Specificity of murine glutathione S-transferase isozymes in the glutathione conjugation of (-)-anti- and (+)-syn-stereoisomers of benzo[g]chrysene 11,12-diol 13,14-epoxide

Ajai Pal, Albrecht Seidel1,2, Hong Xia, Xun Hu, Sanjay K. Srivastava, Franz Oesch1 and Shivendra V. Singh3

Cancer Research Laboratory, Mercy Cancer Institute, Mercy Hospital of Pittsburgh, Pittsburgh, PA 15219, USA and 1Institute of Toxicology, University of Mainz, Obere Zahlbacher Strasse 67, D-55131, Mainz, Germany

1Present address: Biochemical Institute for Environmental Carcinogens, Prof. Dr Gernot Grimmer Foundation, Lurup 4, D-22927 Grosshansdorf, Germany
2To whom correspondence should be addressed
Email: ssingh@mercy.pmhs.org

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants and known to produce tumors at various sites in laboratory animals (1–4). Moreover, these compounds are suspected human carcinogens (4). The tumorigenicity of PAHs is predominantly linked to the formation of diol epoxides, which are generated through cytochrome P450- and microsomal epoxide hydrolase-mediated activation of the parent hydrocarbons (2,3). The diol epoxides implicated as the active metabolites of PAH carcinogens generally have the epoxide function in a bay or a fjord region (5). The diol epoxides of both bay and fjord region classes exist as a pair of optical enantiomers [(+)- and (–)-enantiomer] of two diastereomers (anti- and syn-isomers) (3,5). In the case of bay region diol epoxides, e.g. benzo[a]pyrene 7,8-diol 9,10-epoxide (BPDE), the stereoisomer with (7R,8S)-diol (9S,10R)-epoxide absolute configuration [(+)-anti-BPDE] is significantly more tumorigenic than the other three stereoisomers [refer to Figure 1 for structure and absolute configuration of (+)-anti-BPDE (6,7). For fjord region diol epoxides, e.g. benzo[g]chrysene 11,12-diol 13,14-epoxide (B[g]CDE), both the (–)-anti-stereoisomer with the (11R,12S)-diol (13S,14R)-epoxide absolute configuration and the (+)-syn-stereoisomer with the (11S,12R)-diol (13S,14R)-epoxide absolute configuration (Figure 1) possess high tumorigenic activity (8–10). Moreover, the diol epoxides of the fjord region class are relatively more potent carcinogens than corresponding bay region compounds. For example, it has been demonstrated that racemic anti-B[g]CDE, the activated form of the environmental pollutant benzo[g]chrysene (B[g]C), is a far more potent tumor initiator than anti-BPDE (11). While covalent interaction of the PAH diol epoxides with nucleophilic centers in DNA is a critical event in PAH-induced tumorigenesis (1,3), glutathione (GSH) S-transferases (GSTs) play an important role in their cellular detoxification (12–14).

GSTs are a superfamily of isozymes that catalyze the GSH conjugation of a wide variety of electrophilic compounds, generally leading to their detoxification (15,16). The cytosolic GST activity in mammalian tissues is often due to multiple isozymes, grouped into four major classes, α, μ, π and θ, which exhibit overlapping but distinct substrate specificities (15–18). For example, the α class human and rat GSTs are poor in catalyzing the GSH conjugation of (+)-anti-BPDE (12), which is the activated form of benzo[a]pyrene (BP) (3).

Recently, we have identified a novel α class GST isozyme in tissues of female A/J mouse (previously designated GST 9.5), which is between 9- and 625-fold more efficient than other murine GSTs in the GSH conjugation of (+)-anti-BPDE (19). More recently, we have cloned the cDNAs for both the subunits of GST 9.5 and demonstrated that this isozyme is composed of A1 and A2 type murine GST subunits and that the A1 type subunit is responsible for its exceptional activity towards (+)-anti-BPDE (20). However, the specificity of murine GSTs, especially mGSTA1-1, in catalyzing the GSH conjugation of fjord region diol epoxides is poorly characterized.

