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Differentiation of EDTA-sensitive from EDTA-insensitive human serum esterases hydrolysing phenylacetate

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Abstract

The aim of this study was to differentiate the EDTA-sensitive from the EDTA-insensitive human serum esterases by evaluating their catalytic constants, K_M and V_m, for the hydrolysis of phenylacetate (PA). Measurements were done at 37°C in 0.1 M Tris/HCl buffer pH 7.4 and 8.4. The $K_{M,sen}$ and $K_{M,ins}$ constants were significantly different, 0.97 and 2.7 mM respectively, confirming that two esterases hydrolyse PA. The pH of the medium had no effect on K_M values, and also no effect on $V_{m,sen}$ while $V_{m,ins}$ was two fold higher at pH 8.4 than at 7.4 further confirming the existence of two different enzymes. The stability of the esterases in aqueous media was also studied. EDTA-sensitive activity in buffer without $CaCl_2$ was extremely unstable; the time-course of inactivation followed a two-phase reaction kinetics, indicating that two EDTA-sensitive esterases hydrolyse PA. The EDTA-insensitive activity remained constant in aqueous media under the same experimental conditions.

Keywords: *Arylesterases, catalytic constants,* K_M, V_m, *esterase stability, phenylacetate hydrolysis measurement*

Introduction

Mammalian sera contain EDTA-sensitive and EDTA-insensitive esterases that hydrolyse phenylacetate (PA). The same holds for the hydrolysis of paraoxon (POX; O,O′-diethyl 4-nitrophenyl phosphate). PA is the characteristic substrate of arylesterase (EC 3.1.1.2) and the organophophorus compound POX is the characteristic substrate of paraoxonase (EC 3.1.8.1) [1]. Until 1992, both enzymes were classified as arylesterase [2,3]. The physiological substrates of these enzymes are not known and their substrate specificities overlap. Paraoxonases hydrolyse organophosphorus compounds but also aryl esters and lactones [4]. The majority of studies are however performed with POX as substrate. The rates of POX or PA hydrolysis in the serum are affected by diseases linked to lipid metabolism disorders (atheroscleroses, hypercholesterolemia, diabetes)[5–10].

EDTA-sensitive esterases in mammalian sera require calcium ions for activity, and chelating agents, like EDTA, therefore inhibit both PA and POX hydrolysis. The distribution profile of EDTA-sensitive PA activities in human sera is unimodal [11], while the distribution profile of the EDTA-sensitive POX activities is polymodal [11,12] due to polymorphism in the paraoxonase coding sequence [13,14]. Only about 1–2% of the total activity with PA and up to 10% of the total activity with POX are EDTA-insensitive [11]. The EDTA-insensitive hydrolysis of PA was shown to be due to a serine esterase [15]. The EDTA-insensitive hydrolysis of POX has been attributed to albumin [12,16]. Both EDTA-insensitive activities are unimodally distributed in mammalian sera, and there is no correlation between EDTA-sensitive and EDTA-insensitive activities for either PA or POX hydrolysis [11].

Although EDTA-sensitive and EDTA-insensitive esterases in human sera have been known for a long time, there are few published data on the catalytic constants for the hydrolysis of their substrates. We evaluated K_M and V_m constants for the hydrolysis of

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PA in order to differentiate quantitatively these two esterases. The stability of these esterases in aqueous solutions was also evaluated as a tool of further differentiation, and as an important property when choosing experimental conditions for the study of these enzymes. For the sake of comparison, some data on POX hydrolysis are also included in this paper.

Materials and methods

Enzyme source

The enzyme source was native human serum from 11 healthy adults (5 male and 6 female). Samples were stored at −18°C before assay.

Buffers and substrate

All measurements were done in 0.1 M Tris/HCl buffer pH 7.4 or 8.4. The buffer was prepared by titrating 0.2 M tris (hydroxymethyl) aminomethane with 30% HCl to the required pH. Buffers containing $CaCl_2$ (1.0 mM final concentration) or EDTA (1.0 mM final concentration) were prepared in the same way. The substrate was PA ("Sigma", USA). Stock solutions of PA (200 mM) were prepared in 40% methanol in water.

