Purification and characterization of guinea-pig liver microsomal deacetylase involved in the deacetylation of the *O*-glucoside of *N*-hydroxyacetanilide

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A microsomal deacetylase that catalyses the deacetylation of the *O*-glucoside of *N*-hydroxyacetanilide (GHA) was purified from guinea-pig liver. The activity was located exclusively in the microsomes and not detected in the cytosol. The purified GHA deacetylase was a trimeric protein with a molecular mass of 160 ± 10 (S.D.) kDa composed of subunits of 53 ± 2 kDa; its pI was 4.7. The N-terminal amino acid sequence of GHA deacetylase was similar to those reported for guinea-pig and rat liver microsomal carboxylesterases. The GHA deacetylase showed a comparable hydrolytic activity towards *p*-nitrophenyl acetate (PNPA), although the activities towards *N*-hydroxyacetanilide, acetanilide and some endogenous acylated compounds were very low or not detectable. The deacetylase activity towards GHA was inhibited

by organophosphates but not by *p*-chloromercuribenzoate, suggesting that GHA deacetylase can be classified as a B-esterase. The enzyme exhibited a positive homotropic cooperativity towards GHA. The values of the Hill coefficient, the half-saturating concentration ($[S]_{0.5}$) for GHA, and $V_{\max}$ were 1.59 ± 0.03 , 5.51 ± 0.07 mM and 32.5 ± 1.4 μ mol/min per mg respectively, at the optimum pH of 8.5. The bell-shaped pH dependence of the $V_{\max}/[S]_{0.5}$ profile indicated pK_a values attributed to histidine and lysine residues. The study of stoichiometric inhibition by di-isopropyl fluorophosphate and kinetic analysis with the Monod–Wyman–Changeux model suggests that GHA deacetylase has six substrate binding sites and three catalytically essential serine residues per enzyme molecule.

INTRODUCTION

It is generally accepted that carcinogenic nitro- and aminoaromatics are metabolically activated to their derivatives, which could combine with cellular macromolecules [1,2]. *N*-Hydroxy-*N*-arylamides, formed through oxidative [3] and reductive [4–6] pathways from *N*-arylamides and nitroso-aromatics, respectively, are thought to have important roles as proximate carcinogens, which can be further activated to yield electrophilic ultimate carcinogens. The activation pathways of *N*-hydroxy-*N*-arylamides have been demonstrated to be in conjugation with sulphate or glucuronic acid and by *N,O*-transacylation [7–10].

For the glucuronide, Cardona and King [7] reported that the *O*-glucuronide of *N*-hydroxy-*N*-2-fluorenylamide is deacetylated by guinea-pig liver microsomes to yield a more electrophilic glucuronide that reacts with nucleic acids. We have previously reported the synthesis of and the chemical and mutagenic properties of *O*-glucosides of *N*-hydroxy-*N*-arylamides to investigate the mechanism of the metabolic activation of those carcinogenic *O*-glucuronides [11–13]. In these studies, mutagenic activation of the *O*-glucosides has been shown to be mediated by the deacetylation catalysed by guinea-pig liver microsomes. Furthermore, the N–O linkage of the deacetylated *O*-glucosides is shown to be far more labile than that of the parent *O*-glucosides [12]. These facts indicate that the deacetylation might be a crucial step in the carcinogenic/mutagenic activation of these *O*-glycosides of *N*-hydroxy-*N*-arylamides. It is interesting that no deacetylase activity towards the *O*-glucosides of *N*-hydroxy-*N*-arylamides, including 1-[(*N*-acetyl-*N*-phenylamino)oxy]-1-deoxy- β -D-glucopyranoside (GHA) (Figure 1), was detected in guinea-pig liver cytosol, although the cytosol has been shown to catalyse the deacylation of *N*-hydroxy-*N*-arylamides [14]. Hepatic micro-

somal amidases/carboxylesterases have been reported to hydrolyse xenobiotics containing ester and amide bonds, and to be involved in their toxification and/or detoxification [15,16].

