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High-affinity binding of fatty acyl-CoAs and peroxisome proliferator-CoA esters to glutathione S-transferases

Effect on enzymatic activity

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Acyl-CoAs are present at high concentrations within the cell, yet are strongly buffered by specific binding proteins in order to maintain a low intracellular unbound acyl-CoA concentration, compatible with their metabolic role, their importance in cell signaling, and as protection from their detergent properties. This intracellular regulation may be disrupted by nonmetabolizables acyl-CoA esters of xenobiotics, such as peroxisome proliferators, which are formed at relatively high concentration within the liver cell. The low molecular mass acyl-CoA binding protein (ACBP) and fatty acyl-CoA binding protein (FABP) have been proposed as the buffering system for fatty acyl-CoAs. Whether these proteins also bind xenobiotic-CoA is not known. Here we have identified new liver cytosolic fatty acyl-CoA and xenobiotic-CoA binding sites as glutathione S-transferase (GST), using fluorescent polarization and a acyl-etheno-CoA derivative of the peroxisome proliferator nafenopin as ligand. Rat liver GST and human liver recombinant GSTA1-1, GSTP1-1 and GSTM1-1 were used. Only class alpha rat liver GST and human GSTA1-1 bind xenobiotic-CoAs and fatty acyl-CoAs, with K_d values ranging from 200 nM to 5 μM. One mol of acyl-CoA is bound per mol of dimeric enzyme, and no metabolization or hydrolysis was observed. Binding results in strong inhibition of rat liver GST and human recombinant GSTA1-1 (IC_{50} at the nanomolar level for palmitoyl-CoA) but not GSTP1-1 and GSTM1-1. Acyl-CoAs do not interact with the GSTA1-1 substrate binding site, but probably with a different domain. Results suggest that under increased acyl-CoA concentration, as occurs after exposure to peroxisome proliferators, acyl-CoA binding to the abundant class alpha GSTs may result in strong inhibition of xenobiotic detoxification. Analysis of the binding properties of GSTs and other acyl-CoA binding proteins suggest that under increased acyl-CoA concentration GSTs would be responsible for xenobiotic-CoA binding whereas ACBP would preferentially bind fatty acyl-CoAs.

Keywords: glutathione transferase; acyl-CoA binding; peroxisome proliferators.

Acyl-CoAs are important metabolic intermediates for lipid biosynthesis and fatty acid degradation. They have also been implicated in the modulation of signal transduction systems and other cellular functions, such as membrane fusion and gene regulation [1], where they seem to be involved, among others, in the regulation of transcription factor function [2,3]. Recently, they have been shown to directly modulate the transcriptional activity of the hepatic nuclear factor-4α, an orphan transcription factor that controls the expression of various genes such as those encoding apolipoproteins AI, AII, B and CIII, and vitamin K dependent coagulability factors [4]. Thus, through the action of their acyl-CoA esters, dietary fatty acids might modulate the onset and progression of various diseases, including atherogenesis, hyperlipidaemia, disturbances of blood coagulability and insulin resistance [4]. In view of these important regulatory functions, it has been proposed that although acyl-CoAs are highly concentrated within the cell (5–160 μM), their free unbound concentration is maintained in the low nanomolar range under normal physiological conditions by the buffering action of specific binding proteins, such as acyl-CoA binding protein (ACBP) and fatty acid binding protein (FABP) [1]. This regulation might be potentially disrupted by peroxisome proliferators, such as hypolipidaemic drugs, plasticizers and agrochemicals, which are known to form nonmetabolizable acyl-CoA esters [5–8] and to induce a pleiotrophy of similar effects in treated animals, including nongenotoxic carcinogenesis [9]. The formation of such xenobiotic-CoA derivatives is an early event following exposure to peroxisome proliferators [7,8], and the perturbation of lipid metabolism that they induce, through CoASH sequestration, a rise in endogenous acyl-CoA concentration, activation of protein kinase C, and inhibition of β-oxidation, has been proposed to underlie some of their effects [7–11]. We have previously reported saturable binding sites for some

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Abbreviations: GSH, glutathione; GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene; ACBP, acyl-CoA binding protein; FABP, fatty acid binding protein; N-etCoA, nafenopin-1,N^6-ethenocoenzyme A.

Enzyme: glutathione transferase (EC 2.5.1.18)

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xenobiotic-CoAs, of higher molecular mass than that of ACBP and FABP, in rat liver cytosol [12]. In the present work, we have identified these new binding sites as glutathione transferases (GSTs), which are abundant multifunctional enzymes, generally regarded as some of the major phase II drug metabolizing enzymes [13–15], catalyzing the addition of glutathione (GSH) to electrophilic xenobiotics. In addition to their conjugating activity, they bind various hydrophobic ligands such as bile acids, steroid hormones and neurotransmitters [14,16–18]. GST isoenzymes contribute to the cellular resistance against carcinogens, antitumor drugs, environmental pollutants and the products of oxidative stress, and their expression can be up-regulated by various enzyme-inducing agents such as herbicides and at least a hundred other chemicals [14,15]. The cytosolic GST isoenzymes present different but overlapping substrate specificity and are divided into seven different classes: alpha, mu, pi, theta, sigma [15] kappa [19] and Zeta [20], which in turn are active as homo- or heterodimers containing subunits belonging to the same class [21,22].

For the characterization of the GST isoforms involved in fatty acyl-CoA and xenobiotic-CoAs binding, rat liver isolectric focusing-purified GST classes, and three commercially available recombinant human GST isoforms alpha, mu and pi (GSTA1-1, GSTM1-1 and GSTP1-1) were used. Binding was assessed by fluorescence polarization using a fluorescent peroxisome proliferator CoA ester derivative as ligand and displacement titration. Only GSTA1-1 and rat liver class alpha GSTs bind acyl-CoAs with high affinity, resulting in strong inhibition of GST enzymatic activity measured with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate.

