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DIFFERENT ENZYME KINETICS DURING THE GLUTATHIONE CONJUGATION OF THE FOUR STEREOISOMERS OF THE FJORD-REGION DIOLEPOXIDES OF BENZO[*c*]PHENANTHRENE BY THE μ-CLASS RAT LIVER GLUTATHIONE *S*-TRANSFERASE HTP II

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Abstract—The enzyme-catalysed conjugation of each of the four stereoisomers of *trans*-3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene (B[c]PhDE) with glutathione (GSH) by HTP II, a novel isolated μ-class GSH transferase from the liver of untreated rat, was studied. All four stereoisomers were substrates for GSH transferase HTP II. The enzymatic reaction shows three different types of enzyme kinetics: substrate inhibition for (−)-*anti*-B[c]PhDE with (*R,S,S,R*)-absolute configuration, allosteric behavior using (+)-*anti*-B[c]PhDE with (*S,R,R,S*)-absolute configuration and Henri–Michaelis–Menten kinetics with both the (−)-*syn*- and (+)-*syn*-enantiomers, with (*S,R,S,R*)- and (*R,S,R,S*)-absolute configuration, respectively. When the concentration of these diolepoxides was varied (using 2 mM GSH), the apparent V_{max} values were 1975 nmol/min × mg for (−)-*anti*-B[c]PhDE and about 60 nmol/min × mg for both (−)-*syn*- and (+)-*syn*-B[c]PhDE, with the corresponding K_m values of 1.05 and 0.20 mM. The reaction of (+)-*anti*-B[c]PhDE determined by applying the Hill equation had an estimated V_{max} value of 930 nmol/min × mg. On varying the concentration of GSH, linear Lineweaver–Burk plots were obtained. No competitive effect could be observed using a mixture of (−)-*anti*- and (+)-*anti*-enantiomers, indicating that their binding sites are different and independent. It was also shown, that the binding sites of (+)-*anti*- and both *syn*-enantiomers were different and independent of each other, while there was a small effect on the binding of the *syn*-enantiomers caused by (−)-*anti*-B[c]PhDE. All products of the reaction between GSH and the dihydrodiol epoxides of benzo[*c*]-phenanthrene could be resolved by HPLC and were identified and quantitated using the corresponding synthetic GSH conjugates.

Polycyclic aromatic hydrocarbons (PAH†) are common environmental contaminants several of which are tumorigenic in experimental animals and are suspected to play a role in the chemical carcinogenesis in humans [1–4]. It has been well documented that PAH are not carcinogenic *per se* but require metabolic activation to highly reactive electrophilic intermediates, in particular to vicinal dihydrodiol epoxides located in a "bay-region" of PAH [2, 3]. It is generally believed that the subsequent reaction of these reactive intermediates with nucleophilic centers in DNA is essential for initiation of the carcinogenic process [5]. For instance, benzo[*a*]pyrene (B[a]P) is metabolized in a sequence involving epoxidation of the 7,8-position, hydrolysis to the corresponding *trans*-dihydrodiol and further epoxidation at the 9,10-position to yield the *syn*- and *anti*-diastereomers of *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-B[a]P (B[a]PDE) [2, 3]. *Anti*-B[a]PDE, and in particular the (+)-enantiomer with (*R,S,S,R*)-absolute configuration, is the biologically most active species involved in tumor formation in experimental animals [6, 7]. In general, when tested in mice, bay-region diolepoxides derived from several PAH with (*R,S,S,R*)-absolute configuration and the same conformation of hydroxyl groups seem to be the most tumorigenic intermediates [8, 9].

In recent years, diolepoxides of PAH whose oxirane ring is located in a fjord-region, which is sterically more constricted than a simple bay-region, gained increased attention in carcinogenicity [10] and mutagenicity studies [11, 12]. Benzo[*c*]phenanthrene (B[c]Ph), which is the simplest PAH possessing a fjord-region, was the first example in which both diastereomeric fjord-region diolepoxides (*syn*- and *anti*-configuration) show significant tumorigenic activity [13]. Additionally, its fjord-region diolepoxides are the most tumorigenic diolepoxides yet tested on mouse skin despite their expected and observed very low chemical reactivity [10, 14].

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† Abbreviations: BA, benz[*a*]anthracene; B[a]P, benzo[*a*]pyrene; B[c]Ph, benzo[*c*]phenanthrene; B[c]PhDE, *trans*-3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydro-B[c]Ph; B[a]PDE, *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-B[a]P; GSH, glutathione; GST, glutathione *S*-transferase; PAH, polycyclic aromatic hydrocarbons.

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Further, the fjord-region diolepoxides of B[c]Ph covalently bind to a significantly higher extent to DNA than the bay-region diolepoxides of B[a]P [15]. In order to investigate the molecular basis of the surprisingly high biological activity, the metabolism of all four diolepoxide stereoisomers of B[c]Ph seemed to be an attractive project [16].

The intracellular accumulation of diolepoxides in the intact cell and thus the extent of DNA binding required for tumor formation is determined by several processes such as the elimination of (+)-*anti*-B[a]PDE, the spontaneous hydrolysis to non-reactive products like tetrols, the reduction to triols, the reaction with cellular nucleophilic macromolecules other than DNA, the enzyme-catalysed conjugation with glutathione (GSH) and further metabolism to triol epoxides. Previous studies with subcellular fractions and isolated hepatocytes from rat liver clearly demonstrate that enzymatic conjugation with GSH is the major route of diolepoxide elimination [17, 18].

A growing body of information indicates that GSH *S*-transferases (GSTs; EC 2.5.1.18) play an important part in cellular protection against harmful intermediates generated during metabolism of cytotoxic and carcinogenic compounds [19, 20]. GSTs are a family of homo- and hetero-dimeric combinations of various species- and tissue-specific subunits, catalysing the reaction of GSH with large numbers of compounds which bear an electrophilic center [21, 22]. These enzymes are mainly located in the cytosol, although a microsomal form has been described [23]. The cytosolic isoenzymes fall into three classes, mainly based upon differences in structural, immunological and catalytic properties [24, 25]. They are thought to be derived from four multigene families [24]. The GST nomenclature used in this paper is that recommended by Jakoby *et al.* [26]. The classification α for "basic", μ for "acidic" was originally determined by the isoelectric points of human GST isoenzymes. In the case of rat liver GST isoenzymes, the "acidic fraction" contains μ- and π-class isoenzymes.

