Biochemical Characterization of an Aryl Acetic Ester Hydrolase Isolated from Human Monocytes

William K. W. Lam, Julia Chen, Edwin Taft, and Lung T. Yam

A carboxylic-ester hydrolase was isolated from the leukocytes of a patient with myelomonocytic leukemia. Its relative molecular mass as estimated by sucrose density-gradient sedimentation is about 70 000. The purified enzyme is specific for acetyl esters of aromatic alcohols. It is inhibited by fluoride, but insensitive to eserine or p-chloromercuriphenylsulfonate. Hydrolysis of 1-naphthyl acetate was optimal above pH 6.0; of α-nitrophenyl acetate, above 8.0. The common catalytic site for the two types of substrates on the enzyme was confirmed by competitive inhibition data.

Additional Keyphrases: enzyme activity · leukemia · leukocytes

Many enzymes catalyze the hydrolysis of naphthyl esters. The hydrolysis product, naphthol, forms insoluble colored compounds with azo dyes; therefore, these enzymes are easily detected by histochemical methods. Wachstein and Wolf (1) used "naphthyl AS acetate" (2-acetoxy-3-naphthoic acid anilide) to demonstrate esterase activity among different types of cells in smears of bone marrow and blood. Braunstein (2) observed that only monocytes were stainable with naphthyl acetate as substrate. The chloroacyl esters of naphthol—naphthol-AS or naphthol-ASD—stained mast cells and neutrophilic granulocytes but were inactive for monocytes (3-6). Fischer and Schmalzl (7) showed that the monocyte esterase was fluoride sensitive, while granulocyte esterase was fluoride resistant. The optimal procedures for identification of monocytes and for differential diagnosis of leukemia based on esterase staining were described in our previous reports (5, 6).

The substrate and inhibitor specificity of monocyte esterase described above indicate that the high esterase activity of monocytes is due to enzymes that are not present in other leukocytes. The conclusion is strengthened by the electrophoresis of monocyte extract, which shows only one major activity band (6). The activity in this band was isolated by ion-exchange chromatography, and its biochemical properties are described in this report.

Materials and Methods

Leukapheresis: A 47-year-old man with acute myelomono-
ml of the phosphate buffer. The eluate was collected in 3-mL fractions, and 10-μL portions from each fraction were analyzed for esterase activity, with α-naphthyl butyrate or α-naphthyl acetate as substrate.

Fractions under the second peak (which was inactive to α-naphthyl butyrate, see Fig. 3, ib) were combined and the enzyme was precipitated by ammonium sulfate (40–65% saturation) and dialyzed. The CM-Sepharose chromatography step was repeated in the same manner.

Electrophoresis was done as described previously (6). For spectrophotometry, we used a final volume of 1 mL, including phosphate buffer (0.1 mol/liter, pH 7.5) and substrate (1 mmol/liter). The p-nitrophenol formed was calculated from the absorbance measured at 410 nm. The naphthol formed was estimated from the color produced by adding 2 mL of a color reagent prepared by dissolving 30 mg of Fast Garnet GBC, 4.8 g of lauryl sulfate, 0.93 g of sodium acetate, and 2.35 g of sodium barbitol in 100 mL water, which is then mixed with 40 mL of 0.2 mol/liter HCl. The color developed with α-naphthol was measured at 570 nm; that developed with β-naphthol was measured at 520 nm. One unit (U) of enzyme activity is the activity producing hydrolysis of 1 μmol of substrate per minute in 1 mL of assay medium.

Protein concentration was determined by the method of Lowry et al. (9), with bovine serum albumin as standard. The relative molecular mass of the esterase was estimated by the sucrose density gradient sedimentation method described by Martin and Ames (10).

Results

The distribution of esterase activity at different stages of purification is summarized in Table 1. Most of the esterase activity in the cell suspension was recovered in the soluble fractions after sonic disruption. The activity in the extract was divided into three peaks by DEAE-cellulose chromatography (Figure 1). The first peak was purified by repeated chromatography on a CM-Sepharose column for biochemical characterization.

Distribution of activity in the fractions from DEAE-column chromatography was first determined spectrophotometrically. All of the proteins with low affinity for the column emerged from the column in a broad peak (I) as the extract passed through the column. The esterase within peak I reacted to both α-naphthyl acetate and α-naphthyl butyrate.

As sodium chloride concentration was increased above 0.1 mol/liter a sharp activity peak (peak II) was observed. The enzyme activity in this location had a stronger reactivity toward α-naphthyl butyrate than α-naphthyl acetate.