MATERIALS AND METHODS

Chemicals

Glutathione-Sepharose, 1,N^6-ethenoCoA, acyl-CoAs and other chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA). [^{3}H]-nafenopin was a gift from J. Bar-Tana (Hadassa Medical School, Israel). Nafenopin and tibric acid were provided by CIBA-Geigy AG (Basel, Switzerland), and ciprofibrate by Sterling-Winthrop (New York, USA). Purified (> 95% pure) recombinant human GST alpha, mu and pi isoforms (GSTA1-1; GSTM1-1, and GSTP1-1) were purchased from PanVera Corp. (Madison, WI, USA).

Preparation of GSTs and isoelectric separation of GST isoforms

Fractions containing GST activity, obtained by Sephacryl S-200 column chromatography of rat liver supernatants as previously described [12], were dialyzed against 20 mM phosphate buffer pH 7.0. The dialyzed fractions (60 mL) were then filtered through a glutathione-Sepharose column (0.8 × 3 cm) pre-equilibrated in 20 mM phosphate buffer, pH 7.0. The column was successively washed with 20 mL of the same buffer and with 10 mL of 50 mM Tris/HCl buffer, pH 9.6. Finally, bound GSTs were eluted using 15 mL of 50 mM Tris/HCl buffer, pH 9.6, containing 5 mM glutathione [23–25]. The GST affinity purified fraction was dialyzed against 10 mM phosphate buffer, pH 7, and isoelectric separation of GST isoforms [23,24] was performed using a 110-mL preparative column (LKB, Stockholm, Sweden). GST enzymatic activity was determined spectrophotometrically according to [23] using 1 mM 1-chloro-2,4-dinitrobenzene as substrate and 1 mM glutathione.

Synthesis of Nafenopin-Etheno-CoA and [^{3}H]-nafenopin-CoA

The fluorescent acyl-CoA derivative of the peroxisome proliferator nafenopin, nafenopin-1,N^6-ethenocoenzyme A (N-etCoA) and [^{3}H]-nafenopin-CoA were chemically synthesized starting from nafenopin and 1,N^6-ethenoCoA for the fluorescent derivative, and from [^{3}H]-nafenopin and CoASH for the radioactive derivative. The synthesis was performed according to [26], and the crude derivatives purified and their structure assessed as described previously [5,12]. Similar procedures were used for the synthesis of ciprofibroyl-CoA and tibryl-CoA. A complete report of the synthesis and characterization of the fluorescent derivative as well as their spectral properties will be published elsewhere.

Fluorescence anisotropy titration

Fluorescence measurements were performed using a Perkin Elmer LS50 spectrofluorometer. Excitation was at 310 nm and emission at 410 nm. Fluorescence anisotropy (r) was calculated from polarization (P) values according to the equation $r = 2P/(3-P)$ [27]. All titrations were performed at 25 °C, in buffer A (20 mM phosphate buffer pH 7.4, containing 20% glycerol) in a total volume of 1 mL. After various additions, the final volume increased by a maximum of 10%, but no changes were observed for polarization measurements under those conditions. Data was corrected for changes in concentration. Dilution titration (polarization as a function of varying protein concentration at constant N-etCoA concentration), addition titration (polarization as a function of increasing N-etCoA concentration and fixed protein concentration) and displacement titration (polarization at increasing acyl-CoA concentrations measured in the presence of fixed N-etCoA and protein concentrations) were determined as described in [27]. Determination of the amount of bound and free ligands from anisotropy were calculated as previously described in the literature [27,28]

Other methods

Protein concentration was determined according to [29], using bovine serum albumin as standard. SDS/PAGE was performed according to [30], using 0.1% SDS and 3 and 20% acrylamide for the stacking and resolving gels, respectively.

RESULTS

Binding of [^{3}H]-Nafenopin-CoA to rat liver GST

Using Sephacryl S-200 chromatography of rat liver cytosol, we previously identified a fraction of [^{3}H]-nafenopin-CoA-binding proteins in the 35–50 kDa molecular mass range [12]. As cytosolic GSTs elute in this fraction and are known to bind electrophilic xenobiotics and hydrophobic ligands [13–15] we investigated the possible correlation between [^{3}H]nafenopin-CoA binding and GST activity. Both were found to closely copurify during Sephacryl S-200 and glutathione-Sepharose affinity chromatographies, strongly suggesting that GST is responsible for [^{3}H]nafenopin-CoA binding. Considering that studies with [^{3}H]nafenopin-CoA are limited by its low specific activity [12], we synthesized a fluorescent analog of nafenopin-CoA (see Materials and methods) in order to determine binding