Experiments employing purified GST isoenzymes from various sources showed that certain enzyme forms, in particular the μ-class transferase 4-4 [27, 28] and those belonging to the π-class [29, 30] are highly efficient and selective in detoxifying *anti*-diolepoxides of PAH with (*R,S,S,R*)-absolute configuration. This finding has attracted increasing interest in the elucidation of the toxicological role of μ- and π-class isoenzyme forms of GST. Recently, a very efficient purification procedure for acidic GST isoenzymes from rat liver has been developed in this laboratory [31]. A total of 12 forms with apparent molecular weights between 24 and 27 kDa could be separated. Only one of these isoenzymes is present in considerable amounts in the uninduced rat liver: the novel isolated isoenzyme form HTP II (pI = 6.76), constituting about 0.7% of total cytosolic protein. Further, this is the only isoenzyme which shows a high detoxifying capacity in Ames tests using mutagenic diolepoxides of several PAH as substrates, in particular with *anti*-BA-8,9-diol-10,11-oxide [31].

In the present study we investigated the reaction and the enzyme kinetics of the four stereoisomers of the fjord-region dihydrodiol epoxides of B[c]Ph with GSH catalysed by the μ-class GST isoenzyme HTP II from untreated rat liver. The significance of the results obtained with respect to the detoxification of B[c]Ph by rat liver is discussed.

MATERIALS AND METHODS

Chemicals. Synthesis of (±)-*anti*- and (±)-*syn*-3,4-dihydrodiol-1,2-epoxides of B[c]Ph was performed as described recently [12]. Pure enantiomers of *anti*- and *syn-trans*-3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydro-B[c]Ph (B[c]PhDE) were obtained and characterized with regard to spectral properties and absolute structure as reported previously [32]. The absolute configuration of the optically active isomers is shown (Fig. 1).

The corresponding GSH conjugates of these four different stereoisomers of B[c]PhDE were synthesized as described elsewhere [16, 33], the glutathionyl moiety being attached to the benzylic carbon atom (C-1) by *trans*-opening of the oxirane ring. After purification by HPLC, the absolute purity of the single synthetic GSH conjugate was verified by HPLC, UV spectroscopy, 400 MHz ^{1}H-NMR, mass spectroscopy and elemental analysis [16, 33].

The acetonitrile used was of HPLC grade (J. T. Baker Chemical Co., Philippsburg, NJ, U.S.A.). All other chemicals were of analytical grade.

GSH transferase 4-4. The μ-class transferase 4-4 was purchased in the most purified form available as GST Yb2 antigen from Biotrin International Ltd, a subsidiary of Medlabs Ltd (Unit 1C, Stillorgan Industrial Park, Dublin, Ireland).

GSH transferase HTP II. The purification and characterization of the homodimeric rat liver GST isoenzyme HTP II (pI = 6.76) has already been described [31]. In brief, the purification procedure was based upon the method of Hayes and Chalmers [34]. In a first step, the rat liver cytosolic protein fraction, prepared as described [35], was roughly separated into basic and acidic proteins by radial anion-exchange chromatography using QA-52 cellulose (Whatman, Maidstone, U.K.) in a "Superflo"-column (Sepragen, San Leandro, CA, U.S.A.). The eluate containing the acidic fraction was subjected to affinity chromatography on GSH-sepharose as a very efficient purification step for GSTs. The separation of the acidic GST pool was carried out according to isoelectric points by FPLC-chromatofocusing in the pH range 7.0–5.0 on the polybuffer exchanger Mono P (Pharmacia, Uppsala, Sweden). Final preparative chromatography of single chromatofocused GST fractions on Bio-Gel HTP hydroxylapatite (Bio-Rad Laboratories, Munich, F.R.G.) resulted in electrophoretic pure homo- and heterodimers.

Characterization of the purified enzymes was performed by several methods: (a) separation of the subunits and verification of their purity was achieved by reversed phase HPLC. The RP-HPLC chromatogram of the HTP II isoform shows a single homogeneous peak, demonstrating its homodimeric (subunits Y2-Y2) composition (Fig. 2A). (b) SDS–PAGE of the HPLC pure HTP II isoenzyme, performed according to Schägger and von Jagow