In the present study we have compared catalytic efficiencies of murine GSTs in the GSH conjugation of (–)-anti- and (+)-syn-stereoisomers of B[g]CDE, the two stereoisomers to which the moderate carcinogen B[g]C is metabolically activated in mouse skin (21). The results of the present study indicate that murine GSTs significantly differ in their ability to detoxify
Materials and methods

Female A/J mice (~8 weeks old) were purchased from the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). Reagents including GSH and epoxy-activated Sepharose 6B were purchased from Sigma (St Louis, MO). Individual enantiomers of B[**g**]CDE were prepared from optically active B[**g**]CDE stereoisomers and that mGSTA1-1 is significantly more efficient than other murine GSTs towards both (**-)**- and (++-)**-syn-B[**g**]CDE. In conclusion, our results indicate that mGSTA1-1 plays a major role in the detoxification of both bay region and fjord region PAH diol epoxides.

**Purification of GST isozymes**

Previous studies from our laboratory have shown that >90% of total GST activity in various organs of female A/J mouse is due to mGSTA1-1, mGSTA2-2, mGSTA3-3, mGSTA4-4, mGSTM1-1 and/or mGSTP1-1 (20,23). We therefore selected the above isozymes for their kinetic characterization towards B[**g**]CDE stereoisomers. Recombinant mGSTA1-1 and mGSTA2-2 were expressed and purified by GSH affinity chromatography as described previously (20). Recombinant mGSTA4-4 was a generous gift from Dr Piotr Zimniak (University of Arkansas for Medical Sciences, Little Rock, AR). Other murine GST isozymes were purified from the liver of female A/J mice using a protocol that involves GSH affinity chromatography followed by chromatofocusing, as described previously (23). The GSH affinity chromatography was performed by the method of Simons and Vander Jagt (24) with some modifications described previously (25). Protein content was determined by the Bradford method (26). The homogeneity and classification of the above GST isozymes was ascertained by reverse phase HPLC and western blot analysis, respectively, as described previously (23).

**Determination of GST activity towards B[**g**]CDE stereoisomers**

The purified GST isozymes were dialyzed against 50 mM Tris–HCl (pH 7.5) containing 2.5 mM KCl and 0.5 mM EDTA (TKE buffer) and stored at −20°C until used. The activity of each isozyme towards the model substrate 1-chloro-2,4-dinitrobenzene was determined (27) prior to activity measurements towards B[**g**]CDE stereoisomers. The reaction mixture in a final volume of 0.1 ml contained TKE buffer, 2 mM GSH and the desired concentrations of the B[**g**]CDE stereoisomer (10–320 µM) and GST isoyme protein. The GST activity was determined as a function of varying (–)**-anti-B[**g**]CDE concentration (10–320 µM) at a fixed saturating concentration of GSH (2 mM), each isozyme obeyed Michaelis–Menten kinetics (plots not shown). As shown in Table I, the α class isozyme mGSTA1-1 was significantly more efficient than other murine GSTs in the GSH conjugation of (–)**-anti-B[**g**]CDE. For example, the catalytic efficiency of mGSTA1-1 towards (–)**-anti-B[**g**]CDE was ~6.4-, 16.6- and 7.5-fold higher compared with mGSTA3-3, mGSTP1-1 and mGSTM1-1, respectively. This was mainly due to a relatively higher Vmax (~2.6- to 4.4-fold higher) and considerably lower Km (~60–71% lower) for mGSTA1-1 than for mGSTA3-3, mGSTP1-1 and mGSTM1-1. Even though the Vmax for mGSTA1-1 was slightly lower than that for mGSTA2-2, the former isozyme was expressed and purified by GSH affinity chromatography as described previously (23). The GSH affinity chromatography was performed by the method of Simons and Vander Jagt (24) with some modifications described previously (25). Protein content was determined by the Bradford method (26). The homogeneity and classification of the above GST isozymes was ascertained by reverse phase HPLC and western blot analysis, respectively, as described previously (23).