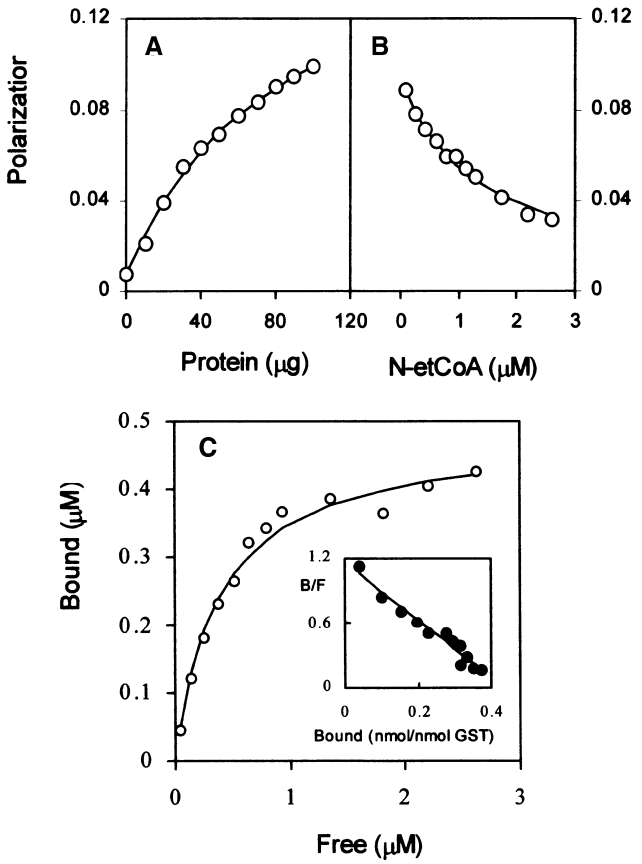

Fig. 1. Polarization titration of rat liver affinity-purified GST with N-etCoA. (a) Dilution titration: Protein concentration was increased at a fixed (0.88 μM) N-etCoA concentration by the addition GST (stock solution: 4.4 mg·mL^{-1} of protein). (b) Addition titration, N-etCoA was varied at a fixed purified GST concentration (0.22 mg·mL^{-1} of protein) by the addition of aliquots of a concentrated N-etCoA solution. (c) Free and bound N-etCoA concentrations were calculated as described in the experimental section. K_d and B_{max} values were obtained by direct fitting of the data using nonlinear regression analysis. Inset, Scatchard plot of the data. Bound N-etCoA is expressed as nmol bound per nmol of GST, considering a molecular mass of 50 kDa for GST. A K_d of 0.386 ± 0.045 μM and a B_{max} of 0.484 ± 0.017 nmol·nmol^{-1} of GST (value ± SD) were obtained. Similar values were obtained with two other GST preparations.

constants and to investigate the possible interaction of GST with other acyl-CoAs. The binding of the fluorescent acyl-CoA derivative, N-etCoA, was studied by fluorescent polarization in the presence of 20% glycerol. The addition of GST to N-etCoA induced a significant increase in the polarization (Fig. 1a) indicating that the movement of N-etCoA was restricted within the acyl-CoA binding site of the enzyme. Experiments in the absence of glycerol indicated that there was binding of the fluorescent substrate to GST, and the saturation of binding sites was found to occur at the same concentration of ligand as in the presence of glycerol. Addition of glycerol to the incubation media in polarization fluorescence studies is a well documented method, as it augments the polarization of the bound ligand and thus diminishes the experimental error on the one hand, and on the other, helps to maintain the stability of the protein during the experiment [31].

Addition titration of N-etCoA, using increasing concentrations of this derivative (Fig. 1b) allowed us to determine

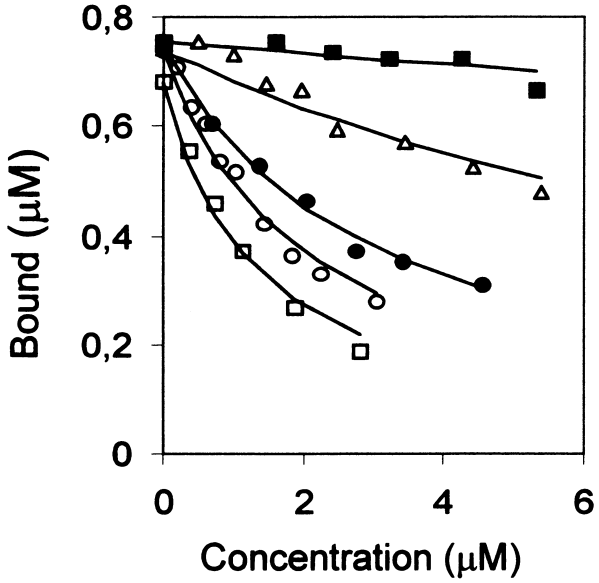

Fig. 2. Displacement titration of N-etCoA by saturated acyl-CoAs, using affinity-purified rat liver GST. The concentration of various acyl-CoAs was varied at fixed concentrations of N-etCoA (0.88 μM) and GST (0.35 mg·mL^{-1} of protein), and the polarization recorded in order to calculate the amount of bound N-etCoA at each acyl-CoA concentration. Octanoyl-CoA (C8 : 0; ■); lauryl-CoA (C12 : 0; △); miristoyl-CoA (C14 : 0;●); palmitoyl-CoA (C16 : 0; ○); stearoyl-CoA (C18 : 0; □). CoASH alone was without effect.

bound and free N-etCoA (Fig. 1c) and to calculate both the K_d and B_{max} for affinity purified GST. A K_d of 0.386 ± 0.045 μM and a B_{max} of 0.484 ± 0.017 nmol of N-etCoA per nmol of GST (considering a molecular mass of 50 kDa for the dimeric enzyme [15]) were obtained by direct fitting to the curve shown in Fig. 1c.

Relative affinity of GST towards acyl-CoAs

The relative affinity of purified GST for saturated-CoAs, unsaturated-CoAs and certain xenobiotic-CoAs was determined by displacement titration of N-etCoA. Figure 2 shows the results observed with saturated acyl-CoAs. The affinity of GST for saturated fatty acyl-CoAs increased almost exponentially as

Table 1. Acyl-CoA binding to GST (or IC_{50} for the displacement of N-etCoA). IC_{50} values were calculated by nonlinear regression analysis from curves as those presented in Fig. 2. Values ± SD.

