Determination of the inhibition constants for the competitive (K_I) and noncompetitive (K_i) inhibitory components further revealed (Table 3) that the K_I value of 0.01 mM was identical to that found for NaF and that the K_i concentration was very close to the observed affinity (K_m) of MonEst species for α-naphthyl butyrate [2]. Consequently, it is concluded that MonEst 'recognizes' with similar affinity esterase substrates and PMSF, and that at lower relative PMSF:substrate concentrations, the more effective (reversible) noncompetitive inhibitory action becomes apparent.

DISCUSSION

Early attempts to categorize enzymes with esterolytic activity were based on their sensitivity (or resistance) to organophosphates, such as diisopropyl fluorophosphate (DFP) and diethyl *p*-nitrophenyl phosphate (E600), and their ability to hydrolyse substrates of different acyl chain lengths [12–15]. On this basis, A-esterases (some, but not all, arylesterases) typically show a preference for acetate substrate hydrolysis and are unaffected by E600 concentrations up to 1 mM. For comparison, B-esterases (carboxylesterases and cholinesterases) hydrolyse acetate and butyrate substrates at similar rates and are inhibited by organophosphate concentrations as low as 10 μM. A third group of esterase (C; acetylesterases) that are organophosphate resistant and also show a preference for acetate may be distinguished from A-esterases by their resistance to sulphydryl blocking agents [16, 17]. In addition, lipases may be differentiated from simple esterases by their activation with taurocholates [16, 18], whilst cholinesterases are distinguished from carboxylesterases by their relative sensitivity to eserine [19]. Despite its attraction, the application of empirical criteria such as these may however create a number of difficulties. For example, some esterases resistant to inhibition at low organophosphate concentrations can show considerable variations in sensitivity at higher concentrations [17], or may show distinct differences in organophosphate inhibition with esterase substrates of different acyl chain length [20]. Furthermore, although sulphydryl blocking agents appear to preferentially inhibit arylesterases, there is some evidence, in the context of esterase inhibition, that these compounds may not affect mercapto groups at all [13, 21].

This current communication has investigated the inhibitor characteristics of the two major myeloid esterase isoenzyme species. The first of these, which is specifically associated with monocytic differentiation (MonEst), was purified to homogeneity [7] using an extensive chromatographic procedure which involved 18 different columns (ion-exchange, hydrophobic interaction, lectin affinity and gel filtration). The resulting pure protein appears

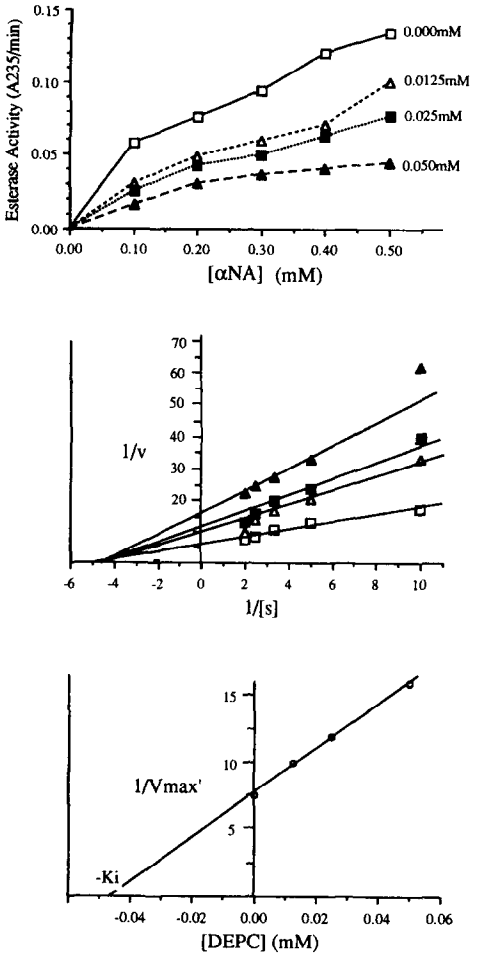

Fig. 4. Effect of diethyl polycarbonate (DEPC) on the observed rate of alpha-naphthyl acetate (αNA) hydrolysis by the purified ComEst species. The rate of αNA hydrolysis was determined by UV spectrophotometry [8] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A_{235}/min with each data point representing a mean of duplicate determinations. The upper diagram shows the ComEst rates of αNA hydrolysis with a range (0.1–0.5 mM) of substrate concentrations in the presence of increasing DEPC (0–0.05 mM). The Lineweaver–Burk plot (centre diagram) indicates that DEPC exerts noncompetitive inhibition on ComEst; a secondary plot of altered $1/V_{max}$ ($1/V'_{max}$) values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (K_i) for inhibition of ComEst by DEPC.