In this paper, to gain insights into the molecular mechanism of enzymic deacetylation, guinea-pig liver microsomal deacetylase was purified by using GHA as the substrate, and its biochemical and kinetic properties were characterized.

MATERIALS AND METHODS

Materials

Sephadex G-150, Phenyl-Sepharose CL-4B, PBE94, Polybuffer74 and concanavalin A (ConA)–Sepharose were purchased from Pharmacia (Uppsala, Sweden); DE52 was obtained from Whatman (Maidstone, Kent, U.K.). *p*-Nitrophenyl acetate (PNPA) and *p*-chloromercuribenzoic acid were purchased from Kishida Chemical Co. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan) respectively. Methyl- α -D-glucopyranoside, triolein, acetanilide and di-isopropyl fluorophosphate were purchased from Wako Chemical Industries (Osaka, Japan). L- α -Phosphatidylcholine dipalmitoyl was purchased from Serdary Research Laboratories (London, Ontario, Canada). Acetyl-D,L-carnitine, diethyl pyrocarbonate and pyridoxal 5'-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bis-(4-nitrophenyl)phosphate and paraoxon were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). *N*-Hydroxyacetanilide, GHA and its deacetylated derivative were synthesized as described previously [11]. All other chemicals were of A. R. grade.

Subcellular fractionation

Male Hartley guinea pigs (from SLC Japan, Shizuoka, Japan) weighing 350–450 g were used for the experiments. All the guinea

Abbreviations used: ConA, concanavalin A; GHA, 1-[(*N*-acetyl-*N*-phenylamino)oxy]-1-deoxy- β -D-glucopyranoside; *h*, Hill coefficient; PNPA, *p*-nitrophenyl acetate; $[S]_{0.5}$, half-saturating concentration.

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Figure 1 Structure of GHA

pigs had been fed with a laboratory chow and water *ad libitum*. After the animals had been killed, the liver was removed, minced, and suspended in 4 vol. of ice-cold 0.25 M sucrose. The liver was homogenized in a Teflon–glass homogenizer with six vertical passes at low speed. The homogenate was filtered through two layers of gauze. Subcellular fractions were obtained by differential centrifugation based on the method of Moroi and Satoh [17]. All fractions were washed once with 1.15% (w/v) KCl and re-centrifuged before enzyme assays. The activities of marker enzymes, i.e. alcohol dehydrogenase, succinate:cytochrome *c* reductase and NADPH:cytochrome *c* reductase, were assayed by the methods reported previously [18–20]. Protein concentration was determined by the method of Lowry et al. [21], with BSA as the standard.

Purification of GHA deacetylase

GHA deacetylase was purified on the basis of the method of Hosokawa et al. [22,23]. Determination of GHA deacetylase activity was performed together with measurement of PNPA hydrolase activity during chromatographic purification. The elution of proteins was monitored by measuring the absorbance at 280 nm. The protein concentration was determined by the method of Bradford [24], with BSA as the standard. All operations were performed at 4 °C.

Step 1: preparation and solubilization of microsomes

Guinea-pig liver microsomes were prepared by the method of Schenkman and Cinti [25] and solubilized with 1% (w/v) saponin as described by Hosokawa et al. [22], in 0.1 M Tris/HCl buffer, pH 8.5, at a final protein concentration of 3.5 mg/ml. The mixture was stirred for 1 h and then centrifuged at 100 000 *g* for 1 h.

Step 2: (NH₄)₂SO₄ precipitation

The solubilized microsomal fraction was brought to 30% (NH₄)₂SO₄ saturation and stirred for 30 min, followed by centrifugation at 7000 *g* for 20 min. (NH₄)₂SO₄ was added to the supernatant to 70% saturation. The mixture was stirred for 1 h and then centrifuged as described above. The pellet was dissolved in a minimum volume of 10 mM Tris/HCl, pH 8.0 (buffer A).