Enzyme activity measurements and calculation of catalytic constants for substrate hydrolysis

All activities were measured spectrophotometrically at 270 nm at 37°C (thermostated instruments) [17]. The optical path was 1.0 cm, and the total assay volume was 3.0 mL. The final substrate concentrations were 0.2, 1.0 and 5.0 mM PA.

Serum samples were diluted 60- or 120- fold with saline (0.9% NaCl in water) at room temperature (about 25°C) and aliquots added to the medium containing buffer and substrate at 37°C. The final serum dilution during activity measurements was 600- or 1200-fold. The time of assay was 2–3 min (in the absence of EDTA) or 10 min (in the presence of EDTA). The increase in absorbance was linear during the time of the assay.

Activities were corrected for the rates of spontaneous substrate hydrolysis, and expressed in micromoles of hydrolysed PA per minute per millilitre of serum. The used ε_M values ($M^{-1}\ cm^{-1}$) were 1510 at pH 7.4 [17] and 1323 at pH 8.4 (this paper).

Rates of substrate hydrolysis measured in buffer containing $CaCl_2$ represent the total enzyme activity, $v_{tot.}$ Rates of substrate hydrolysis measured in buffer containing EDTA represent the EDTA-insensitive enzyme activity, v_{ins}. The difference between v_{tot} and v_{ins} represents the EDTA-sensitive enzyme activity, v_{sen},:

$$v_{sen} = v_{tot} - v_{ins} \quad (1)$$

To minimize experimental errors of measurement, the v_{tot} and v_{ins} activities in a given sample were always measured on the same day.

The Michaelis constants (K_M) and maximum reaction rates (V_m) were calculated from the linearized Michaelis-Menten equation:

$$s/v = (K_M/V_m) + (s/V_m) \quad (2)$$

where v denotes the enzyme activity and s the substrate concentration.

ε_M for phenol at pH 8.4

The absorbance of phenol (six concentrations; 50–500 μM) was measured (each concentration 8 times) at 270 nm in buffer without $CaCl_2$ or EDTA. The ε_M (± SD) calculated from the slope of the calibration line was $1323.04 \pm 0.03\ M^{-1}\ cm^{-1}$.

Spontaneous hydrolysis of PA

The rates of spontaneous hydrolysis of PA (0.2, 1.0 and 5.0 mM) were measured at 37°C in buffer without $CaCl_2$ or EDTA, in buffer with 1.0 mM $CaCl_2$, and in buffer with 1.0 mM EDTA. The rate constants of spontaneous PA hydrolysis, k_{PA}, were calculated from the equation:

$$\Delta P/\Delta t = k_{PA}\cdot(PA) \quad (3)$$

where ΔP is the increase in phenol concentration over time Δt, and (PA) is the initial PA concentration. As the Δt was short, the decrease in PA concentration need not to be taken into account. The rates of spontaneous PA hydrolysis were the same in all three buffers, and the obtained rate constants (mean ± SD; 18 individual assays) were:

$$k_{PA} = (5.2 \pm 0.3)10^{-4}\text{min}^{-1} \text{ at pH } 7.4$$

$$k_{PA} = (26.7 \pm 0.0)10^{-4}\text{min}^{-1} \text{ at pH } 8.4$$

Stability of esterases hydrolyzing PA in aqueous media

The stability was studied in saline at room temperature and in buffer at 37°C. The enzyme activities were measured with 5.0 mM PA as substrate.

When stability was studied in saline, serum was diluted into saline (60 or 120 times) and kept at room temperature (about 25°C) up to 90 min. At given time intervals aliquots were withdrawn, added into buffer containing substrate at 37°C, and the activities were measured. The buffer contained either $CaCl_2$ or

EDTA. The v_{sen} activities were calculated from Equation (1).