**Results and discussion**

Non-enzymatic (spontaneous) GSH conjugation of (–)**-anti-B[**g**]CDE was not detectable. On the other hand, this reaction was accelerated to varying degrees in the presence of different GST isozymes. When GST activity was determined as a function of varying (–)**-anti-B[**g**]CDE concentration (10–320 µM) at a fixed saturating concentration of GSH (2 mM), each isozyme obeyed Michaelis–Menten kinetics (plots not shown). As shown in Table I, the α class isozyme mGSTA1-1 was significantly more efficient than other murine GSTs in the GSH conjugation of (–)**-anti-B[**g**]CDE. For example, the catalytic efficiency of mGSTA1-1 towards (–)**-anti-B[**g**]CDE was ~6.4-, 16.6- and 7.5-fold higher compared with mGSTA3-3, mGSTP1-1 and mGSTM1-1, respectively. This was mainly due to a relatively higher Vmax (~2.6- to 4.4-fold higher) and considerably lower Km (~60–71% lower) for mGSTA1-1 than for mGSTA3-3, mGSTP1-1 and mGSTM1-1. Even though the Vmax for mGSTA1-1 was slightly lower than that for mGSTA2-2, the former isozyme was
Table I. Kinetic constants for murine GSTs towards (–)-anti- and (+)-syn-B[\(\gamma\)]CDE stereoisomers [values for (+)-syn-B[\(\gamma\)]CDE are given in parentheses]

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Class</th>
<th>(V_{\text{max}}) (nmol/min/mg)</th>
<th>(K_{\text{m}}) ((\mu)M)</th>
<th>(k_{\text{cat}}) (per s)</th>
<th>Catalytic efficiency (k_{\text{cat}}/K_{\text{m}}) (per mM/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGSTA1-1</td>
<td>(\alpha)</td>
<td>61 ± 4(b) (159 ± 10)</td>
<td>6 ± 3 (15 ± 4)</td>
<td>0.05 (0.13)</td>
<td>8.3 (8.7)</td>
</tr>
<tr>
<td>mGSTA2-2</td>
<td>(\alpha)</td>
<td>69 ± 7 (180 ± 18)</td>
<td>14 ± 6 (47 ± 17(b))</td>
<td>0.05 (0.15)</td>
<td>3.6 (3.2)</td>
</tr>
<tr>
<td>mGSTA3-3</td>
<td>(\alpha)</td>
<td>24 (35)</td>
<td>14 (26)</td>
<td>0.02 (0.03)</td>
<td>1.4 (1.2)</td>
</tr>
<tr>
<td>mGSTA4-4</td>
<td>(\alpha)</td>
<td>nd(b) (nd)</td>
<td>16 (22)</td>
<td>0.02 (0.03)</td>
<td>1.2 (1.4)</td>
</tr>
<tr>
<td>mGSTP1-1</td>
<td>(\pi)</td>
<td>14 ± 2(b) (54 ± 7)</td>
<td>21 ± 11 (20 ± 12)</td>
<td>0.01 (0.04)</td>
<td>0.5 (2.0)</td>
</tr>
<tr>
<td>mGSTM1-1</td>
<td>(\mu)</td>
<td>26 ± 2(b) (31 ± 4(b))</td>
<td>18 ± 8(b) (44 ± 20)</td>
<td>0.02 (0.03)</td>
<td>1.1 (0.7)</td>
</tr>
</tbody>
</table>

\(a\)Data are means ± SD of three independent experiments except for mGSTA3-3, where \(n = 2\); both values for mGSTA3-3 are given.

\(b\)Significantly different from mGSTA1-1; \(P < 0.05\).

\(c\)Activity not detected.