Step 3: Sephadex G-150 chromatography

The above solution was applied to a Sephadex G-150 column (2.0 cm × 80 cm) equilibrated with buffer A. Elution was performed with the same buffer at a flow rate of 16 ml/h. The fractions containing GHA deacetylase activity were pooled.

Step 4: DE52 chromatography

The pooled fractions were chromatographed on a DE52 column (2.5 cm × 20 cm) equilibrated with buffer A. After washing with 110 ml of buffer A, the column was eluted with a gradient formed by the linear mixing of 250 ml of buffer A (starting buffer) with 250 ml of buffer A containing 0.2 M NaCl at a flow rate of 30 ml/h, and then with a 200 ml linear gradient of 0.2–0.6 M NaCl in buffer A at a flow rate of 50 ml/h. The fractions containing GHA deacetylase activity were combined and dialysed twice against 2 litres of 25 mM imidazole/HCl buffer, pH 7.4.

Step 5: chromatofocusing

The dialysed fractions were applied to a chromatofocusing gel PBE96 column (0.9 cm × 30 cm) equilibrated with the dialysis buffer at a flow rate of 10 ml/h. After the elution of unbound proteins, adsorbed proteins were eluted with a 9-fold dilution of Polybuffer74, adjusted to pH 4.0.

Step 6: Phenyl-Sepharose CL-4B chromatography

(NH₄)₂SO₄ was added to the pooled active fractions obtained in step 5 to a final concentration of 1 M (NH₄)₂SO₄. The solution was loaded on a Phenyl-Sepharose CL-4B column (2.0 cm × 15 cm) that had been equilibrated with buffer A containing 1 M (NH₄)₂SO₄ at a flow rate of 18 ml/h. The column was eluted by linear mixing of 150 ml of buffer A containing 1 M (NH₄)₂SO₄ (starting buffer) with 150 ml of buffer A containing 50% (v/v) ethylene glycol, and then with the latter buffer. The pooled fractions containing GHA deacetylase activity were diluted with 20 mM Tris/HCl buffer, pH 8.0, containing 0.1 M NaCl, 0.1 mM MgCl₂ and 0.5 mM CaCl₂ (buffer B) and were concentrated with a Centricon-30 (Amicon, Beverly, MA, U.S.A.).

Step 7: ConA-Sepharose chromatography

The concentrated solution was loaded on a ConA-Sepharose column (1.3 cm × 1.5 cm) equilibrated with buffer B. After the column had been washed with buffer B containing 0.5 M methyl- α -D-glucopyranoside, GHA deacetylase was eluted with buffer B containing 0.7 M methyl- α -D-glucopyranoside. The pooled fractions containing GHA deacetylase activity were diluted with buffer A, concentrated with a Centricon-30 and then stored at –80 °C.

Analysis of purity and molecular mass

The native molecular mass of the GHA deacetylase was determined by gel filtration on a TSK gel G3000 SW column (0.75 cm × 60 cm) (Tosoh, Tokyo, Japan) that had been equilibrated and eluted with 100 mM potassium phosphate buffer, pH 7.0, containing 100 mM (NH₄)₂SO₄, at a flow rate of 0.5 ml/min. The subunit molecular mass of the enzyme was estimated by SDS/PAGE on a 12.5% (w/v) gel by the method of Laemmli [26]. The molecular mass standards used for SDS/PAGE and gel filtration were SDS/PAGE MW standard (low range) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and MW-Marker (HPLC) (Oriental Yeast Co., Tokyo, Japan) respectively.

N-terminal amino acid sequence analysis

The purified GHA deacetylase was subjected to SDS/PAGE and then electrotransferred from the gel to a ProBlott membrane (Applied Biosystems, Foster, CA, U.S.A.) in accordance with

the method of Matsudaira [27]. Electrophoretic blotting was performed for 90 min in 10 mM 3-cyclohexylamino-1-propanesulphonic acid buffer, pH 11, containing 10% (v/v) methanol at a constant current of 1.5 mA/cm², with a semi-dry blotting system AE-6675 (Atto, Tokyo, Japan). To reveal the protein band, the membrane was stained with 0.2% Ponceau S and 1% (v/v) acetic acid. After washing with water, the membrane piece containing the bound GHA deacetylase was subjected to protein sequencing analysis directly with an Applied Biosystem Protein Sequencer, model 477A/120A, at Hokkaido University (Sapporo, Japan).