Acyl-CoA	IC_{50} (μM)
Tibryl-CoA	9.91 ± 1.58
Ciprofibroyl-CoA	3.94 ± 0.43
Nafenopin-CoA	0.65 ± 0.08
Octanoyl-CoA (C8 : 0)	67.70 ± 58.80
Lauryl-CoA (C12 : 0)	11.62 ± 1.21
Myristoyl-CoA (C14 : 0)	3.17 ± 0.12
Palmitoyl-CoA (C16 : 0)	2.03 ± 0.24
Stearoyl-CoA (C18 : 0)	1.39 ± 0.26
Arachidoyl-CoA (C20 : 4)	2.89 ± 0.37
Oleoyl-CoA (C18 : 1)	1.49 ± 0.11
Linoleoyl-CoA (C18 : 2)	1.19 ± 0.18

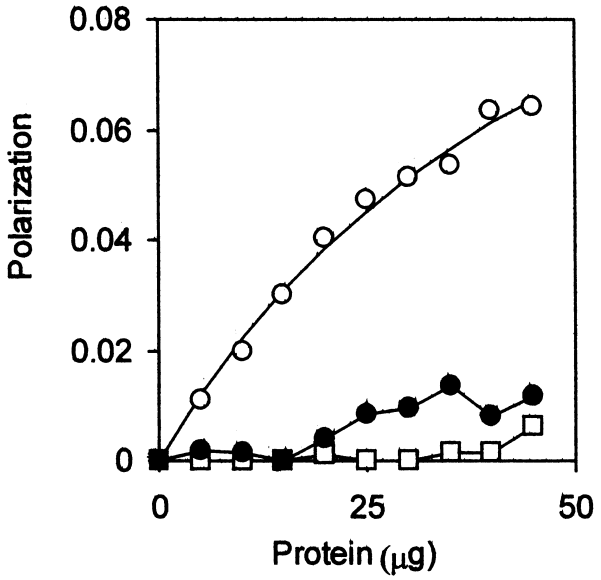

Fig. 3. Polarization titration by dilution of human recombinant GSTs. Protein concentration was increased at a fixed (1 μM) N-etCoA concentration by the addition of human recombinant GSTs (stock solution: 1–2 $mg \cdot mL^{-1}$ of protein). GSTA1-1 (○); GSTP1-1 (□); GSTM1-1 (●).

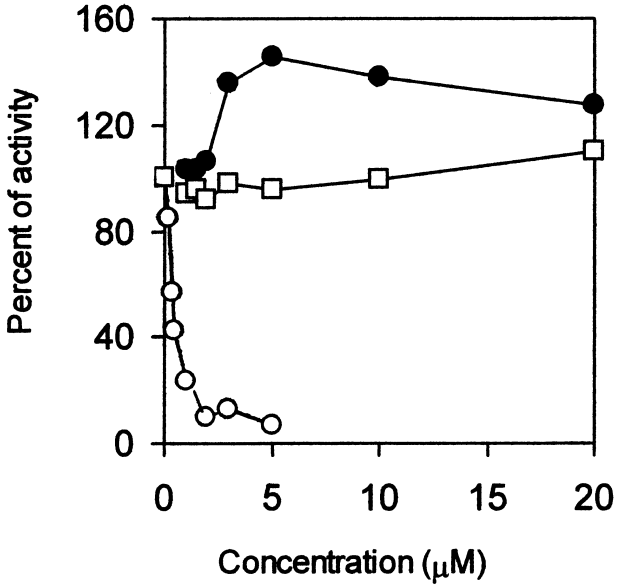

Fig. 5. Effect of palmitoyl-CoA on human recombinant GSTs. Enzyme activities were measured using CDNB, as described in Materials and methods. GSTA1-1 (○); GSTP1-1 (□); GSTM1-1 (●).

the fatty acyl-chain length increased. Table 1 lists the IC_{50} values obtained for the displacement of N-etCoA calculated from the data in Fig. 2, for saturated acyl-CoAs, and the values for some unsaturated acyl-CoAs and xenobiotic-CoAs calculated from similar experiments as those reported in Fig. 2 (not shown). The relative affinity for unsaturated acyl-CoAs was of the order observed for long chain saturated acyl-CoAs.

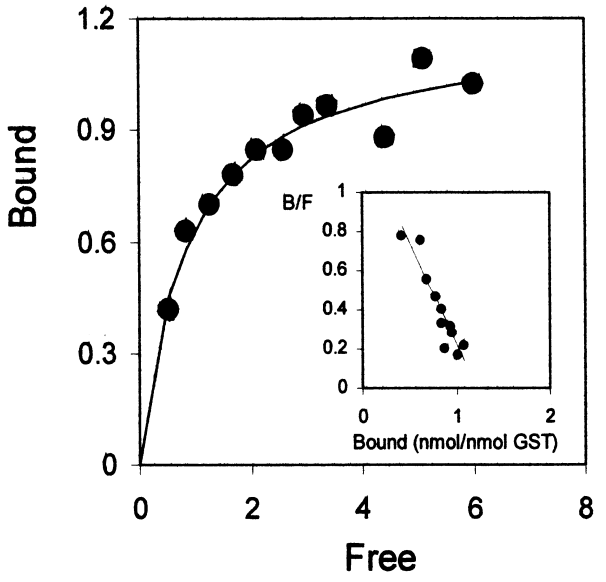

Fig. 4. Binding of N-etCoA to GSTA1-1. The protein concentration of GSTA1-1 was fixed and the concentration of N-etCoA was varied by the addition of aliquots of a concentrated N-etCoA solution. Free and bound N-etCoA concentrations were calculated from the data, as described in the Experimental section. Scatchard plots is presented in the insert, with bound N-etCoA expressed as nmol per nmol of GST (considering a molecular mass of 50 kDa). A K_d of 0.85 ± 0.13 μM and a B_{max} of 1.1 ± 0.045 were obtained by direct fitting of the data using nonlinear regression analysis.

Xenobiotic-CoAs presented IC_{50} values in the low μM range. Ciprofibrate or lauric acid (C12:0) alone did not induce a significant displacement of N-etCoA alone, up to 20 μM. Experiments with long chain fatty acids were complicated by their low solubility.