The results of the present study reveal that the \(\alpha\) class murine GSTs markedly differ in their ability to detoxify B[\(\gamma\)]CDE. For example, \(\alpha\) class isozyme mGSTA4-4, which shares ~59% amino acid sequence identity with mGSTA1-1 (20,31), is virtually inactive towards both the (–)-anti- and (+)-syn-B[\(\gamma\)]CDE stereoisomers. Even though mGSTA2-2 and mGSTA3-3, which respectively share ~96 and 69% amino acid sequence identities with mGSTA1-1 (20,32,33), can catalyze the GSH conjugation of both (–)-anti- and (+)-syn-B[\(\gamma\)]CDE, their catalytic efficiencies are significantly lower compared with mGSTA1-1. These observations, taken together with the results of previous studies, suggest that \(\alpha\) class murine GSTs may have a unique role in the detoxification of xenobiotics. For example, while mGSTA1-1 seems to be important in the detoxification of PAH diol epoxides (20; present study), this isozyme may have a limited role in cellular protection against 4-hydroxynonenal, which is the toxic end product of lipid peroxidation. Instead, the majority of the GST-mediated detoxification of 4-hydroxynonenal may be carried out by mGSTA4-4, as the specific activity of this isozyme towards 4-hydroxynonenal (55 \(\mu\)mol/min/mg protein) is ~162-, 83- and 50-fold higher compared with mGSTA1-1, mGSTA2-2 and mGSTA3-3, respectively (16,20). On the other hand, mGSTA3-3 may play a major role in cellular protection against other oxidative products such as lipid hydroperoxides (16). For example, the specific activity of mGSTA3-3 towards cumene hydroperoxide (12 \(\mu\)mol/min/mg protein) is ~9-, 4- and 17-fold higher compared with mGSTA1-1, mGSTA2-2 and mGSTA4-4, respectively (16,20).

Previous studies have shown that the diol epoxides of the fjord region class are relatively more potent mutagens in bacterial and mammalian cells (34) and more potent carcinogens in animal models than corresponding diol epoxides of the bay region class (6–11). For example, it has been shown that racemic anti-B[\(\gamma\)]CDE is a far more potent tumor initiator compared with anti-BPDE (11). The results of the present study, taken together with our previous studies, reveal that the catalytic efficiencies of murine GSTs is significantly lower towards B[\(\gamma\)]CDE stereoisomers (present study) compared with corresponding bay region diol epoxides (20). For example, the catalytic efficiency of mGSTA1-1 towards anti-BPDE stereoisomer with the \(\{(\gamma,\delta)\}\)-diol (9S,10R)-epoxide absolute configuration \([(\gamma)-anti-stereoisomer] (131 mM/s; 20) is ~16-fold higher than towards the B[\(\gamma\)]CDE stereoisomer with a similar absolute configuration \([(\gamma)-anti-B[\(\gamma\)]CDE] (present study). Likewise, the catalytic efficiencies of mGSTA2-2 and mGSTP1-1 towards (–)-anti-BPDE are significantly higher.

~2.3-fold more efficient than mGSTA2-2 in the GSH conjugation of (–)-anti-B[\(\gamma\)]CDE. mGSTA4-4, which also belongs to class \(\alpha\), was virtually inactive towards this substrate.

Similarly to (–)-anti-B[\(\gamma\)]CDE, non-enzymatic conjugation of the (+)-syn-epimer of B[\(\gamma\)]CDE with GSH could not be detected. Adherence to Michaelis–Menten kinetics was also observed for each isozyme when GST activity was measured as a function of varying (+)-syn-B[\(\gamma\)]CDE concentration (10–320 \(\mu\)M) at 2 mM GSH concentration (plots not shown). The kinetic constants for murine GSTs in the GSH conjugation of (+)-syn-B[\(\gamma\)]CDE are also summarized in Table I (parentheses). Similarly to the (–)-anti-epimer, mGSTA1-1 was significantly more efficient (~2.7- to 12.4-fold) than other murine GSTs in the GSH conjugation of (+)-syn-B[\(\gamma\)]CDE. While catalytic efficiencies for \(\alpha\) class isozymes mGSTA1-1, mGSTA2-2 and mGSTA3-3 towards (–)-anti- and (+)-syn-B[\(\gamma\)]CDE were comparable, mGSTP1-1 was 4-fold more efficient towards (+)-syn-stereoisomer than towards (–)-anti-B[\(\gamma\)]CDE (Table I). On the other hand, mGSTM1-1 was ~1.6-fold more efficient towards (–)-anti-B[\(\gamma\)]CDE compared with the (+)-syn-stereoisomer.