Enzyme assays

GHA deacetylase activity was determined by HPLC. Enzyme sample (20 μ l) and 100 μ l of 0.1 M Tris/HCl buffer, pH 8.0, were preincubated at 37 °C, and the reaction was started by the addition of 20 μ l of an aqueous solution of GHA (final concentration 2 mM). The reaction mixture was incubated at 37 °C for up to 10 min; the reaction was then terminated by heating in boiling water for 5 s. After the addition of Celite (no. 545; approx. 10 mg), the mixture was centrifuged at 2000 *g* for 1 min. Determination of the deacetylated product was performed on a LiChrosorb RP-8 column (7 μ m; 4 mm $\times$ 250 mm) (Cica-Merck, Tokyo, Japan). Elution was performed with water/methanol (4:1, v/v) at a flow rate of 0.8 ml/min, and the absorbance at 240 nm was monitored. The retention times of GHA and its deacetylated product were 8.5 and 7.0 min respectively. One unit of the enzyme activity was expressed as the formation of 1 μ mol of the deacetylated product/min under the incubation condition used. The hydrolysis of PNPA was measured spectrophotometrically by monitoring the formation of *p*-nitrophenol [28]. The hydrolyses of acetanilide and *N*-hydroxyacetanilide were measured colorimetrically in accordance with the reported procedures [29,30]. Acetyltransferase activity was determined by the method previously described [31]. In the reaction mixture, *p*-aminoazobenzene (0.1 or 0.5 mM) as an acetyl acceptor and both *N*-hydroxyacetanilide and GHA (each 1 or 5 mM) as acetyl donors were used. The hydrolyses of glyceride, phospholipid and acetyl-D,L-carnitine were assayed with the estimation of glycerol (Glycerol F-kit; Boehringer Mannheim, Mannheim, Germany), non-esterified fatty acid (NEFA C-test; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and L-carnitine (L-carnitine assay kit; Boehringer Mannheim) respectively.

pH dependence of kinetic parameters

The initial velocities of the deacetylation of GHA catalysed by the purified GHA deacetylase were measured at various concentrations of GHA (0.79, 1.06, 1.27, 1.58 and 2.11 mM), in a pH range of 6.5–9.0 at 37 °C. At pH 8.7 and 9.0 a non-enzymic formation of the deacetylated product was observed, and therefore the enzymic activity was calculated by subtracting the non-enzymic activity from the observed total activity.

Inhibition studies

Effects of bis-(4-nitrophenyl)phosphate, paraoxon and *p*-chloromercuribenzoate on the deacetylation of GHA catalysed by GHA deacetylase were investigated in 0.1 M *N,N*-bis-(2-hydroxyethyl)glycine buffer, pH 8.5, at 37 °C. Inactivation of GHA deacetylase by diethyl pyrocarbonate (7 mM) and pyridoxal 5'-phosphate (2 mM) was performed essentially in accordance with the methods of Carrillo and Vallejos [32] and Tsubaki et al. [33] respectively. Inactivation of GHA deacetylase by di-isopropyl fluorophosphate was performed on the basis of the method of Hayashi et al. [34]. The enzyme (98 μ g/ml) was

incubated with various concentrations of di-isopropyl fluorophosphate in 0.1 M Tris/HCl buffer, pH 8.0, at 37 °C for 5 h, and the residual activity was determined.

Computer analysis

All linear curve fitting was done by computerized least-squares fits. Initial velocities at different substrate concentrations were fitted to the Hill equation and analysed by the non-linear least-squares curve-fitting program MULTI-LINES FITTING to determine $V_{\max}$, half-saturating concentration ($[S]_{0.5}$) and Hill coefficient (*h*) values. $V_{\max}$ and $[S]_{0.5}$ at various pH values were fitted to the Dixon plot to determine the pK_a values. To obtain further information on the molecular mechanisms of GHA deacetylase, the Monod–Wyman–Changeux model [35] was applied to the enzyme kinetics by linear and non-linear curve fitting.