Binding of Net-CoA to liver human recombinant GSTs

The B_{max} lower than one mole of N-etCoA per mole of dimeric GST, determined for N-etCoA binding to affinity purified GST (Fig. 1c) suggests that GST isoforms with no binding activity are present in the affinity-purified GST preparation. As separation of pure GST isoforms is difficult owing to the complexity of the GST families and subfamilies, three commercially available recombinant human GST alpha, mu and pi isoforms (GSTA1-1, GSTM1-1, and GSTP1-1) homodimers were investigated for acyl-CoA binding. As shown in Fig. 3, only the alpha GST human homodimer GSTA1-1 binds Net-CoA, whereas very low or no binding is observed for the other isoforms. A B_{max} near 1 mol of Net-CoA bound per mol of GST, and a K_d of 0.85 ± 0.13 μM was determined by addition and dilution titration for GSTA1-1 (Fig. 4), a result which is in good agreement with values obtained for rat liver affinity-purified GST. Furthermore, GSTA1-1 enzyme activity was found to be strongly inhibited by palmitoyl-CoA, whereas no effect was observed for GSTP1-1 and GSTM1-1 was partially activated (Fig. 5).

The molecular mechanism of GSTA1-1 inhibition by palmitoyl-CoA was studied at fixed inhibitor concentration and increasing concentration of substrates (Fig. 6). A mixed type of inhibition with changes both in V_{max} and K_m was observed for the inhibition of GSTA1-1 by palmitoyl-CoA when the glutathione concentration was varied at fixed CDNB concentration (Fig. 6A), whereas a decrease in V_{max} with no changes in the K_m was determined when the CDNB concentration was varied at fixed GSH concentration (Fig. 6B). Thus, palmitoyl-CoA changes both V_{max} and the affinity of GSTA1-1 for GSH (mixed type of inhibition)

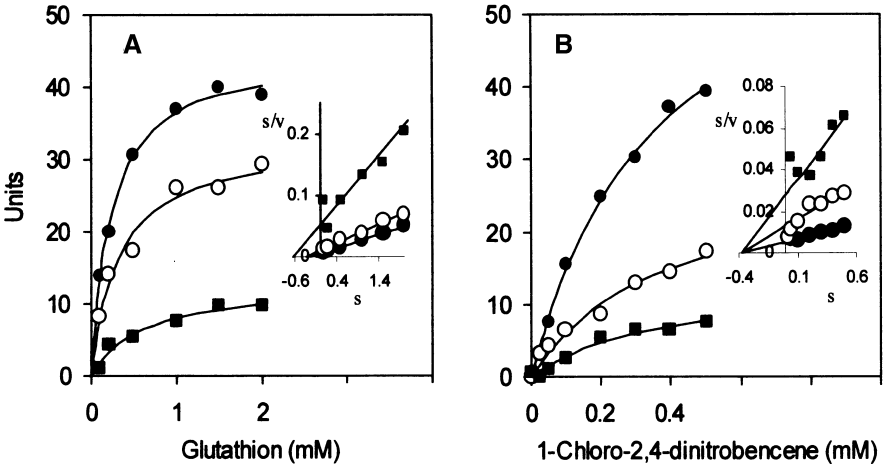

Fig. 6. Mechanism of GSTA1-1 inhibition activity by palmitoyl-CoA. (A) Inhibition by palmitoyl-CoA at increasing concentration of glutathione and fixed concentration (0.5 mM) of CDNB, in the absence (●) and presence of 0.5 μM (○) and 2 μM (■) of palmitoyl-CoA. K_m values of 0.23 ± 0.02; 0.32 ± 0.07; and 0.60 ± 0.2 and B_{max} values of 45.0 ± 1.02; 32.7 ± 2.2; and 12.9 ± 1.6 were determined for palmitoyl-CoA 0, 0.5 and 2 μM, respectively, by direct fitting to the curves. (B) Inhibition by palmitoyl-CoA at increasing concentration of CDNB and fixed concentration (1 mM) of glutathione, in the absence (●) and presence of 1 μM (○) and 2 μM (■) of palmitoyl-CoA. K_m values of 0.37 ± 0.04; 0.37 ± 0.12; and 0.38 ± 0.16 and B_{max} values of 69,6 ± 4.5; 28.8 ± 4.9; and 13.5 ± 3.2 were determined for palmitoyl-CoA 0, 1 and 2 μM, respectively, by direct fitting to the curves. Hanes plots of V, S against s are presented in the insert of each figure.

whereas it also changes the catalytic properties of the enzyme for CDNB (decreased V_{max}) but with no apparent changes in the enzyme affinity for this substrate (noncompetitive inhibition). This behaviour suggest that palmitoyl-CoA does not interfere with the binding site of CDNB and therefore it binds to an specific and different binding site. Other saturated acyl-CoAs were also found to inhibit GSTA1-1 activity (Table 2). Long chain fatty acyl-CoA presented the lower IC_{50} values, a behaviour with follows closely the IC_{50} pattern for acyl-CoA binding-displacement of Net-CoA by saturated acyl-CoAs for rat liver GST previously determined (Table 1), further suggesting that acyl-CoA binding to GST is responsible for the inhibition of enzyme activity. Unsaturated fatty acyl-CoAs and xenobiotics-CoA also inhibited GSTA1-1 although with higher IC_{50} values that saturated long-chain acyl-CoAs.

As for GSTA1-1, acyl-CoAs were also found to inhibit affinity purified rat liver GST, although with higher IC_{50} (3–7 μM for palmitoyl-CoA and 20–50 μM for nafenopin-CoA and ciprofibroyl-CoA). In all cases, palmitic acid, nafenopin, ciprofibrate and also CoASH were without effect (not shown), suggesting that the inhibition was specific for acyl-CoAs. We have already determined that rat liver cytosolic fractions with GST enzymatic activity do not metabolize nafenopin-CoA [12]. In order to further investigate this finding, affinity purified GST or GSTA1-1 were incubated in the presence of glutathione and either palmitoyl-CoA or nafenopin-CoA for 50 min, under the same assay conditions. No changes in the concentration of these acyl-CoAs was found, as estimated by HPLC [12], showing that GST does not metabolize or hydrolyze acyl-CoAs, and that the effect of these compounds on GST enzymatic activity is probably a direct consequence of acyl-CoA binding.