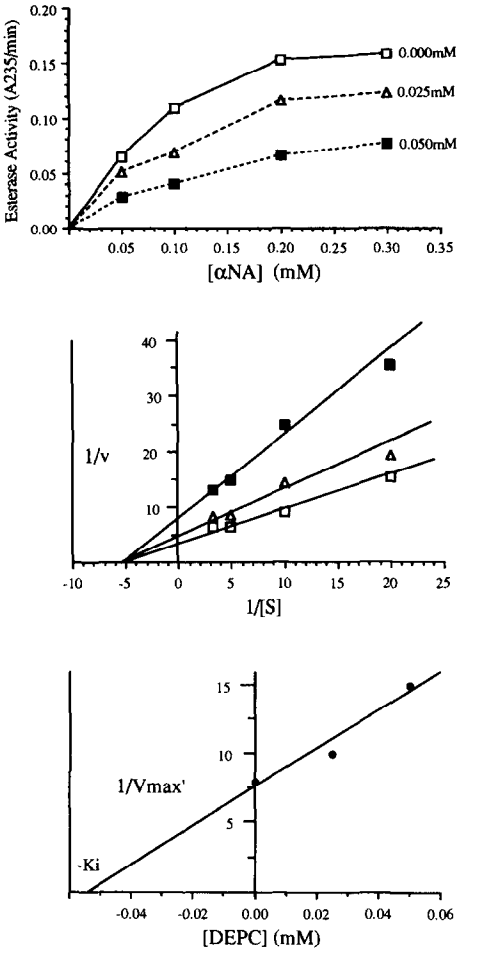

Fig. 5. Effect of diethyl polycarbonate (DEPC) on the observed rate of alpha-naphthyl acetate (αNA) hydrolysis by the purified MonEst species. The rate of αNA hydrolysis was determined by UV spectrophotometry [8] at each inhibitor concentration as detailed in Materials and Methods; reaction rates are shown as increases in A_{235}/min with each data point representing a mean of duplicate determinations. The upper diagram shows the ComEst rates of αNA hydrolysis with a range (0.05–0.3 mM) of substrate concentrations in the presence of increasing DEPC (0–0.05 mM). The Lineweaver–Burk plot (centre diagram) indicates that DEPC exerts noncompetitive inhibition on MonEst; a secondary plot of altered $1/V_{max}$ ($1/V'_{max}$) values versus inhibitor concentration (lower diagram) was used to determine the inhibition constant (K_i) for inhibition of MonEst by DEPC.

to exist as an enzymatically active trimer which migrates as a single charge species by Native-PAGE and irreversibly forms a 63 kDa monomer in the presence of SDS. The second myeloid esterase species studied (ComEst) was purified to a highly enriched state, contaminated only by a small number of non-esterase proteins, using a 13 column chromatographic sequence. This esterase fraction was shown to exist as an homogenous and active 68 kDa monomer in the presence of SDS and comprised four main charge species by Native-PAGE (corresponding to the four isoenzyme components as defined by IEF). A number of previous studies have claimed purification of the MonEst species but examination of their data indicate a number of discrepancies. For example, the most recent of these [22] reported the purification of monocytic carboxylesterase to homogeneity, with apparent K_m values (pH 7.5) of 0.16 mM (V_{max}, 7.5 units/mg protein) and 0.33 mM (V_{max}, 14.0) for α-naphthyl acetate and butyrate respectively. However, as the IEF analysis of this purified esterase species revealed pI values ranging from 7.5 to 7.8, clearly differing from a range of 5.5 to 6.2 for the