When stability was studied in buffer, serum was diluted into saline (60 or 120 times), and aliquots diluted further into buffer at 37°C. At given time intervals, the substrate was added, and the activity measured at 37°C. Buffer composition, serum dilution and time of inactivation are given under *Results and discussion*.

When the time-course of inactivation followed first-order kinetics, the rate constants of inactivation k were calculated from the equation:

$$\ln a_0/a_t = k{\cdot}t \tag{4}$$

where a_0 and a_t are the enzyme activities at time zero and at time t. When the time course of inactivation followed two-phase reaction kinetics, the rate constants of inactivation k_1 and k_2 were calculated from the equation

$$y_t = \mathrm{A}{\cdot}e^{-k_1{\cdot}t} + \mathrm{B}{\cdot}e^{-k_2{\cdot}t} \tag{5}$$

where y_t denotes percentage of enzyme activity at time t, and A and B are percentages of total activity (y_0) that decrease at rate constants k_1 and k_2, respectively (total activity at time zero $y_0 = 100\% = \mathrm{A} + \mathrm{B}$).

Statistical analysis

To test differences between two constants (K_M or V_m), Paired t-test and the Wilcoxon signed rank test were used (confidence intervals 95%). These tests were performed using GraphPad Prism 4.00 (GraphPad Software, San Diego, California, USA).

Results and discussion

Catalytic constants for the hydrolysis of PA

The Michaelis constants and maximum reaction rates for the hydrolysis of PA are shown in Table I.

In each serum sample and at both pH values, $K_{M,sen}$ was significantly lower than $K_{M,ins}$. The level of significance (p-value) at pH 7.4 was 0.0001 (t-test) and 0.002 (Wilcoxon rank test) and at pH 8.4 it was 0.0008 (t-test) and 0.0039 (Wilcoxon rank test). The differences between K_M values at pH 7.4 and 8.4 were not significant (p-values range from 0.12 to 1.0).

These results confirm that EDTA-sensitive and EDTA-insensitive esterases in human sera are two different enzymes, where the EDTA-sensitive enzyme has a higher affinity (lower K_M) for PA than the EDTA-insensitive enzyme. Mean $K_{M,sen}$ value obtained at pH 7.4 and 8.4 is 0.97 mM and the mean $K_{M,ins}$ is 2.7 mM (cf. Table I).

EDTA-insensitive enzyme activities, v_{ins}, amounted in all serum samples and at both pH values to only 1–2% of the total enzyme activities, v_{tot}. As this is a very small percentage, the v_{sen} activities are almost the same as the v_{tot} activities (cf. Equation (1)). Consequently, there is no significant difference between either $K_{M,sen}$ and $K_{M,tot}$ or between $V_{m,sen}$ and $V_{m,tot}$ (cf. Table I). $V_{m,sen}$ were the same at both pH values (p-value: 1.0 in both tests), while $V_{m,ins}$ was 1.8 times higher at pH 8.4 than at pH 7.4 (p-value:

Table I. Michaelis constants (K_M) and maximum reaction rates (V_m) for the hydrolysis of phenylacetate (PA) and paraoxon (POX) in the absence (v_{tot} activity; $K_{M,tot}$, $V_{m,tot}$) and in the presence of 1.0 mM EDTA (v_{ins} activity; $K_{M,ins}$, $V_{m,ins}$). EDTA-sensitive activity (v_{sen}; $K_{M,sen}$, $V_{m,sen}$) was calculated according to Equation (1). Activities were measured at pH 7.4 and 8.4. K_M and V_m constants were calculated according to Equation (2). Numbers are mean values obtained on serum samples from ten individuals.