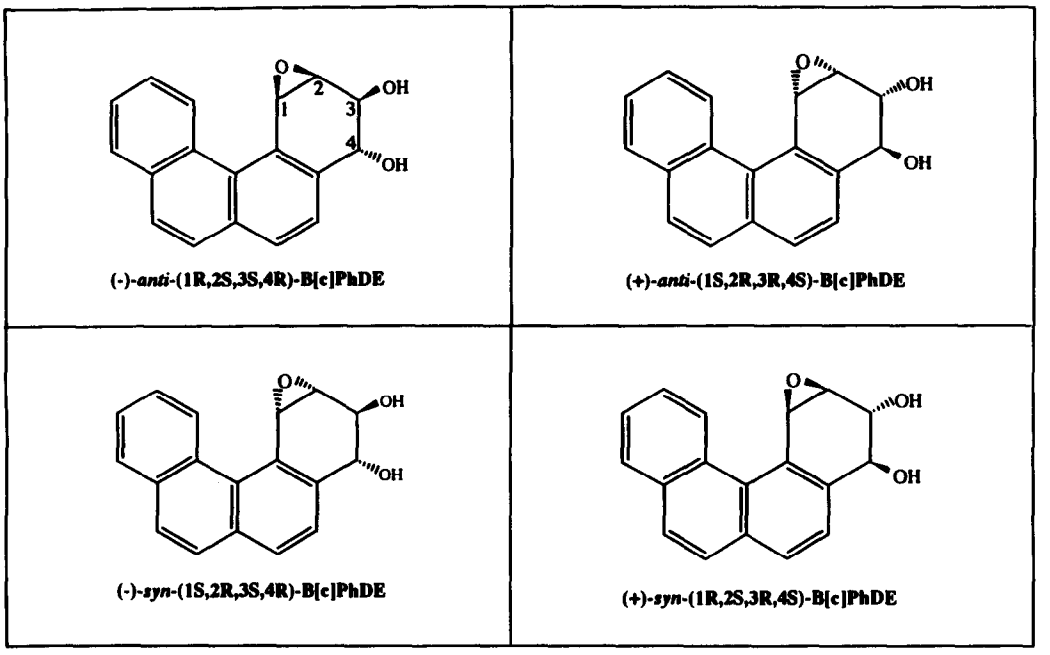

Fig. 1. Chemical structure of the *anti-* and *syn*-diastereomers of B[c]PhDE. The absolute configuration of the optically active isomers of the diastereomeric fjord-region 3,4-dihydrodiol-1,2-epoxides of B[c]-Ph are shown. In the *anti*-diolepoxide enantiomers the benzylic hydroxyl group and the epoxide oxygen are located on the opposite faces of the molecule, whereas they are located on the same face of the molecule in the *syn*-diolepoxide series. When their bay-regions are aligned, it is (−)-*anti*-(1*R*, 2*S*, 3*S*, 4*R*)-B[c]PhDE which is superimposable on the ultimate carcinogenic (+)-*anti*-diolepoxide isomers of B[a]P, BA and chrysene, all of which have the analogous (*R,S,S,R*)-absolute configuration.

[36], revealed a monomeric molecular weight of approximately 26.0 kDa (Fig. 2B). (c) An isoelectric point of 6.76 for the native HTP II, determined by isoelectric focusing in the pH range 4–7, was deduced according to simultaneously separated marker proteins (Fig. 2C). Moreover, the gels presented in Fig. 2B and C demonstrate the uniqueness of the isoenzyme HTP II as compared to additional purified homo- and heterodimeric GSTs belonging to the same enzyme preparation. (d) The high detoxifying capacity of the isoenzyme HTP II in Ames tests using mutagenic diolepoxides of several PAH as substrates, in particular with *anti*-BA-8,9-diol-10,11-oxide, was not observed with the other isoenzyme forms of the same enzyme preparation [31]. (e) N-terminal amino acid sequence analysis, substrate specifities with diagnostic substrates and immunological studies with polyclonal antibodies against the prominent and well-characterized rat liver GSTs demonstrated HTP II as belonging to μ-class GSTs [31].

Protein concentration was determined using the Bradford microassay procedure according to Redinbaugh and Campbell [37], with bovine serum albumin as standard. The enzyme solutions were dialysed against TKE-buffer (50 mM Tris–HCl, 25 mM KCl and 0.5 mM EDTA, pH 7.5) and stored at −20°.

Incubation of GSH transferase HTP II with GSH and the four diolepoxide stereoisomers as substrates. Prior to incubation, a solution containing 205 μL TKE-buffer, 25 μL GSH (20 mM in TKE-buffer) and 10 μL GST HTP II (0.5 mg/mL in TKE-buffer) was preincubated in an 1.5 mL Eppendorf microtest tube for 30 sec at 37°. The reaction was initiated by addition of 10 μL (−)-*anti*-B[c]PhDE in dimethyl sulfoxide and mixing using a Vortex mixer (Model Vortex Genie K 550-GE at position 4 for 1 sec). When the dependence of enzymatic activity on the concentration of the respective diolepoxide was studied, the concentration of (−)-*anti*-, (+)-*anti*-, (−)-*syn*- and (+)-*syn*-B[c]PhDE was varied between 50 and 2000 μM, at 2 mM GSH and 20 μg protein/mL. However, when the dependence of enzymatic activity on GSH concentration was studied, this co-substrate was varied between 25 μM and 2 mM at fixed concentrations of 300 μM (−)-*anti*-B[c]PhDE and 1000 μM (+)-*anti*-B[c]PhDE. Incubations were carried out for 60 sec at 37° in a total volume of 250 μL. In spite of the short incubation time reproducibility between repeated experiments was at least within 10%, usually around 5%. The reaction was stopped by rapid mixing with 250 mL ice-cold acetone (Vortex mixer position 4 for 10 sec). This step was followed by extraction with 3 × 500 μL ethyl acetate saturated with TKE-buffer (Vortex mixer position 4 for 10 sec and Eppendorf Centrifuge Model 5412 for 30 sec). This procedure resulted in an upper organic phase, a lower aqueous phase and a white, very thin layer of denatured protein at the interphase. The organic phases containing unreacted diolepoxide, and tetrols resulting from their