Binding of N-etCoA to rat liver GST classes

Results with human liver recombinant homodimers suggest that only class alpha isoforms containing the A1 subunit bind and are inhibited by acyl-CoAs. However heterodimers and homodimers of different class alpha subunits constitute the alpha class (at least GSTA1–2, GSTA1-3, GST3-3, GSTA3-5, GSTA4-4 [14,15,21,22]). In order to verify whether other class alpha isoforms bind acyl-CoAs, the isoenzymes of rat liver GST were separated by isoelectric focusing. Four peaks of GST activity of different isoelectric points (P_1-P_4) corresponding to the main isoforms present in rat liver [13–15] were thus obtained, as already reported by other authors [32,33]. The electrophoretic pattern of the four fractions together with that of affinity purified GST are shown in Fig. 7. Using the electrophoretic terminology, P_1, P_2 and P_3 containing the type Y_a subunit (at least the A1,A2, A5 and P1 subunits [15]) and type Y_c subunits (at least the A3 and A5 subunits) correspond to class alpha GSTs, whereas P_4 containing Y_b subunits (at least the M2, M3, M4 and M5) corresponds to class mu GSTs. Polarization as a function of varying protein concentration and constant N-etCoA concentration (dilution titration), and at fixed protein concentration and increasing N-etCoA concentration (addition titration) were determined for each of the GST subfamilies, as already shown for the affinity-purified GST and GSTA1-1, in order to determine equilibrium parameters. Similar K_d values were obtained for N-etCoA for the different peaks (0.38 ± 0.02 μM; 0.24 ± 0.01 μM; 0.57 ± 0.04 μM and 0.46 ± 0.04 μM, respectively, for P_1, P_2, P_3 and P_4). However, only the more basic class alpha GSTs (P_1 and P_2) showed a B_{max} of near 1 nmol of N-etCoA bound per nmol of GST (0.97 ± 0.01 and 1.04 ± 0.02, respectively, for P_1 and P_2, considering a molecular mass of 50 000 kDa for the dimer).

Table 2. IC_{50} for the inhibition of GSTA1-1 activity by acyl-CoAs. Values ± SD, were calculated by nonlinear regression analysis from curves as that presented in Fig. 5.

Acyl-CoA	IC_{50} (μM)
Arachidoyl-CoA(C20 : 0)	0.70 ± 0.11
Stearoyl-CoA (C18 : 0)	0.97 ± 0.39
Palmitoyl-CoA (16 : 0)	0.44 ± 0.18
Myristoyl-CoA (C14 : 0)	9.37 ± 2.03
Lauryl-CoA (C12 : 0)	15.21 ± 9.78
Oleoyl-CoA (18 : 1)	3.85 ± 1.84
Linoleoyl-CoA (18 : 2)	2.67 ± 0.58
Ciprofibroyl-CoA	54.10 ± 14.10
Nafenopin-CoA	24.30 ± 7.90

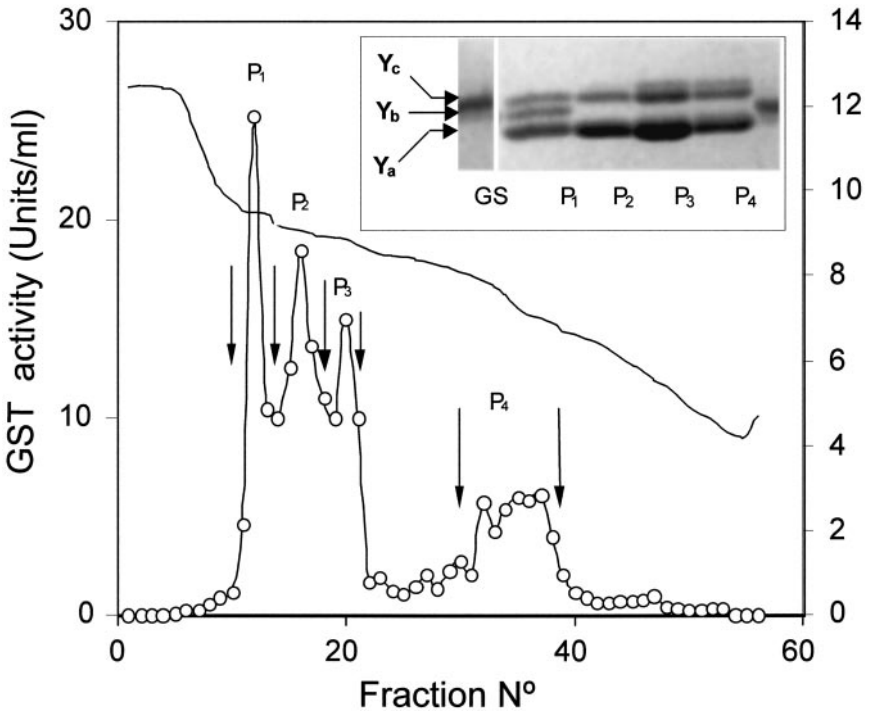

Fig. 7. Isoelectric focusing of affinity purified GST. Affinity purified GST (8.8 mg protein) was submitted to isoelectric focusing in a preparative column as described in Materials and methods. Fractions were collected and pH (3/4) and GST activity (○-○) were determined for each fraction. These were then pooled into four fractions: P_1–P_4 as shown by the arrows. Isoelectric points of 9.49; 9.10; 8.90; and 6.6–7.4 were determined for P_1, P_2, P_3 and P_4, respectively. Inset, SDS/PAGE of affinity purified GST and of the four fractions isolated by isoelectric focusing. Positions of the three GST subunit families Y_a, Y_b and Y_c are indicated [15].

The class mu GST (P_4) bound less than 0.1 nmol of N-etCoA per nmol of GST, i.e. a 10-fold lower binding capacity than the basic class alpha GST isoforms (B_{max} = 0.09 ± 0.01 nmol·nmol^{-1} GST), whereas an intermediate behavior was found for the more acidic class alpha GST (P3), which probably also contains class pi GST isoforms (B_{max} = 0.57 ± 0.01 nmol·nmol^{-1} GST). These results suggest that most class alpha GST isoforms are capable of binding at least 1 mole of N-etCoA per mole of dimeric GST, and suggest that dimers other than GSTA1-1 bind acyl-CoAs.