	PA		POX*
	pH 7.4	pH 8.4	pH 7.4
$K_{M,sen}$/mM			
Mean ± SD	0.86 ± 0.29	1.08 ± 0.60	–
Range	0.50–1.54	0.47–2.15	
$K_{M,ins}$/mM			
Mean ± SD	2.59 ± 0.43	2.71 ± 0.84	–
Range	1.76–3.10	1.85–4.35	
$K_{M,tot}$/mM			
Mean ± SD	0.86 ± 0.29	1.10 ± 0.60	1.02 ± 0.23
Range	0.51–1.54	0.47–2.15	0.59–1.29
$V_{m,sen}$/(μmol min^{-1} mL^{-1})			
Mean ± SD	178 ± 72	179 ± 77	–
Range	84.6–311	89–341	
$V_{m,ins}$/(μmol min^{-1} mL^{-1})			
Mean ± SD	1.88 ± 0.56	3.69 ± 1.22	–
Range	1.12–3.20	2.42–6.59	
$V_{m,tot}$/(μmol min^{-1} mL^{-1})			
Mean ± SD	180 ± 72	182 ± 78	0.14 ± 0.10
Range	85.6–313	92–344	0.04–0.28

* Unpublished data and data from [21] (cf. [11] for POX hydrolysis measurement).

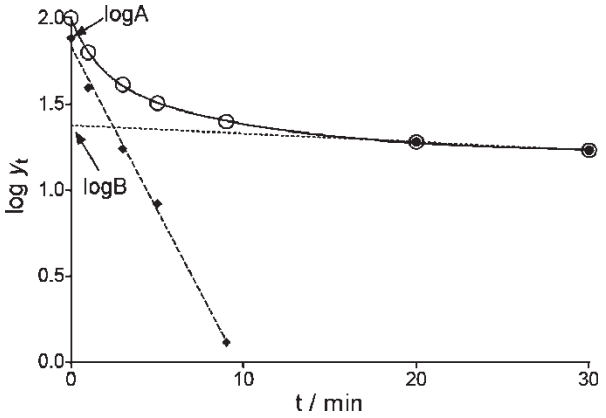

Figure 1. Time-course of inactivation of EDTA-sensitive esterase hydrolysing phenylacetate (PA) at 37°C in buffer pH 8.4 without $CaCl_2$ or EDTA (open circles; ○). Serum dilution during inactivation was 2160-fold. Inactivation followed two-phase reaction kinetics. Activities that inactivated slower were calculated from the line drawn through the full circles (•). These activities were subtracted from the measured activities (○) in order to obtain the fast-inactivating activities (full squares; ■). The slopes of the two lines are the rate constants k_1 and k_2 in Equation (5). A and B are the percents of total activity (cf. Equation (5)) that inactivate faster and slower, respectively. The calculated constants are given in Table II.

0.002 in both tests). This difference between $V_{m,sen}$ and $V_{m,ins}$ further confirms the difference between the two enzymes.

No data seem available on the K_M of EDTA-insensitive PA hydrolysis. Available data refer to $K_{M,tot}$, and that constant does not differ from $K_{M,sen}$ as discussed above. A recent study on purified arylesterase from human serum reports that K_M for the hydrolysis of PA is 0.86 mM (Tris buffer, pH 7.4, 25°C)[18], while a study on lyophilised human serum reports a K_M of 0.28 mM (Tris buffer, pH 7.3–7.4, 37°C) [19]. An earlier study on human plasma reports a K_M of 1.1 mM (Tris buffer, pH 7.5, 25°C) [20]. Our values on $K_{M,tot}$ and $K_{M,sen}$ fit into that fairly broad range (cf. Table I).

For the sake of comparison, Table I shows $K_{M,tot}$ and $V_{m,tot}$ constants for the hydrolysis of POX in ten serum samples from healthy individuals. The rates of POX hydrolysis were measured under the same experimental conditions as described for the hydrolysis of PA in this paper. $K_{M,tot}$ for POX is of the same order of magnitude as $K_{M,tot}$ for PA, while $V_{m,tot}$ for POX is about three orders of magnitude lower than $V_{m,tot}$ for PA.

Stability of PA-hydrolyzing esterases in aqueous media

The calculated rate constants of inactivation in saline at room temperature and in buffer at 37°C are summarized in Table II.