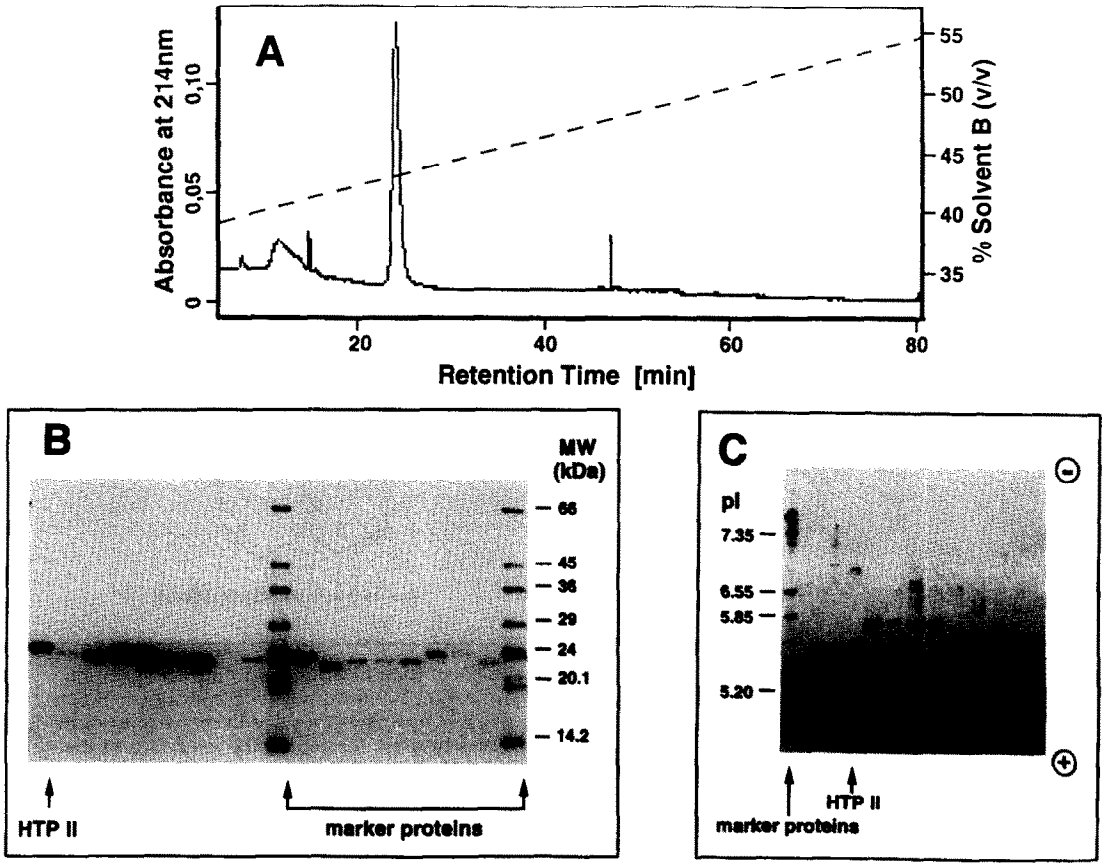

Fig. 2. Chromatographic properties of acidic GSTs and their subunits isolated from liver cytosol of untreated male Sprague–Dawley rats. (A) Reversed phase HPLC of GSH transferase HTP II: 30 μg of the pure isoenzyme were applied to a Dynamax 300 Å column (Rainin Instruments Company Inc., Emeryville, CA, U.S.A.), which was equilibrated with solvent A (0.1% trifluoroacetic acid in 1% aqueous acetonitrile) and elution was performed with a linear gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile) at a flow rate of 1.5 mL/min. (B) SDS–PAGE of HPLC-separated subunits of hydroxylapatite-purified acidic GSTs. HPLC subunit peaks were collected, lyophilized, dissolved in sample buffer and subjected to electrophoresis in a 10% SDS–polyacrylamide gel according to Schägger and von Jagow [36]. Marker proteins are taken from the "SDS-7 kit" (Sigma Chemical Co., St Louis, MO, U.S.A.). Protein bands were visualized by staining with Serva Blue T (Serva Chemicals, Heidelberg, F.R.G.) Subunit Y2 of the homodimeric GSH transferase HTP II is shown in the first lane. The other occurring bands represent the subunits of additional purified homo- and heterodimeric GSTs of the same preparation. (C) Isoelectric focusing of native hydroxylapatite-purified GSTs in an immobilized pH gradient 4–7 (Servalyt Precote, Serva Chemicals, Heidelberg, F.R.G.). Samples were microdialysed against distilled water using Millipore membranes (25 nm pore size) and loaded onto the gel in 10 μL volume corresponding to 2.5 μg protein. Isoelectric point marker proteins (IEF Calibration Kit pH 5–10.5, Pharmacia, Uppsala, Sweden) were applied in a total volume of 8 μL/lane. GSH transferase HTP II is shown in lane 4, all other lanes indicate additional GSTs belonging to the same enzyme preparation.

hydrolysis were removed with a syringe and discarded. The aqueous phase containing the water-soluble GSH conjugates in a total volume of 210 μL was filtered using Millex GV_4 filter units (Millipore Corporation, Bedford, MA, U.S.A.) prior to HPLC. The recovery of the GSH conjugates of B[c]PhDE was determined by incubating and extracting known amounts of synthetic GSH conjugates as standards and bovine serum albumin instead of the GST isoenzyme HTP II. The recovery was found to be 98%. As internal control, the whole incubation and extraction procedure was validated by determining the known reaction rate of GSH transferase 4-4 with the substrate (±)-*anti*-B[a]PDE [27, 28].

Separation of the GSH conjugates. The GSH conjugates of the (+)- and (−)-enantiomers of *anti*- and *syn*-B[c]PhDE were resolved by HPLC under isocratic conditions on a reversed phase RP-18 column (LiChrosorb RP-18, 5 μM, 4 mm i.d. × 10 mm, Knauer GmbH, Berlin, F.R.G.) at room temperature (Fig. 3). The solvent system used was helium-degassed 12% (v/v) acetonitrile/22.5 mM ammonium acetate adjusted to pH 3.5 with acetic acid. The solvent was delivered at a constant