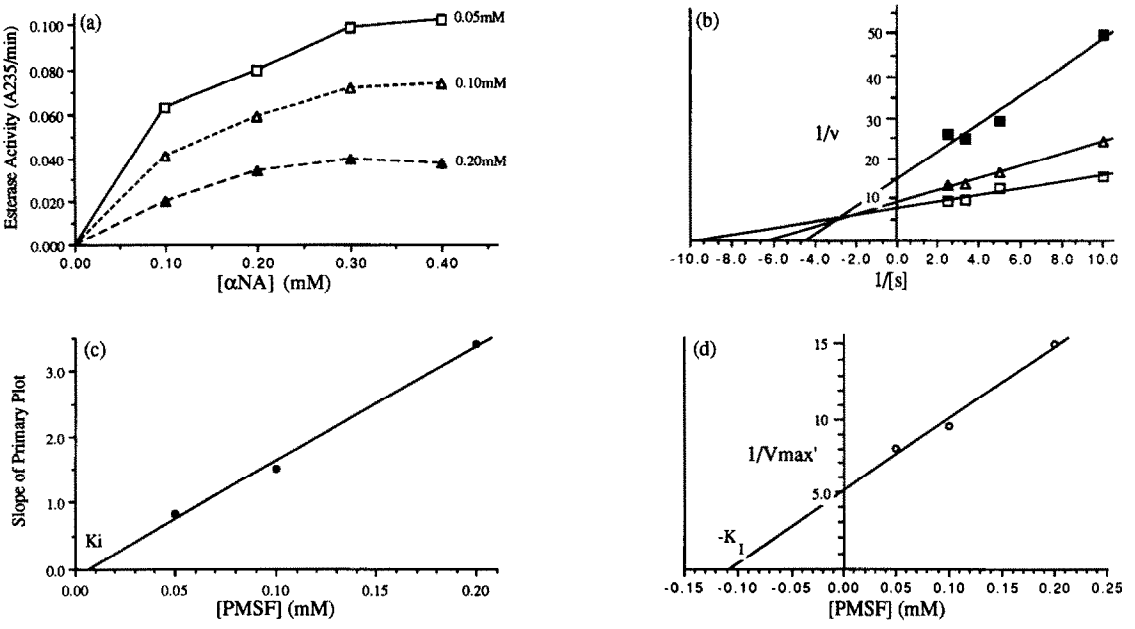

Fig. 6. Relationships between phenylmethylsulphonyl fluoride (PMSF) inhibitor concentrations and observed rates of alpha-naphthyl acetate (αNA) hydrolysis by the purified MonEst species (a). The rate of αNA hydrolysis was determined by UV spectrophotometry [8] at each inhibitor concentration as detailed in Material and Methods; reaction rates are shown as increases in A_{235}/min with each data point representing a mean of duplicate determinations. The Lineweaver–Burk plot (b) indicates that PMSF exerts mixed competitive/noncompetitive inhibition on MonEst. Secondary plots of slope of primary plot (b) and altered $1/V_{max}$ ($1/V'_{max}$) values versus inhibitor concentration were used to determine inhibition constants for the competitive [K_I (c)] and noncompetitive [K_i (d)] inhibition, respectively, of MonEst by PMSF.

MonEst species which is widely accepted by many independent investigators [1, 23, 24], we consider that these results are more in keeping with the characteristics of the myeloid ComEst species or a previously described monocytic acetic ester hydrolase [25].

Many organic and inorganic substances are capable of modifying enzyme activity, and inhibitors in biological systems often serve as control mechanisms. As pH can have a significant influence on the inhibition characteristics of any given enzyme, preliminary studies were carried out to determine the substrate reaction rates of both myeloid esterase species throughout a wide pH range. The results showed that the assay of the MonEst species for butyrate esterase activity was not possible above pH 7.0, because of unacceptable rates of spontaneous hydrolysis, and that in the pH range 6.0–7.0 there appeared to be little significant difference in the reaction rate. Although acetate esterase activities could be measured at a higher pH, the reaction rates were generally similar through the pH range of 6.0 to 9.0. The UV spectrophotometric esterase assay used in this study, which measures the generation of α-naphthol at 235 nm, was originally optimized in MES at pH 6.4 [8]. As the histochemical demonstration of ComEst and MonEst with α-naphthyl acetate and butyrate are also typically carried out at an acidic pH, a pH of 6.3 was selected for the inhibition studies reported in this present communication.

Enzyme inhibition can be either a reversible or irreversible process. With irreversible inhibition, the inhibitor and enzyme usually become covalently linked whereas reversible inhibition results from a relatively loose (e.g. electrostatic) association. Of the 17 inhibitors examined in this present study, two (SDS and DEPC) caused >50% inactivation of ComEst, and two further inhibitors (NaF and iodoacetamide) induced partial (20–50%) inhibition. In contrast, a total of six agents (PMSF, DCIC, NaF, SDS, TPCK and DEPC) exerted significant (>50%) inhibitory effects on MonEst isoenzymes. In terms of assessing differences between the active sites of ComEst and MonEst, these observations provided a number of important insights.

Sodium fluoride (NaF) is widely used in histochemical studies for the differentiation of esterase species. NaF inhibited both groups of isoenzymes reversibly in a noncompetitive manner, indicating that the fluoride ion may bind to an amino acid residue which is unlikely to be part of the active site and, consequently, does not affect its affinity for the substrate. However, the amino acid–fluoride complex is sufficiently close to the active site to conformationally induce a change in the catalytic ability of the enzyme, resulting in a reduced rate of substrate turnover (i.e. the K_m remains constant whereas the V_{max} decreases with increasing NaF concentration). Although the type of inhibition exerted by NaF appears similar for both esterase species, the magnitude of inhibition was markedly

different in that MonEst isoenzymes showed a 128-fold increased sensitivity to NaF as compared to ComEst. Although a commonly observed alternative mode of NaF inhibition is to bind to an essential metal ion (usually Mg^{2+}) required for enzymatic activity, our observations that other metal chelators (EDTA and 1,10-phenanthroline) did not cause significant inhibition tends to exclude this possibility.