The results of the present study indicate that mGSTA1-1 is significantly more efficient than other classes of murine GSTs, including those of class \(\alpha\), in the GSH conjugation of both the (–)-anti- and (+)-syn-stereoisomers of B[\(\gamma\)]CDE, which are the activated metabolites of the environmental pollutant B[\(\gamma\)]C (21,30). The catalytic efficiency of mGSTA1-1 towards B[\(\gamma\)]CDE stereoisomers is ~2.3- to 16.6-fold higher than those of other murine GSTs examined in the present study. We have shown previously that mGSTA1-1 is ~3- to 655-fold more effective than mGSTA2-2, mGSTA3-3, mGSTA4-4, mGSTP1-1 and mGSTM1-1 in catalyzing the GSH conjugation of bay region diol epoxide (–)-anti-BPDE (20), another environmentally relevant PAH diol epoxide (1–4). Taken together, these observations suggest that mGSTA1-1 may play a major role in the GSH conjugation (detoxification) of not only bay region compounds but also the PAH diol epoxides of the fjord region class (e.g. B[\(\gamma\)]CDE).

Previous studies with human GST isozymes have also demonstrated that the catalytic efficiency of hGSTA1-1 is between 1.3- and 5.9-fold higher compared with hGSTM1-1 and hGSTP1-1 towards the (–)-anti- and (+)-syn-B[\(\gamma\)]CDE stereoisomers (13). Unlike mouse GSTA1-1, however, human GSTA1-1 is relatively more effective towards the (+)-syn-B[\(\gamma\)]CDE compared with the (–)-anti-epimer of B[\(\gamma\)]CDE (13). Mouse GSTA1-1 is equally efficient in the GSH conjugation of the (–)-anti- and (+)-syn-B[\(\gamma\)]CDE isomers (Table I).
compared with the corresponding enantiomer of B[\(g\)]CDE (20: present study). On the other hand, some isoforms, such as mGSTA3-3 and mGSTM1-1, exhibit comparable catalytic efficiency towards both above diol epoxides. These observations suggest that relatively higher carcinogenic potency of fjord region compounds compared with diol epoxides of the bay region class may, at least in part, be due to a relatively lower capacity of GSTs to detoxify the former compounds. On the other hand, differences in the DNA adduction profile for bay region and fjord region diol epoxides may also be important in their differential carcinogenic potency. For example, it has been shown that while guanine residues in DNA are the principal site of reaction for bay region dihydrodiol epoxides (e.g. anti-B[\(g\)]-BPDE), both adenine and guanine residues are modified by the dihydrodiol epoxides of the fjord region class, such as anti-B[\(g\)]CDE (35–37).

In conclusion, the results of the present study indicate that murine GSTs, particularly the isoforms of class \(\alpha\), significantly differ in their ability to detoxify B[\(g\)]CDE stereoisomers and that the \(\alpha\) class isoforms mGSTA1-1 is significantly more efficient than other classes of murine GSTs in the GSH conjugation of both stereoisomers \([\text{anti-}]+\text{(+)-syn-}]\) of B[\(g\)]CDE. Thus, it seems reasonable to conclude that mGSTA1-

\(\alpha\) plays a major role in the detoxification of both bay region and fjord region PAH diol epoxides.

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