RESULTS

Subcellular distribution of GHA deacetylase

The activities of GHA deacetylase and of known marker enzymes were determined in the subcellular fractions obtained from the guinea-pig liver. As shown in Table 1, GHA deacetylase activity was not detected in the cytosol fraction, as reported previously [11,13]. From the distribution pattern of the marker enzymes, GHA deacetylase is shown to be located in the microsomes.

Purification of GHA deacetylase

The GHA deacetylase of guinea-pig liver microsomes was purified on the basis of the method of Hosokawa et al. [22,23]. The GHA deacetylase was purified to approx. 550-fold and with 14% yield (Table 2). The decline in activity of the purified enzyme was within 5% in 1 year when stored at –80 °C. The pI of GHA deacetylase was shown to be 4.7 by using chromatofocusing column chromatography. The ConA–Sephacryl step was effective in purifying the GHA deacetylase, although the recovery of the deacetylase decreased. As shown in Figure 2, the ratio of GHA deacetylase activity to PNPA-hydrolysing activity in the GHA deacetylase fractions obtained from the column was almost constant.

Purity and molecular mass of GHA deacetylase

The purity and molecular mass of the final preparation of GHA deacetylase were assessed by SDS/PAGE (Figure 3, upper panel). The enzyme preparation gave a single protein band with a molecular mass of 53 ± 2 kDa. The native molecular mass of the

Table 1 Subcellular distribution of GHA deacetylase and marker enzymes in guinea-pig liver

Values are means $\pm$ S.D. (*n* = 3) expressed as percentages of the recovered activities. Abbreviation: n.d., not detected.

Enzyme	Enzyme activity (% of recovered activity)			
	Cytosol	Microsomes	Mitochondria	Nuclei
GHA deacetylase	n.d.	80 $\pm$ 7	11 $\pm$ 5	9 $\pm$ 2
NADPH:cytochrome <i>c</i> reductase	9 $\pm$ 1	78 $\pm$ 8	11 $\pm$ 5	9 $\pm$ 2
Succinate:cytochrome <i>c</i> reductase	3 $\pm$ 1	3 $\pm$ 1	74 $\pm$ 11	21 $\pm$ 5
Alcohol dehydrogenase	77 $\pm$ 4	8 $\pm$ 2	5 $\pm$ 1	10 $\pm$ 1

Table 2 Purification of GHA deacetylase from microsomes of guinea-pig liver

	Total protein (mg)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	Yield (%)	Purification (fold)
Microsomes	524	14.2	0.0271	100	1
Solubilization	53.0	12.2	0.230	86	8.5
$(\text{NH}_4)_2\text{SO}_4$ precipitation	20.2	9.91	0.491	70	18
Sephadex G-150	8.42	9.18	1.09	65	40
DE52	3.25	7.82	2.40	55	89
Chromatofocusing	1.38	5.31	3.87	37	140
Phenyl-Sepharose	0.528	4.60	8.71	32	320
ConA-Sepharose	0.129	1.92	14.9	14	550

Figure 2 Elution patterns of GHA deacetylase and PNPA hydrolase on ConA-Sepharose

GHA deacetylase (●) and PNPA hydrolase (○) activities are expressed as $\mu\text{mol}/\text{min}$ per ml. The broken line indicates the ratio of GHA deacetylase activity to PNPA activity. The arrow indicates the start of the elution with buffer B containing 0.7 M methyl- α -D-glucopyranoside.

preparation was estimated to be 160 ± 10 kDa by gel filtration on TSK gel G3000 SW (Figure 3, lower panel). These results suggest that GHA deacetylase is a homotrimeric protein.