<table>
<thead>
<tr>
<th>Table 1. Analytical Recovery of Esterase Activity During Purification of the Aryl Acetic Ester Hydrolase</th>
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<tbody>
<tr>
<td>Purification step</td>
</tr>
<tr>
<td>1. Sonic disruption</td>
</tr>
<tr>
<td>a. soluble fraction</td>
</tr>
<tr>
<td>b. particulate</td>
</tr>
<tr>
<td>2. DEAE-column chromatog.</td>
</tr>
<tr>
<td>Peak I</td>
</tr>
<tr>
<td>(40–50% saturation)</td>
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<tr>
<td>3. 1st CM-column</td>
</tr>
<tr>
<td>Peak IA</td>
</tr>
<tr>
<td>Peak IB</td>
</tr>
<tr>
<td>4. 2nd CM-column</td>
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</tbody>
</table>

All assays were done in 1 mL at 37°C, with 1 mmol/liter substrate.

The third peak had relatively little reactivity toward α-naphthyl butyrate as compared to naphthyl acetate.

As described earlier (2), a crude extract of leukocytes showed only one electrophoretic band, strongly reactive toward α-naphthyl acetate (Figure 2A), but only weakly toward α-naphthyl butyrate (Figure 2B). The same phenomena were observed in the enzymic activity corresponding to Peak I (Figure 2C, D). The second peak showed a sharp electrophoretic band (Figure 2E) in the same location as that for Peak I and a diffused activity band with slower electrophoretic mobility. The sharp band contained material that reacted with either α-naphthyl acetate or α-naphthyl butyrate (Figure 2F). The third peak showed two bands, which reacted only toward naphthyl acetate (Figure 2G and H). Our present interest is in Peak I only.

It was mentioned earlier that spectrophotometric analyses of Peak I showed similar reactivity toward α-naphthyl acetate and α-naphthyl butyrate. However, electrophoretic analyses showed much stronger reactivity toward naphthyl acetate (Figure 2C) than toward naphthyl butyrate (Figure 2D). This was explained later by the results of chromatography on CM-Sepharose.

Peak I (Figure 1) was further separated into two peaks (IA and IB) on the first CM-Sepharose column (Figure 3). According to spectrophotometric analyses, Peak IA is slightly
more reactive toward α-naphthyl butyrate than α-naphthyl acetate. When material corresponding to Peak IA was subjected to electrophoresis, no activity band was observed (Figure 2, I and J). Peak IA must be a labile enzyme. Electrophoresis of Peak IB showed one strong activity band, reactive only toward α-naphthyl acetate (Figure 2K, L).

Rechromatography of Peak IB on another CM-Sepharose column yielded a single activity peak with much improved specific activity. The active fractions were combined for biochemical characterization.

The purified enzyme is specific for acetyl esters of aromatic alcohols. The acetyl group cannot be replaced by a carboxyl group with more than two carbons. The alcohol group can be either naphthol or nitrophenol. Inactive substrates tested include acetylcholine, naphthyl-ASD-chloroacetate, α-naphthyl-leucylamide, α-nitrophenyl propionate, o-nitrophenyl sulfate, o-nitrophenyl-α-D-glucoside, p-nitrophenyl-N-acetyl-α-D-glucosamide, o-nitrophenyl caproate, and α-naphthyl palmitate. The $K_m$ and $V_{\text{max}}$ of the active substrates are shown in Table 2.

The pH-activity relationship is shown in Figure 4. When o-nitrophenyl acetate is used as substrate the enzyme has very little activity below pH 6 and maximal activity above pH 8.0. The optimal pH for the hydrolysis of α-naphthyl acetate is shifted to the acidic side. p-Nitrophenyl acetate is very unstable above pH 7.5, therefore Figure 5 shows only the results for o-nitrophenyl acetate.

The different pH optima of the two substrates led us to wonder if the enzyme preparation contained two protein species, one acting on each of the two different substrates. This possibility was ruled out by the competitive inhibitory effect of o-nitrophenyl acetate (Figure 6) on the hydrolysis of α-naphthyl acetate. When o-nitrophenyl acetate was added to the assay medium for α-naphthyl acetate, naphthol pro-

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**Table 2. Kinetic Properties of Aryl Acetic Ester Carboxylic Ester Hydrolase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mmol/liter)</th>
<th>$V_{\text{max}}$ (μmol/min)</th>
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<tbody>
<tr>
<td>o-Nitrophenyl acetate</td>
<td>1.70 ± 1.30</td>
<td>2.68 ± 1.1</td>
</tr>
<tr>
<td>α-Nitrophenyl acetate</td>
<td>0.95 ± 0.10</td>
<td>11.8 ± 0.5</td>
</tr>
<tr>
<td>α-Naphthyl acetate</td>
<td>1.34 ± 0.14</td>
<td>14.5 ± 1.0</td>
</tr>
<tr>
<td>β-Naphthyl acetate</td>
<td>1.64 ± 0.51</td>
<td>8.6 ± 1.5</td>
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</table>

All assays were done in 1 ml at 37 °C. At least 10 different substrate concentrations (0.1 to 2.0 mmol/liter) were used. The mean value and standard error was calculated by use of the computer program described by Bliss and James (22).
duction was competitively inhibited by o-nitrophenyl acetate, which indicated that both substrates compete for the same active site of one enzyme. The $K_i$ for nitrophenyl acetate, estimated by the graphical method of Dixon (11), was about 1 mmol/liter, which is very similar to the $K_m$ value when it was used as the substrate (Figure 6).