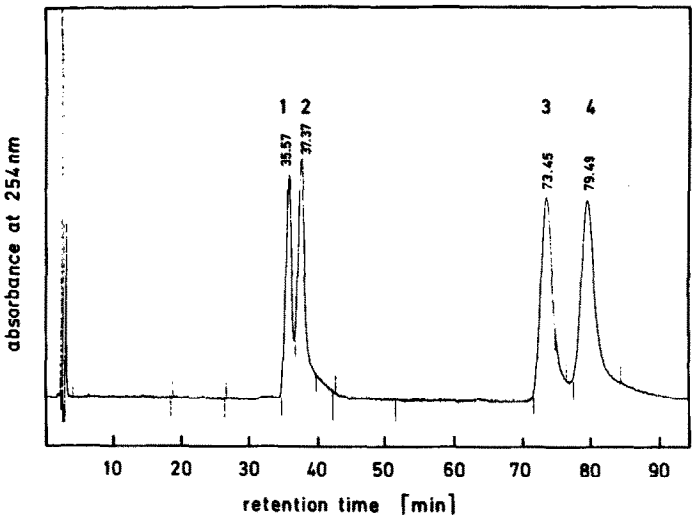

Fig. 3. HPLC separation profile of the GSH conjugates of the *anti-* and *syn*-diastereomers of B[c]-PhDE. A mixture of all four stereoisomers was injected onto a Reversed Phase LiChrosorb RP-18 column. The separation was conducted isocratically as described in Materials and Methods, using 13% acetonitrile as the organic component of the aqueous solvent system. The four stereoisomeric compounds were identified as peak 1 (−)-*anti*-, peak 2 (+)-*anti*-, peak 3 (−)-*syn*- and peak 4 (+)-*syn*-B[c]PhDE, respectively.

flow rate of 1.0 mL/min (Waters Model 680 Automated Controller and Waters Model 510 HPLC Pump, Waters Associates, Milford, MA, U.S.A.). If the *syn*-diastereomers of B[c]PhDE were used as substrates, the polarity of the solvent system was enhanced by using 14% (v/v) acetonitrile under otherwise identical conditions. UV absorbance in the eluate was monitored at 254 nm using a Waters Model 484 Tunable Absorbance Detector (Waters Associates, Milford, MA, U.S.A.) equipped with a 1 cm 10 μL flow-through cell.

Quantification of the GSH conjugates. The resulting GSH conjugates of the four stereoisomers of B[c]-PhDE were quantified by integration of UV_{254} absorbance after HPLC separation and comparison with synthetic standards. Stock solutions of the standards were prepared by weighing out 1 mg of each lyophilisate and dissolving in 100 mL 50% (v/v) methanol/50% (v/v) H_2O. After separation on HPLC as described above, the UV_{254} signals were integrated by a Waters Integrator Model 745 Data Module using method number 0 peak area integration. For each of the four GSH conjugates a linear calibration curve was obtained by plotting the peak area value versus the amount of GSH conjugate. The detection limit was 10 ng or 0.017 nmol.

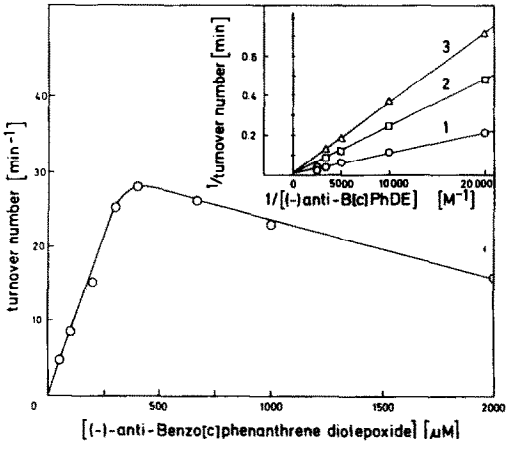

Fig. 4. Course of the enzymatic conjugation of GSH with (−)-*anti*-B[c]PhDE. The diolepoxide substrate was varied at a constant GSH concentration of 2 mM. The single data points are derived from at least three separate incubation experiments, which were quantified three times each. The inset shows Lineweaver–Burk plots at three different GSH concentrations: line 1 is the curve for 2 mM GSH (○), line 2 for 250 μM GSH (□) and line 3 for 100 μM GSH (△).

RESULTS

Conjugation of GSH with (−)-anti-B[c]PhDE

The enzymatic reaction of the GSH conjugation of the (−)-*anti*-diastereomer of B[c]PhDE catalysed by rat liver isoenzyme GSH transferase HTP II was linear during the initial phase. After approaching maximal velocity at a substrate concentration of about 400 μM, the course of the reaction showed substrate inhibition (Fig. 4). All possible controls were performed to prove that the effect of the inhibition is due only to the substrate (−)-*anti*-B[c]-PhDE. Most substrate inhibitions result from combination of a substrate with the wrong enzyme form and, in general, are apparent only at high substrate concentrations, as in the observed case, or

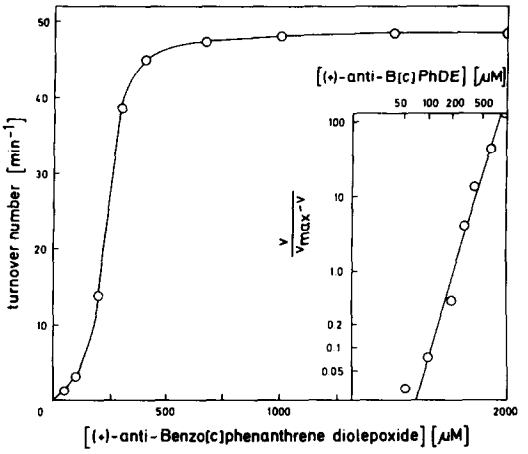

Fig. 5. Course of the enzymatic conjugation of GSH with (+)-*anti*-B[c]PhDE. The diolepoxide substrate was varied at a constant GSH concentration of 2 mM. The single data points are derived from at least three separate incubation experiments, which were quantified three times each. The inset shows the replot of the data in the form of a Hill plot.

when the reaction is studied in the non-physiological direction. Kinetic parameters for the initial phase of the reaction were estimated from results of experiments where the diolepoxide substrate was varied between 50 and 400 μM at a constant GSH concentration of 2 mM. A linear Lineweaver–Burk plot was obtained (Fig. 4, inset, line 1) and the apparent V_{max} and K_m values were evaluated by linear regression analysis. The apparent V_{max} value was 103 min^{-1} (1975 nmol/mg × min) with a corresponding K_m value of 1.05 mM. Using these data, the value of the catalytic efficiency was calculated from the ratio V_{max}/K_m to be 0.097 $min^{-1} \times \mu M^{-1}$ (0.0016 $sec^{-1} \times \mu M^{-1}$). To determine the kinetic mechanism of the initial phase of the reaction of GSH with (−)-*anti*-B[c]PhDE in detail, the initial velocity at three fixed concentrations of GSH was plotted in the form $1/V$ versus 1/[substrate] (Fig. 4, inset). Experiments were also performed in which the concentration of the nucleophilic substrate GSH was varied at a constant concentration (300 μM) of (−)-*anti*-B[c]PhDE. A linear Lineweaver–Burk plot was obtained (data not shown). The kinetic parameters were obtained by linear regression analysis as $V_{max} = 25\ min^{-1}$ and $K_m = 0.21$ mM.