N-terminal amino acid sequence

The N-terminal amino acid sequence of GHA deacetylase is shown in Table 3, together with the known sequences of guinea-pig and rat liver microsomal carboxylesterases [36]. The sequence of the GHA deacetylase was similar to those of the listed carboxylesterases in this region, although these N-terminal amino acids were different from each other.

pH dependence of kinetic parameters in the deacetylation of GHA

The experiment on the saturation kinetics of GHA deacetylase by GHA exhibited a sigmoidal curve, indicating a positive homotropic co-operativity. A representative pattern is shown in Figure 4. To investigate the kinetic behaviour in detail, we focused

Figure 3 Purity and molecular mass of GHA deacetylase

Upper panel: analysis by SDS/PAGE. Lane a, standard molecular mass marker proteins (phosphorylase b, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21 kDa; lysozyme, 14 kDa); lane b, purified GHA deacetylase. Lower panel: analysis by TSK gel G3000 SW. Symbols: ●, purified GHA deacetylase; ○, marker proteins [glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome c (12 kDa)].

on the influence of pH, because the kinetic properties of some allosteric enzymes are significantly affected by pH [37–39]. A homotropic allosteric effect was observed in the pH range studied, with $h > 1$ (Figure 5). The h values were independent of pH between 7.0 and 8.0 and the average value was 1.74 ± 0.03 . The GHA deacetylase exhibited positive co-operativity towards PNPA as well, although h was relatively low (1.21 ± 0.04 at

Table 3 Comparison of the N-terminal amino acid sequence of GHA deacetylase with those reported for guinea-pig and rat liver microsomal carboxylesterases

The amino acid sequence of 18 of the first 20 residues of the GHA deacetylase was 72% identical with the reported sequence of the guinea-pig GPL1. The sequences other than that of GHA deacetylase are taken from [36].

Enzyme	Sequence
GHA deacetylase	EKLSXPXVVDTKYKGKVLGMYEH
Guinea-pig GPL1	XXPSXPXVVDXKYKGKVLGKY
Rat RL1	DPSSPPVVDTVKGKVLGKYVSL
Rat RL2	XPSXPPVNVXVKGKVLGKYV
Rat RH1	YPSSPPVNVTVKGKVLGKYV

Figure 4 Positive homotropic co-operativity of GHA deacetylase towards GHA

GHA deacetylase activity was measured in 0.1 M Tris/HCl buffer, pH 8.0, and $1/v$ was plotted against $1/[GHA]^{1.71}$.

Figure 5 pH dependence of h in the deacetylation of GHA catalysed by GHA deacetylase

The buffers used were 0.1 M 3-(*N*-morpholino)propanesulphonic acid for pH 6.5–7.8, and 0.1 M *N,N*-bis-(2-hydroxyethyl)glycine for pH 7.8–9.0. An appropriate amount of NaCl was added to keep the ionic strength constant (0.1 M).

pH 8.0; results not shown). As shown in Figure 6, kinetic parameters of the GHA deacetylase varied significantly with pH. The $[S]_{0.5}$ for GHA increased from 1.34 ± 0.02 mM at pH 6.5 to 5.51 ± 0.07 mM at pH 8.5, and decreased again above pH 8.5 (Figure 6, upper panel). Figure 6 (lower panel) shows the curve of $\log V_{\max}/[S]_{0.5}$ against pH, indicating the pH optimum at approx. 8.5; $V_{\max}$ was 32.5 ± 1.4 μ mol/min per mg at this pH.

Figure 6 pH dependence of kinetic parameters of GHA deacetylase

Upper panel, $[S]_{0.5}$; lower panel, $V_{\max}/[S]_{0.5}$. The values of $[S]_{0.5}$ and $V_{\max}$ were obtained from non-linear least-squares analysis at each pH. The solid lines indicate the theoretical curves given by the estimated pK_a values.