The enzyme was insensitive to 0.1 mmol/liter p-chloromercuriphenylsulfonate and eserine. Competitive inhibition by fluoride was observed ($K_i = 12$ mmol/liter) (Figure 7).

Centrifugation of a mixture of the purified enzyme and hemoglobin showed similar sedimentation properties for the two proteins. The esterase activity sedimented slightly faster than that of hemoglobin (Figure 8). We estimate the relative molecular mass of the esterase to be about 70,000.

Discussion

Carboxylic-ester hydrolase includes many enzymes that share similar catalytic activity toward synthetic substrates. These enzymes have been reviewed by Latner (12), Krisch (13), Shnitka (14), and Masters and Holmes (15). Because there are so many of these enzymes and because of the overlapping substrate specificity, their subdivision has been difficult.

The requirement of an aromatic alcohol group for the present enzyme favors its classification with the group of aryl-ester hydrolases. This group of esterases has been isolated from serum (16, 17) and liver (18-20), but it was not specific to acetyl esters. On the other hand, the hydrolases specific to acetic esters, described by Bergman et al. (21) and Jansen et al. (22), were not specific to the aromatic alcohol group. The present report describes an enzyme specificity requiring both the acetyl and aryl groups. Therefore, it should be classified as an aryl acetic ester hydrolase (EC 3.1.1.2). The lack of reactivity toward carboxylic esters of choline and the insensitivity to eserine clearly rule out any relationship of this enzyme to choline esterases. The unique substrate and inhibitor specificity, as well as its electrophoretic mobility, clearly show that the enzyme described in this report is a distinct protein species, different from those described previously (12-22).

The high esterase activity observed in one type of cells could be the result of a high concentration of all esterases, or to a disproportionate activity of one or more specific enzymes related to the specialized function of the cell. Previous studies show that monocyte esterase has properties different from those of other leukocytes. The biochemical characteristics of the purified enzyme also point up the unique features of the monocyte esterase. Therefore, the high esterase activity in monocytes clearly is attributable to the occurrence of a few specific enzymes rather than to a general increase in all esterases, as the name "nonspecific esterase" implies.

The criteria for "nonspecific esterase" previously observed in monocytes were sensitivity to fluoride and insensitivity to eserine inhibition (6, 7). Enzymes with these properties were observed by electrophoresis among the cationic proteins. On staining for enzyme activity with $\alpha$-naphthyl-ASD-chloroacetate, at least nine bands could be seen in the extract of normal leukocytes, but only band 5 was reactive toward naphthyl acetate (6). It is the major band shown by monocytes, but is absent from granulocytes and lymphocytes. Our previous report (6) showed that when the acidity of the staining solution was reduced to pH 6.5, the intensity of band 5 was unchanged, while the other bands disappeared. This was consistent with the pH optimum-curve of the purified enzyme described in Figure 4, which shows the same activity for naphthyl acetate between pH 6 and 8. All properties of the purified enzyme described in this report, including electrophoretic mobility and substrate and inhibitor specificity, are identical with the previous data for "nonspecific esterase" of monocytes.

This study has been reproduced in four cases of myelomonocytic leukemia, and one case of acute monocytic leukemia. The enzyme was barely detectable in three cases of chronic granulocytic leukemia, and not detectable in two cases of chronic lymphocytic leukemia. The trace amount of band 5 observed in leukocytes of granulocytic leukemia is probably due to contamination by monocytes in the preparation. The number of monocytes in lymphocytic leukemia is usually negligible.

Our column chromatographic studies demonstrate the presence of two esterases (Peaks I and II) with identical electrophoretic mobility. Both are reactive toward $\alpha$-naphthyl acetate, but only the activity corresponding to Peak II is reactive toward $\alpha$-naphthyl butyrate. Another esterase, Peak IA (Figure 3) is inactive after electrophoresis (Figure 2 I, J). Therefore, electrophoresis of the crude extract shows only one band, with strong reactivity toward naphthyl acetate but weak reactivity toward naphthyl butyrate. The characterization of Peak IA and II will be described in the future.

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References