DISCUSSION

In this study, we found that the alpha class of GST bind acyl-CoA and xenobiotics-CoA without metabolizing or hydrolyzing them. Binding affinity was maximal for long-chain saturated acyl-CoAs and results in strong inhibition of GST enzymatic activity. Cytosolic GSTs subunits are known to contain a GSH binding site (G-site), to which the adjacent subunits contributes with an aspartic acid residue, and a and second substrate-binding site or H-site (reviewed in [15]). Acyl-CoAs do not appear to interact with the H-site, as at fixed GSH and increased CDNB concentration palmitoyl-CoA inhibit GST activity without changing the affinity for CDNB (noncompetitive inhibition). On the other hand, at constant CDNB and increasing GSH concentration, palmitoyl-CoA inhibits GST changing both the affinity (K_m) and the V_{max} (mixed type of inhibition), suggesting interference with the G-site probably at an intersubunit domain. This suggestion is further supported by a maximal binding of one molecule of acyl-CoA per mol of dimeric GST observed with both the pure GSTA1-1 homodimer recombinant protein and with the basic rat liver class alpha GSTs. On the other hand, as rat liver basic GSTs are constituted by homo- and heterodimers [15,21,22], binding of one molecule of acyl-CoA per mol of dimeric enzyme in fractions P_1 and P_2, suggest that most class alpha isoforms do bind acyl-CoA. The binding of less than one mol of acyl-CoA per mol of the less basic class alpha GSTs (P3), is probably due to the presence of pi and mu class isoforms, which do not bind acyl-CoAs. Owing to commercial unavailability the class theta GST isoforms were not investigated, however, they represent a low proportion of total GST in the liver [15].

These observations may have physiological relevance. ACBP and FABP has been proposed as the main mechanism of buffering and transporting acyl-CoAs within the cell [1]. However, the growth rate of *Saccharomyces cerevisiae* is unaffected in cells carrying a disrupted ACBP gene [34], suggesting that ACBP may not be the only protein buffering acyl-CoAs, and that FABP and GSTs may replace their function. The relative importance of each acyl-CoA binding in buffering acyl-CoAs and xenobiotic-CoAs might be related to their intracellular concentrations as well as to their relative affinities for these ligands.

ACBP binds fatty acyl-CoAs with a K_d in the nanomolar range [1]. We have found that ACBP is also capable of binding nafenopin-CoA and ciprofibroyl-CoA with a K_d in the micromolar range as does GST, whereas FABP although capable of binding fatty acyl-CoAs [1], do not bind xenobiotic-CoAs (C. Silva and M. Bronfman, unpublished results). The concentration of endogenous acyl-CoAs has been reported to be in the range of 5–160 μM, and about 50 μM in fed rat liver [1]. In spite of this high concentration, it has been suggested that under normal physiological conditions, the free cytosolic concentration of acyl-CoA esters is maintained in the low nmolar range and that it is unlikely to exceed 200 nM under the most extreme conditions, because of the relatively high concentration of ACBP (50 μM) found in the cytosol [1]. Moreover, FABP, is likely to collaborate with ACBP in buffering fatty acyl-CoAs under extreme conditions despite its higher K_d for acyl-CoAs (in the μM range) due to its high (300 μM) cytosolic concentration [1]. GST is also a highly concentrated cytosolic protein, but shows a lower affinity for fatty acyl-CoAs than ACBP. Therefore, under physiological conditions, endogenous acyl-CoAs would be preferentially