In both media, v_{ins} activities were stable under the experimental conditions listed in Table II. The v_{sen} activities however decreased with time at rates depending on the media. The half-time of v_{sen} inactivation was 6.4 h in saline at room temperature diluted up to 2400 times, and 1.6 h in buffer containing $CaCl_2$ at 37°C diluted up to 2160 times. In both media, the time-course of inactivation followed first-order reaction kinetics.

The activities were extremely unstable in buffer pH 8.4 at 37°C without $CaCl_2$ or EDTA (Table II). When PA is added to that medium, the measured activities reflect both v_{sen} and v_{ins} activities. As v_{ins} was shown to be stable, the decrease in activity reflects v_{sen}. The decrease deviated from first-order kinetics already after several minutes (Figure 1).

By applying Equation (5) to these results, we have graphically assessed two rate constants over a 30-minute inactivation time (Table II). About 80% activity decreased with a half time between 2–3 minutes and the remaining 20% had a half time of about 70 minutes. Although the time of inactivation was very fast before addition of substrate, the enzyme activity during assay was constant (i.e. the increase in absorbance during the time of assay was linear), indicating that addition of substrate had stabilized the enzyme. The same very fast decrease in activity was observed in buffer pH 7.4 without $CaCl_2$ or EDTA (results not shown). Deviations from first-order kinetics were observed within 9 minutes inactivation time. As only screening experiments were done on two serum samples, no rate constants were calculated.

Table II. Inactivation of esterases hydrolysing phenylacetate (PA) in buffer at pH 8.4 and in saline. Constants of inactivation were calculated from Equation (4) or (5). Each constant is the mean value (± SD) obtained on serum samples from ten individuals. The v_{sen} activities were calculated according to Equation (1).

Medium	Temp.	Serum dilution	Time of inactivation	Determined activity	Constants
Buffer without EDTA or $CaCl_2$	37°C	1080 or 2160-fold	30 min	$_{sen}$	$k_1 = 0.30\ (\pm 0.05)\ min^{-1}$ $A = 78\ (\pm 4)\%$ $k_2 = 0.010\ (\pm 0.008)\ min^{-1}$ $B = 22\ (\pm 4)\%$
Buffer with $CaCl_2$ (1.0 mM)	37°C	2160-fold	30 min	v_{sen}	$k = 0.0072\ (\pm 0.0007)\ min^{-1}$
Buffer with EDTA (1.0 mM)	37°C	540-fold	30 min	v_{ins}	$k \sim 0$
Saline (0.9% NaCl)	~25°C	1200 or 2400-fold	90 min	v_{sen}	$k = 0.0018\ (\pm 0.0002)\ min^{-1}$
		600-fold	90 min	v_{ins}	$k \sim 0$

The above results indicate that two EDTA-sensitive enzymes hydrolyze PA. This was not revealed in buffer containing $CaCl_2$ (cf. Table II). Inactivation in the presence of $CaCl_2$ was slower than without $CaCl_2$ (cf. Table II), and deviations from first-order kinetics did not show up within a 30 minute inactivation time. A study conducted on undiluted human serum samples at 50°C showed that inactivation of esterases measured with PA, POX or beta-naphtylacetate as substrates deviated from first-order kinetics, and these activities all refer to EDTA-sensitive enzymes [22].

Conclusion

This study has confirmed that EDTA-sensitive and EDTA-insensitive hydrolysis of PA in human sera is due to different enzymes, and it was shown that PA has a higher affinity for the EDTA-sensitive than EDTA-insensitive enzyme ($K_{M,sen} < K_{M,ins}$). EDTA-insensitive activity (v_{ins}) has been shown to be stable in aqueous media under conditions where the EDTA-sensitive activity (v_{sen}) was inactivated. The kinetics of v_{sen} inactivation indicated that human sera have two EDTA-sensitive enzymes hydrolyzing PA. However, we cannot attribute these activities to either of two paraoxonase iso-enzymes, PON1 and PON3, present in human sera. The results obtained on the stability of enzymes in aqueous media provide a guideline for choosing reliable experimental conditions for the study of enzymes hydrolyzing PA and other substrates of these esterases.

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