Inhibition studies

From the bell-shaped $V_{\max}/[S]_{0.5}$ profile (Figure 6, lower panel), the pK_a values of 7.3 ± 0.1 and 9.3 ± 0.3 were calculated by computer fitting to the Dixon plot. To determine the amino acid residues attributed to these pK_a values, inactivation experiments with active-site-directed reagents were performed. Figure 7 shows time courses of the inactivation of the GHA deacetylase by diethyl pyrocarbonate (for histidine residues) [32] and pyridoxal

Figure 7 Inactivation of GHA deacetylase by diethyl pyrocarbonate and pyridoxal 5'-phosphate

To 0.1 M phosphate buffer, pH 7.0, containing the enzyme, either diethyl pyrocarbonate (●, final concentration 7 mM) or pyridoxal 5'-phosphate (○, final concentration 2 mM) was added and preincubated at 24 °C. At appropriate time intervals the inactivation reaction was stopped by the methods described previously [31,32] and the remaining GHA deacetylase activity was determined by the assay method described in the Materials and methods section.

Table 4 Effect of organophosphates on GHA deacetylase

After the enzyme had been preincubated for 3 min with either paraoxon or bis-(4-nitrophenyl) phosphate in 0.1 M *N,N*-bis-(2-hydroxyethyl)glycine buffer, pH 8.5, at 37 °C, the activity remaining was determined in the presence of 5.2 mM GHA. The activities are expressed as percentages of control.

Addition	Concentration (M)	Activity remaining (%)
None (control)		100
Paraoxon	10^{-7}	29
	10^{-6}	6
Bis(4-nitrophenyl)phosphate	10^{-4}	29
	10^{-3}	13

Figure 8 Inactivation of GHA deacetylase by di-isopropyl fluorophosphate

After preincubation with various amounts of di-isopropyl fluorophosphate, the remaining activity was determined by the assay method described in the Materials and methods section.

5'-phosphate (for lysine residues) [33]. *p*-Chloromercuribenzoate (final concentration 1 mM, for cysteine residues) had no effect on the activity (results not shown). As shown in Table 4, paraoxon caused almost complete inhibition of the GHA deacetylase at 1 μ M. Bis-(4-nitrophenyl)phosphate also showed an inhibitory effect. Figure 8 shows the inactivation of GHA deacetylase by different concentrations of di-isopropyl fluorophosphate. The results indicated that GHA deacetylase contains three specific serine residues per enzyme molecule if the molecular mass of GHA deacetylase is 160 kDa.

To estimate the number of substrate-binding sites in GHA deacetylase, analysis with a linear plot of the Monod–Wyman–Changeux model proposed by Horn and Bornig [40] was performed. As shown in Figure 9, a representative pattern of the plot at pH 8.0, a straight line with a slope of $-(n-1)$ was obtained, where n is the number of protomers. Over a pH range of 7.5–8.7, the average value of n was 6.15 ± 0.21 , indicating that the number of protomers could be assumed to be six. The interpretation of the allosteric kinetics of GHA deacetylase is based on the assumption that the oligomeric protein exists in two different states, i.e. R (relaxed) and T (tight), and that an exclusive substrate binding occurs in the R state [35,40]. This result of six substrate binding sites indicates that the protein is a hexamer, a trimer of dimers or a dimer of trimers. This, together with the estimate of molecular mass (Figure 3) and the result of the stoichiometric inhibition by di-isopropyl fluorophosphate (Figure 8), indicates that the GHA deacetylase is a trimeric

Figure 9 Plot of $\log(a/Y - a - 1)$ against $\log(a + 1)$

The plot derives from the equation $\log(a/Y - a - 1) = \log L - (n-1) \log(a+1)$, where a , L and Y represent $[S]/[S]_{0.5}$, the allosteric constant and $v/V_{\max}$ respectively; $[S]$ is the concentration of the substrate (GHA). The value of n was found to be 6.19 ± 0.15 .

Table 5 Substrate specificities of GHA deacetylase

Activities were measured under the same conditions as those used for the deacetylation of GHA.