*Conjugation of GSH with (+)-*anti*-B[c]PhDE*

The kinetic parameters were obtained from experiments where the diolepoxide substrate was varied at a constant GSH concentration of 2 mM (Fig. 5). The data show a sigmoidal velocity course of the enzymatic reaction. In general, sigmoidal velocity curves of enzymatic reactions are caused almost exclusively by allosteric enzymes, indicating that the GST isoenzyme HTP II may act, using this particular enantiomer as substrate, as an allosteric enzyme. The maximal velocity of the reaction was estimated from the plot V versus [substrate] as $V_{max} = 48.5\ min^{-1}$ (930 nmol/mg × min). To calculate the other kinetic constants the Hill equation $V/V_{max} = [S]^n/K' + [S]^n$ as a simplified velocity equation for allosteric enzymes was used. In this equation the term n equals the number of substrate binding sites per molecule of enzyme and the term K' represents a constant comprising all interaction factors and the intrinsic dissociation constant K_s. The experimental velocity data were analysed using the Hill plot, the logarithmic form of the Hill equation (Fig. 5, inset).

If the cooperativity of substrate binding is not very high the term n will no longer equal the actual number of substrate binding sites. In this case, the term n in the Hill equation should be designated n_H which is the slope of the Hill plot where $\log (V/V_{max}) = 0$, that is where $V = 0.5\ V_{max}$. The next highest integer above this apparent term n_H represents the minimum number of actual sites. Our experimental data yield a slope of the Hill plot $n_H = 3.4$. Therefore, we are in effect saying that the enzyme behaves as if it possesses exactly 3.4 substrate binding sites with very strong positive cooperativity. Thus, there are at least four sites with relatively strong cooperativity but there could just as well be eight sites with poor cooperativity, or many sites that act in highly cooperative tetramers. When $V = 0.5\ V_{max}$, the constant K' was calculated from the relationship $K' = [S]_{0.5} \times n$ as $K' = (215\ \mu M)^4 = 2.13 \times 10^{-15}\ M^4$.

Experiments were also performed in which the GSH concentration was varied at a constant saturating concentration (1000 μM) of (+)-*anti*-B[c]PhDE. A linear Lineweaver–Burk plot was obtained (data not shown), the kinetic parameters being $V_{max} = 33\ min^{-1}$ and $K_m = 0.25$ mM.

*Conjugation of GSH with (−)-*syn*- and (+)-*syn*-B[c]PhDE*

Formation of GSH conjugates with both enantiomers of *syn*-B[c]PhDE was observed. Linear Lineweaver–Burk plots were obtained (data not shown), the kinetic parameters being identical for the two compounds. The apparent V_{max} and K_m values were estimated by linear regression analysis. When the concentration of the diolepoxides was varied between 50 and 2000 μM at a constant GSH concentration of 2 mM, the apparent V_{max} value was 3.25 min^{-1} (62.5 nmol/min × mg) with a corresponding K_m value of 0.20 mM. The catalytic efficiency of the GST isoenzyme HTP II in the GSH conjugation of both *syn*-B[c]PhDEs was calculated from the ratio V_{max}/K_m to be 0.016 $min^{-1} \times \mu M^{-1}$. Thus, the value of the catalytic efficiency is about 16% and the value of V_{max} is about 3% compared with the substrate turnover of the (−)-*anti*-diastereoisomer under otherwise identical conditions.

Some experiments were carried out varying the GSH concentration at saturating concentrations of the *syn*-diolepoxides (data not shown). The results suggest that the course of the observed conjugation reaction is that expected for GSH transferases, which are proposed to follow a kinetic mechanism called

by Cleland "Ordered Bi Bi" (Mannervik [22], Cleland [38]).

Study of competitive effects during the conjugation of GSH with various mixtures of the four stereoisomers of B[c]PhDE

Due to the favorable chromatographic properties of the GSH conjugates, it was possible to determine the enzymatic turnover of all four stereoisomeric diolepoxides of B[c]Ph separately in one single experiment: the products formed, i.e. the different GSH conjugates, show different retention times on reversed phase HPLC (Fig. 3). Using various substrate concentrations of a racemic mixture of the (±)-*anti*-diastereoisomer of B[c]PhDE, or a mixture of equal amounts of, separately synthesized, (−)-*anti*- and (+)-*anti*-diastereoisomers at a fixed concentration (300 μM), the turnover rates and the kinetic behavior of the single compounds were not changed. No competitive effect could be observed.

For studying the effect of the *syn*-dihydrodiolepoxides of B[c]Ph, a concentration of 300 μM was chosen, where their turnover rate (2.5 min^{-1}) is at the end of the linear phase of the reaction and close to the maximal velocity. Using (−)-*anti*-B[c]PhDE (100 μM) and either the (−)-*syn*- or the (+)-*syn*-diolepoxide as substrates, the turnover rate of the (−)-*anti*-diolepoxide was not changed, whereas the turnover rate of both *syn*-diolepoxides was enhanced by a factor of 2. In contrast, using (+)-*anti*-B[c]PhDE (200 μM) and either the (−)-*syn*- or (+)-*syn*-diolepoxide as substrates, the turnover rates remained unchanged. Using a mixture of equal amounts of the (−)-*syn*- and (+)-*syn*-enantiomers, the formation of the two different GSH conjugates was increased by a factor of 1.2 ± 0.2.