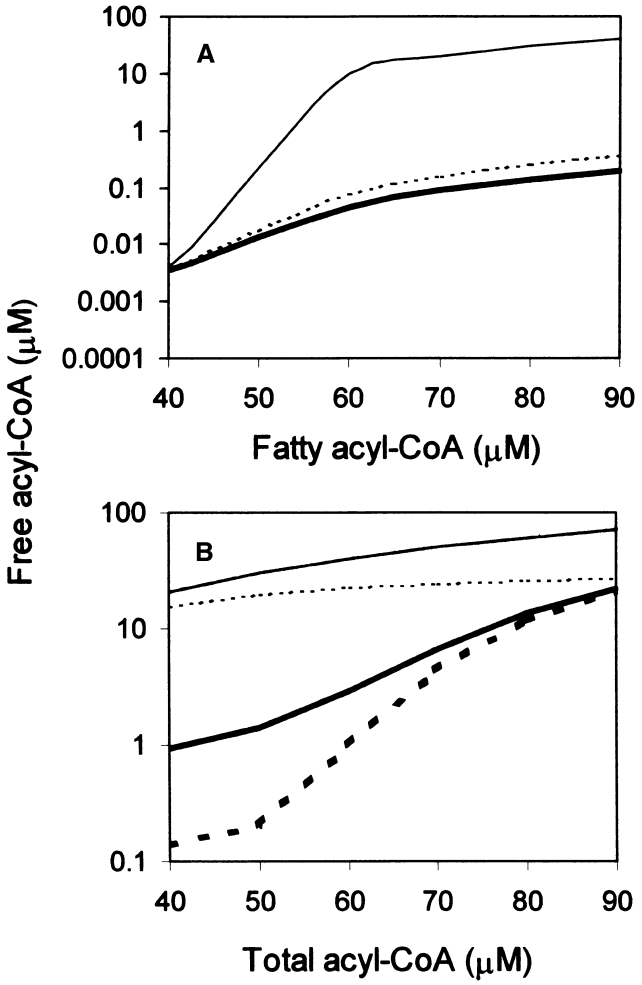

Fig. 8. Simulation of acyl-CoA binding to ACBP, FABP and GST. Equilibrium and total balance equations for each of the binding proteins and ligands were simultaneously resolved by numerical optimization, using the Solver algorithm function of MICROSOFT EXCEL 7.0 (Microsoft Corporation, USA) with a lineal estimation, progressive derivatives and a Newton algorithm. It is assumed that the K_d for acyl-CoAs is 1 nM for ACBP and 1 μM for GST and FABP, and the K_d for xenobiotic-CoAs is 1 μM for ACBP and either 1 μM or 0.1 μM for GST. FABP is considered to have no affinity for xenobiotic-CoAs. The binding protein concentrations were fixed at 50, 50 and 150 μM for ACBP, GST and FABP, respectively (see text). (A) Simulation of free acyl-CoA concentration at increased total acyl-CoA concentration in the absence of xenobiotic-CoAs and in the presence of ACBP alone (thin line); ACBP and FABP (dotted line), and ACBP, FABP and GST (heavy line). (B) Simulation of free acyl-CoA concentrations (the sum of fatty and xenobiotic acyl-CoAs, ordinate) in the presence of 30 μM xenobiotic-CoA and increasing fatty acyl-CoA concentrations as indicated (abscissa). ACBP alone (thin line); ACBP and FABP (dotted line), and ACBP, FABP and GST (heavy line, K_d of GST for xenobiotic-CoA of 1 μM; heavy dotted line, K_d of GST for xenobiotic-CoA of 0.1 μM)

bound by ACBP, but this may not be the case for xenobiotic-CoAs. We have previously shown that a high concentration of xenobiotic-CoAs, up to 30 μM for ciprofibroyl-CoA, can be readily attained in the liver of drug-treated animals [6]. Thus, in the case of xenobiotic-CoAs, ACBP and GST appear to have similar binding affinities, yet due to the higher affinity of ACBP for fatty acyl-CoAs, this protein will become saturated when the concentration of these ligands increases and will not be available for binding xenobiotic-CoAs. Under these conditions, it is likely that GST will buffer xenobiotic-CoAs.

Figure 8 shows a simulation of the free intracellular acyl-CoA concentration as a function of total acyl-CoA concentration in the presence of the various binding proteins. We assume that: (a) endogenous acyl-CoAs bind to ACBP with a Kd of 1 nM, and to GST and FABP with a K_d of 1 μM; (b) xenobiotic-CoAs bind to ACBP with a K_d of 1 μM, and to GST with a K_d of 1 μM or lower (0.1 μM), as the K_d of GST for xenobiotic-CoAs may be assumed to be significantly lower than the reported IC_{50} (Table 1); (c) FABP has no appreciable affinity for xenobiotic-CoAs; and (d) the intracellular concentrations of these binding proteins are 50 μM for ACBP, 50 μM for GST (calculated from the number of binding sites in glutathione-Sepharose isolated GST, which is about half of the total liver GST concentration [15]) and 150 μM for FABP (it is assumed that 50% of cytosolic FABP is available for acyl-CoA-binding, while the other 50% is saturated with fatty acids [35]). As proposed by Færgeman and Knudsen [1], in the presence of only fatty acyl-CoAs, ACBP alone will maintain low levels of free, unbound, acyl-CoAs when the acyl-CoAs/ACBP ratio is below 1, but concentrations will increase rapidly, rearing to toxic levels, when the ratio exceeds 1 (Fig. 8A). The additional presence of FABP will consequently decrease acyl-CoA levels below the 1 μM mark at high acyl-CoA concentrations, and the additional presence of GST will not induce major changes. However, as shown in Fig. 8B, in the additional presence of 30 μM of xenobiotic-CoA, the situation changes dramatically. When only ACBP is present, the total unbound acyl-CoA concentration (fatty acyl-CoAs + xenobiotic-CoA) will increase over the 10 μM level, even at low fatty acyl-CoA concentrations, because of the low affinity of ACBP for xenobiotic-CoAs. In this case, the additional presence of FABP will produce only a minimal decrease in the levels of total unbound acyl-CoAs as we assume that it has no apparent affinity for xenobiotic-CoAs. In contrast, the additional presence of GST, assuming a K_d of either 0.1 μM or 1 μM for xenobiotic-CoA, will decrease the total unbound acyl-CoA concentration below the 10 μM mark by binding xenobiotic-CoAs, even at a high fatty acyl-CoA concentration. This behavior is a consequence of the high affinity of ACBP for fatty acyl-CoAs and of the similar and relatively low affinity of GST for both xenobiotic and fatty acyl-CoA esters. The calculations given in Fig. 8 suggest that in peroxisome proliferator-treated animals, ACBP will buffer the drug-induced increased concentration of fatty acyl-CoAs, whereas class alpha GSTs will mediate the transport of the nonmetabolizable CoASH esters of these drugs. Therefore, metabolic pathways and signaling systems in which acyl-CoAs are intermediates or regulators [6,8] will be protected from xenobiotic-CoA induced disturbance. Overall, these considerations suggest that xenobiotic-CoA binding to class alpha GSTs may have a physiological importance, and are consistent with the role of GSTs isoenzymes as major drug-processing proteins through their binding or metabolism.

On the other hand, the inhibition of GST enzymatic activity by acyl-CoA binding may also have physiological relevance. GSTA1-1 bind acyl-CoAs more avidly *in vitro* than their substrates, judging by IC_{50} for acyl-CoA inhibition one order of magnitude lower that the K_m determined for either GSH or the substrate DNTB. Therefore, class alpha GST isoenzymes activity may be strongly modulated *in vivo* by the intracellular acyl-CoA concentration. Because these isoforms are abundant in the liver [13–15], this inhibition may have important consequences in xenobiotic detoxification under increased intracellular acyl-CoA concentration, as occurs after hypolipidaemic drug treatment [9]. The inhibition of detoxification

under these conditions may be one of the factors involved in the liver carcinogenic properties of peroxisomal proliferators.

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