Substrate	Concentration (mM)	Activity (μ mol/min per mg)
GHA	2	14.5 ± 0.4
PNPA	2	11.0 ± 0.1
<i>N</i> -Hydroxyacetanilide	2	0.3 ± 0.0
Acetanilide	2	0.0
Triolein	5	0.0
Acetyl-D,L-carnitine	5	0.0
L- α -Phosphatidylcholine dipalmitoyl	5	0.0

protein composed of subunits with two substrate-binding sites and one catalytically essential serine residue per subunit.

Substrate specificity

Catalytic activities of GHA deacetylase towards several substrates are summarized in Table 5. Of the substrates tested, GHA was the most effective; PNPA was comparable with GHA. However, the activities towards *N*-hydroxyacetanilide, acetanilide and some endogenous acylated compounds, i.e. triolein, acetyl-D,L-carnitine and L- α -phosphatidylcholine dipalmitoyl, were very low or not detectable. To confirm whether GHA deacetylase possesses an acetyltransferase activity [9], the activity was examined by using *p*-aminoazobenzene as the acetyl acceptor. GHA and *N*-hydroxyacetanilide were unable to participate as acetyl donors under the conditions used (results not shown).

DISCUSSION

We have described the purification and characterization of the GHA deacetylase found in microsomes of guinea-pig liver. The subcellular distribution of GHA deacetylase (Table 1) showed a predominant localization in microsomes. It is interesting that GHA deacetylase shows a high deacetylation activity for GHA but not for *N*-hydroxyacetanilide and acetanilide (Table 5).

Purified GHA deacetylase could be useful in the preparation of labile *O*-glycosides of *N*-hydroxyarylamines, to investigate their carcinogenicity/mutagenicity.

Several findings of the enzymic characterization suggest that GHA deacetylase is a carboxylesterase. The fact that GHA deacetylase showed a high affinity for ConA–Sephadex indicates that the enzyme is a glycoprotein, like many mammalian carboxylesterases [15,16]. GHA deacetylase could be classified as a B-esterase, defined by Aldridge [41], on the basis of the result that activity was inhibited by organophosphates (Figure 8 and Table 4) but not by *p*-chloromercuribenzoate. The pI and pH optima of B-esterases have been reported to be generally in the range of 4.7–6.5 and 7–9 respectively [15]. It is also reported that mammalian liver carboxylesterases consist of units with a molecular mass of approx. 60 kDa [15]. These findings do not contradict those obtained for GHA deacetylase. The N-terminal amino acid sequence of GHA deacetylase (Table 3) is similar to those reported for guinea-pig and rat carboxylesterases [36]. Several kinds of deacetylase activity in guinea-pig liver microsomes have been reported [29,36,42–50]. Among these enzymes, the following are thought to resemble GHA deacetylase: the deacetylase of *N*-hydroxy-*N*-2-fluorenylacetylamine with a molecular mass of 200 kDa [29], and GPH1, a trimeric protein of pI 5.3 composed of subunits with a molecular mass of 57 kDa [36].

GHA deacetylase was shown to exhibit a positive homotropic co-operativity towards GHA (Figure 4) as well as PNPA, although it has not been reported, to our knowledge, that carboxylesterases exhibit sigmoidal saturation kinetics towards their substrates. The bell-shaped pH dependence of $V_{\max}/[S]_{0.5}$ provided two distinct pK_a values (Figure 6). The essential groups attributed to these pK_a values were indicated, by inactivation experiments with active-site-directed reagents, to be histidine and lysine residues (Figure 7). The ionization behaviour of the imidazole and ϵ -amino groups might participate in the catalytic function of the GHA deacetylase. Because the deacetylation of *O*-glucuronides of *N*-hydroxy-*N*-arylacetamides is regarded as a crucial step in metabolic activation, attention should be given to the tissue distribution of GHA deacetylase and the physiological function of the deacetylase. Its endogenous substrate or substrates have not been identified. Interest has been shown in sugar chains in esterases, and glycosylation-dependent activity of carboxylesterase has been reported [51]. It would be of interest to determine whether intrinsic sugar chains affect the kinetic properties of the deacetylase. For this it would be necessary to study GHA deacetylase from toxicological and physiological viewpoints.

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