DISCUSSION

The enzymatic reaction of the GSH conjugation of the four different diastereomers of B[c]PhDE catalysed by the isoenzyme HTP II shows three different types of enzyme kinetics, depending on the diastereomer used: (a) substrate inhibition using (−)-*anti*-B[c]PhDE as substrate, (b) allosteric behavior using (+)-*anti*-B[c]PhDE as substrate and (c) Henri–Michaelis–Menten kinetics with both the (−)-*syn*- and (+)-*syn*-diastereomers.

Using (−)-*anti*-B[c] PhDE as substrate, the conjugation reaction is initially linear and shows a maximum velocity of 103 min^{-1}. The maximum turnover is reached at a substrate concentration of about 400 μM. Using higher substrate concentrations results in inhibition of the enzyme reaction. Our data indicate that a "second" substrate molecule B can bind only to the enzyme until a "first" substrate molecule A binds and promotes a conformational change in the enzyme that exposes the binding site to the "second" substrate molecule B.

$$E + S \underset{}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$
$$\Big\downarrow +S$$
$$ESS$$

Our data are fully consistent with this kinetic mechanism, called "Ordered Bi Bi" by Cleland [38]. However, the initial velocity data alone can not discriminate between random and ordered mechanisms [39]. Nevertheless, we suggest the reaction to be Ordered Bi Bi, based on the observation that all other glutathione *S*-transferases examined so far follow exclusively a kinetic mechanism of this sort [22, 40]. The observed substrate inhibition was surprising and its relevance to detoxification of the (−)-*anti*-diastereomer of B[c]PhDE is not yet known.

Using (+)-*anti*-B[c]PhDE as substrate, a sigmoidal velocity course of the enzymatic reaction was observed. This indicates, that the binding of a "first" substrate molecule A induces structural or electronic changes that result in altered affinities for the vacant sites. In this case, the velocity curve will no longer follow Henri–Michaelis–Menten kinetics and the enzyme will be classified as an allosteric enzyme. Our experimental data show that the binding of one substrate molecule facilitates the binding of the next substrate molecule by increasing the affinities of the vacant binding sites. This phenomenon has been called "cooperative binding" or "positive cooperativity" with respect to substrate binding. This type of reaction observed with the (+)-*anti*-diastereomer of B[c]PhDE is just the opposite of the reaction observed with the (−)-*anti*-diastereomer. In all likelihood, the multiple substrate binding sites of allosteric enzymes reside in different protein subunits. Only if the sites are identical and independent of each other, does the presence of substrate at one site have no effect on the binding properties of the vacant sites and will yield normal hyperbolic velocity curves. In contrast, our data indicate that each of the two subunits of the GSH transferase HTP II has one (or more) binding site(s) for the substrate (+)-*anti*-B[c]PhDE and that these sites are not independent of each other.

In order to calculate the other kinetic constants of the reaction the Hill equation has been used. It has to be kept in mind that the Hill equation is not a "simplified" velocity equation for allosteric enzymes, but is rather a phenomenologic tool to describe sigmoidal velocity curves. Therefore, the assumption that the Hill coefficient (n or n_{app} or n_H) will equal the number of binding sites, is theoretical. The Hill coefficient does not represent a definitive number of cooperative binding sites but describes the course of a sigmoidal reaction curve relative to other sigmoidal reaction curves. Thus, our calculation of $n_H = 3.4$, the slope of the Hill plot at the point where $V = 0.5\ V_{max}$, and the conclusion that there are at least four sites with relatively strong cooperativity, may not reflect reality. The only certain conclusion derived from our experiments is that each of the subunits of GSH transferase HTP II has at least one binding site for (+)-*anti*-B[c]-PhDE and that these sites are not independent of each other.

Using the (−)-*syn* as well as the (+)-*syn*-diastereomer of B[c]PhDE as substrate, the enzymatic formation of GSH conjugates shows simple Henri–Michaelis–Menten kinetics. The course of the observed conjugation reactions is that expected

for GSH transferases, which are proposed to follow a kinetic mechanism of the sort entitled by Cleland "Ordered Bi Bi" [38]. Although the turnover of the *syn*-diastereomers is not very high (V_{max} = 3.25 min^{-1}) compared to the (−)-*anti*-diastereomer of B[c]PhDE, which has (*R,S,S,R*)-absolute configuration (V_{max} = 103 min^{-1}), it shows that the studied enzyme is not just highly specific for *anti*-diastereomers. The observation of the fairly high turnover of the *syn*-diastereomers of the fjord-region diolepoxides of B[c]Ph by the μ-class GSH transferase HTP II is significantly different from those of simple bay-region dihydrodiolepoxides. The fact, that a turnover of the *syn*-diastereomers of B[c]PhDE takes place may be due to the catalytic properties of the enzyme itself or to the structural and electronic properties of a dihydrodiolepoxide located in a fjord-region. In a recent study, it was shown, that a human GSH transferase π catalyses conjugation with GSH more effectively with the racemic *syn*-dihydrodiolepoxides of chrysen, benzo[*c*]chrysen, dibenz[*a,h*]anthracen and picen compared to the *anti*-dihydrodiolepoxides [41]. No difference in the kinetic course of the catalytic reaction with the (−)-*syn*- and the (+)-*syn*-diastereomer could be observed, indicating that both substrates use the same binding site, or use different and independent binding sites with the same properties.

Using a mixture of (−)-*anti*- and (+)-*anti*-diastereomers as substrate the turnover rates and the kinetic behavior of the single compounds were not changed. No competitive effect could be observed. Both findings suggest that the binding sites of (−)-*anti*- and (+)-*anti*-diastereomers are different and independent of each other. It looks almost as if we are dealing with two different enzymes, but this is really not the case. Analysis of the protein chemistry of the studied enzyme, i.e. chromatofocusing, HPLC, western blotting, peptide mapping, two-dimensional SDS–gel electrophoresis, as presented in Ref. 31 and partly in Fig. 2, clearly shows that it consists of only one absolutely pure enzyme form. A probably too simple explanation may be that the GST isoenzyme HTP II consists of two enzyme forms with different affinities (binding sites) towards the *anti*-diastereomers, but with identical properties regarding their protein chemistry.