Assessing the presence of serine residues in enzyme active sites is commonly achieved by examining the inhibitory effect of organophosphates. The mechanisms of organophosphate inhibition appear to be due to the ability of these compounds to compete with normal substrate acyl groups. Instead of becoming acylated, some esterases undergo phosphorylation and the reaction is unable to proceed to the azo-dye coupling stage [26, 27]. Although organophosphate inhibition of esterases has been widely interpreted as evidence for the presence of an active serine residue, there is some evidence to suggest that these inhibitors may also react with a group (?imidazole) near the active site and that steric hindrance can subsequently prevent substrate binding [10, 28]. Two inhibitors used in this present study, PMSF and DCIC, were shown to specifically inactivate the MonEst isoenzymes whilst causing little significant inhibition of ComEst. As these agents inactivate serine residues by sulphonylation and acylation, respectively, these observations clearly indicate a significant difference between the MonEst and ComEst species with regards to the presence of a serine residue in their active sites. Further characterization of the effects of PMSF on MonEst revealed that the mixed competitive–noncompetitive inhibition of MonEst was almost certainly due to a combination of fluoride-induced (noncompetitive) inhibition at low PMSF concentrations and irreversible (competitive) inhibition by sulphonylation at PMSF concentrations exceeding 5.0 mM.

This study also showed that DEPC reversibly inhibited both species of esterase isoenzyme, TPCK irreversibly inhibited MonEst but not ComEst, and TLCK was non-inhibitory. The mechanisms of irreversible inhibition by DEPC, TPCK and TLCK are broadly similar in that these compounds can all bind to the enzyme active site, and alkylate essential histidine residues, by mimicking the substrate. The patterns of inhibition with these agents therefore provide additional information regarding the nature of histidine involvement. The inability of TLCK to cause significant inhibition suggests that the active site sequences of both MonEst and ComEst differ from trypsin but, because of its irreversible inhibition by TPCK, MonEst may share certain similarities to chymotrypsin (even though MonEst is not affected by the chymotrypsin inhibitor NCDC). Further analysis of DEPC inhibition kinetics revealed that both ComEst and MonEst species were inhibited in a noncompetitive manner, similar to the effect exerted by NaF. On the basis of irreversible inhibition by TPCK, it is concluded that MonEst activity is dependent on the presence of histidine residues in the active site whereas for ComEst, this is less likely.

Iodoacetamide inactivates enzymes containing essential cysteine residues by alkylation. Consequently, the lack of significant inhibition of either esterase isoenzyme group by this compound tends to rule out the involvement of cysteine residues within their active sites. This interpretation is further substantiated by the failure of HMBA, which affects -SH groups in a non-specific manner, to inactivate these esterase species and supports previous conclusions [15] that there is no evidence that sulphydryl groups are essential for activity of acetylesterases or carboxylesterases. Analysis of two metal-chelating agents (EDTA and 1,10-phenanthroline) also confirmed that ComEst and MonEst were not metalloproteins, and neostigmine which is a potent inhibitor of cholinesterases also failed to induce significant inhibition of either species.

Enzyme assays in the presence of 1% SDS caused marked inhibition of both esterase isoenzyme species. However, subsequent gel filtration of SDS-treated esterase fractions demonstrated clearly that inhibition of ComEst species was reversible whereas for MonEst, this inhibition was irreversible. One obvious explanation for this difference in inhibition between the two esterase forms is provided by our earlier studies [7] which showed that the normally occurring active trimeric MonEst form migrates electrophoretically as an inactive monomer following SDS treatment (i.e. irreversible dissociation). In distinct contrast, the ComEst species occurs as a monomer which retains its ability to hydrolyse αNA following SDS treatment. These findings further suggest that trimeric association of MonEst monomers is electrostatic in nature and that the ionic binding of SDS prevents subunit recombination.

In summary, these results confirm our earlier conclusions from molecular and substrate studies that the ComEst and MonEst myeloid esterase species are in fact two different enzymes. The results of this current investigation into the effects of a wide range of inhibitors, including a detailed assessment of inhibition types, on ComEst and MonEst activity have further characterized the nature of these enzymes and provide a basis whereby their differential use may lead to modified esterase histochemical procedures with a greater degree of specificity. Furthermore, as MonEst is a highly specific marker of monocytic differentiation and is widely considered to have a fundamental (although as yet unproven) role in monocyte-associated functions, such as antigen processing and cellular cytotoxicity, these results are also important with respect to elucidating the biological function of MonEst and the consequences of its potential pharmacological modulation.

Acknowledgements—We would like to thank Professor A. J. Turner (Department of Biochemistry and Molecular Biology, University of Leeds) for his helpful comments. These studies were supported by the Friends of the Leukaemia Unit at Leeds General Infirmary; D.P. is supported by the Yorkshire Cancer Research Campaign.

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