Using (−)-*anti*-B[c]PhDE together with either the (−)-*syn*- or (+)-*syn*-diolepoxide as substrates the turnover rate of the (−)-*anti*-diolepoxide was not changed, whereas the turnover rate of both *syn*-diolepoxides was enhanced by a factor of 2. This indicates that the binding of the (−)-*anti*-diolepoxide may change the conformation of the enzyme to a form which facilitates the binding and/or the enzymatic turnover of the *syn*-diolepoxides. Thus, the presence of (−)-*anti*-diolepoxide will enhance the detoxifying enzymatic reaction towards the *syn*-diolepoxides. That the enzymatic turnover of both *syn*-diolepoxides is affected in the same way is a further indication that the *syn*-diolepoxides use the same binding site. In contrast, using (+)-*anti*-B[c]-PhDE together with either the (−)-*syn*- or (+)-*syn*-diolepoxides as substrates, no change in the turnover rates was observed compared to the experiments with individual substrates. This observation is a strong indication that the binding sites of (+)-*anti*- and of the *syn*-diolepoxides are different and independent of each other. Using a mixture of equal amounts of the (−)-*syn*- and (+)-*syn*-diastereomers, the formation of the two different GSH conjugates was increased by a factor of 1.2 ± 0.2. This result is within the range of the detection limit of the used method. This observation is also consistent with the finding that there is no difference in the kinetic behavior of the (−)-*syn*- and (+)-*syn*-diastereomers.

The μ-class GSH transferase 4-4, isolated from rat liver, has been previously found to be the most active of the purified and characterized rat hepatic transferases in the GSH conjugation of (±)-*anti*-B[a]PDE [28]. In this case, GSH transferase 4-4, and all other transferases tested with significant activity, were found to be highly specific for the (+)-enantiomer of *anti*-B[a]PDE, a model compound for bay-region dihydrodiolepoxides with (*R,S,S,R*)-absolute configuration [28]. Studying the enzymatic reaction of GSH transferase 4-4 with the corresponding *syn*-enantiomers, no catalytic turnover was observed (Jernström B, personal communication). In contrast, this study showed that the novel isoenzyme GSH transferase HTP II isolated from rat liver [31] does not express this high degree of enantioselectivity with B[c]PhDE, a model compound for fjord-region dihydrodiolepoxides of PAHs: GSH transferase HTP II is able to detoxify all four different enantiomeric diolepoxides of B[c]-Ph (Table 1). However, GSH conjugation, using the four different stereoisomeric diolepoxides of B[c]Ph as substrates, shows three different enzyme kinetics.

In the case of (−)-*anti*-B[c]PhDE, with (*R,S,S,R*)-absolute configuration, the turnover is remarkably high compared to GSH transferase 4-4 with (+)-*anti*-B[a]PDE (see Table 1). Surprisingly, at higher substrate concentrations, the substrate itself acts as the inhibitor of GSH conjugation. The reason(s) for this substrate inhibition is not clear. In contrast, in the case of the (+)-*anti*-B[c]PhDE, the kinetic behavior follows strictly an allosteric pattern, showing that binding of substrate molecules facilitates the binding of the next substrate molecules, causing an increase in the catalytic turnover. The course of the sigmoidal velocity curve is rather steep, indicating that the kinetic properties of the enzyme act as a switch with an on/off function, depending on the amount of substrate available for the enzyme. The binding sites for (−)-*anti*-, (+)-*anti*- and the common binding site for the *syn*-diastereomers are different and independent of each other. Thus, GSH transferase HTP II is acting like several enzymes. As might be expected, the affinity of GSH transferase HTP II for GSH was similar for all four diastereomers.

The fjord-region diolepoxides of B[c]Ph combine high mutagenic and carcinogenic activity with low chemical reactivity [12]. In the study of Glatt *et al.* [12], the B[c]PhDE differ from the other investigated diolepoxides in that their oxirane ring is located in a fjord-region, which is sterically more constricted than a simple bay-region. The same study showed that the unusual biological activity of B[c]PhDE is to some extent paralleled by other fjord-region diolepoxides [12]. Therefore, the question arises as

Table 1. Apparent kinetic parameters obtained for rat liver GSH transferase HTP II and GSH with the four stereoisomers of B[c]PhDE as the variable substrates

GSH transferase	Diolepoxide	Apparent V_{max} (min^{-1})	Apparent V_{max} (nmol/mg × min)	K_m (mM)	Catalytic efficiency* (sec^{-1} μM^{-1})
HTP II	(−)-*anti*-B[c]PhDE	103.0	1975	1.05	0.0016
HTP II	(+)-*anti*-B[c]PhDE	48.5	930	*K'*†	
HTP II	(−)-*syn*-B[c]PhDE	3.25	62.5	0.20	0.00026
HTP II	(+)-*syn*-B[c]PhDE	3.25	62.5	0.20	0.00026
4-4‡	(±)-*anti*-B[a]PDE	—	560	0.011	0.042

GSH transferase HTP II was incubated with GSH and the four different stereoisomers of B[c]PhDE as substrates. The formed GSH conjugates were measured as described in Materials and Methods.

* Catalytic efficiency derived from V_{max}/K_m.

† *K'* derived from the Hill equation (see Fig. 5).

‡ Data from Robertson and Jernström [28] for comparison. As internal control, these data were reproduced using the method described in Materials and Methods.

to whether the unique GSH conjugation of the four stereoisomers of B[c]PhDE by GSH transferase HTP II is also found with other fjord-region dihydrodiolepoxides. Thus, the study of the GSH conjugation of other fjord-region diolepoxides of PAH by GSH transferase HTP II is an interesting project. However, the results of this study suggest a remarkable role for the novel μ-class isoenzyme GSH transferase HTP II in the control of various genotoxic metabolites, not only of fjord-region diolepoxides of PAH.

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