Inactivation of a Diol-Epoxide and a K-Region Epoxide with High Efficiency by Glutathione Transferase X

Hansruedi Glatt, Thomas Friedberg, Philip L. Grover, Peter Sims, and Franz Oesch

Institute of Pharmacology of the University, Obere Zahlbacher Strasse 67, 0-6500 Mainz, Germany [H. G., T. F., F. O.], and Chester Beatty Research Institute, Fulham Road, London, SW3 6JB, England [P. L. G., P. S.]

ABSTRACT

Four glutathione transferases (EC 2.5.1.18), glutathione transferases A, B, and C and a hitherto unknown form, termed X, were purified to apparent homogeneity from rat liver cytosol. They were investigated for their abilities to inactivate two mutagenic epoxides derived from the polycyclic aromatic hydrocarbon benz(a)anthracene, the K-region epoxide benz(a)anthracene 5,6-oxide and the diol-epoxide r-8,9-dihydroxyf-10,11-oxyl-8,9,10,11-tetrahydrobenz(e)anthracene. Mutagenic activity was determined using Salmonella typhimurium his" strain TA100. Glutathione alone had little if any influence on the mutagenicity of the diol-epoxide but significantly decreased the mutagenic effect of the K-region epoxide. This inactivation was enhanced by the addition of glutathione transferases. Both epoxides were inactivated by glutathione in the presence of each of the four enzymes, but with varying efficiencies. Inactivation of the K-region epoxide (in terms of its mutagenicity in the presence of glutathione) required extremely little enzyme, about 1000 times less than for the diol-epoxide. On a molar basis, glutathione transferase X (followed by C > A ≥ B) was clearly the most efficient enzyme in inactivating both substrates and also more efficient than were three other purified enzymes (micromosomal epoxide hydrolase, cytosolic epoxide hydrolase, and dihydrodiol dehydrogenase) previously investigated in this test system. Taking into account the amounts of enzyme present in rat liver, the glutathione transferases C and X were most effective in inactivating the epoxides examined. Thus, the newly discovered glutathione transferase X appears to be of substantial significance in the inactivation of two structural prototypes of epoxides derived from polycyclic aromatic hydrocarbons, a K-region epoxide and a non-bay-region vicinal diol-epoxide.

INTRODUCTION

While the role of drug-metabolizing enzymes in the activation of carcinogens and mutagens has been investigated intensively in the past 2 decades, much less is known about the enzymes involved in their inactivation, and practically nothing is known about the part played by individual enzyme forms in these inactivation processes. This may, however, be of considerable importance, because different enzyme forms can often distinguish between closely related chemical compounds and can therefore play a pivotal role in the control of mutagenic, carcinogenic, and cytotoxic effects. This is particularly true of the glutathione transferases, a group of enzymes that catalyze the conjugation of a wide variety of electrophiles with glutathione (6, 19). Because typical mutagens and tumor-initiating agents are electrophilically reactive, glutathione transferases are capable of playing an inactivating role with many potential mutagens and carcinogens.

In rat liver cytosol, at least 9 different glutathione transferases can be distinguished. The forms AA, A, B, C, D, and E are named in the reverse order of their elution from a carboxymethylcellulose column (19). Ligandin, earlier considered to be identical with Form B, was so termed after its ability to bind organic anions [a property which it, however, shares with other glutathione transferases (21)]. An eighth form has been named glutathione transferase M because of its ability to react with menaphthyl sulfate (13). We have recently discovered and purified an additional enzyme with distinct properties, which we have termed glutathione transferase X (10, 12). It is a homodimer as are the glutathione transferases AA, A, and ligandin. Combination of one subunit from glutathione transferase A and one subunit from glutathione transferase X yields glutathione transferase C (10), analogous with the fact that glutathione transferase B is the heterodimer derived from glutathione transferase AA and ligandin (21, 26).

The new Form X and the glutathione transferases A, B, and C, which are known to be abundant forms in rat liver (24), have been purified to homogeneity and investigated for their abilities to inactivate 2 mutagens, BA 5,6-oxide3 and BA-8,9-diol-10,11-oxide. These epoxides are representative of 2 important classes of polycyclic aromatic hydrocarbon metabolites, K-region epoxides and vicinal diol-epoxides. Although K-region epoxides are potent mutagens in bacteria (2, 17), in mammalian systems they are less active (23, 29, 33, 39, 43) and appear to contribute little to the total DNA binding that results from metabolic activation in mammalian cells (5, 38). This apparent discrepancy may be due to the rapid detoxication of K-region epoxides by epoxide hydrolases (4, 25) and by glutathione transferases (3, 32, 41), enzymes that occur in all mammalian tissues and cells thus far investigated (27, 35, 36) and in most subcellular compartments (11, 20, 40).

Vicinal diol-epoxides seem to be responsible for most of the DNA-binding, carcinogenic, and mutagenic effects that are induced by polycyclic aromatic hydrocarbons, but relatively little is known about their inactivation. They appear to be poor substrates for epoxide hydrolases (15, 44, 45), but mutagenicity and DNA-binding experiments that have been carried out using trans-7,8-dihydro-7,8-dihydroxybenzo(a)pyrene and an activating system have shown that some inactivation occurs when glutathione is added (14, 16, 22). With r-7,8-dihydroxyf-9,10-oxide3, 7,8,9,10-

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1 This work was supported by the Deutsche Forschungsgemeinschaft and by grants to the Chester Beatty Research Institute, Institute of Cancer Research; Royal Cancer Hospital from the Medical Research Council; and the Cancer Research Campaign.

2 To whom requests for reprints should be addressed.

Received January 17, 1983; accepted August 25, 1983.

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3 The abbreviations used are: BA 5,6-oxide, (±)-benz(a)anthracene 5,6-oxide; BA-8,9-diol-10,11-oxide, (±)-r-8,9-dihydroxyf-10,11-oxyl-8,9,10,11-tetrahydrobenz(e)anthracene.
tetrahydrobenzo(a)pyrene and BA-8,9-diol-10,11-oxide, glutathione conjugates have been found to be formed in enzyme-catalyzed reactions (7,8). Bioactivated benzo(a)pyrene (18) and BA-8,9-diol-10,11-oxide (15) can also be inactivated by apparently homogeneous dihydrodiol dehydrogenase (42), although it will be shown in this report that, both on a molar and on a tissue-equivalent basis, glutathione transferase X is more efficient in the inactivation of the diol-epoxide than is dihydrodiol dehydrogenase. One reason why so little is known of this important class of reactive metabolites is that many of them have a short half-life in an aqueous environment. Low stability is not, however, a property of all vicinal diol-epoxides. BA-8,9-diol-10,11-oxide, which is a non-bay-region diol-epoxide, has a half-life of 75 hr (15) and is therefore useful for metabolic studies. In addition to serving as a model for less stable diol-epoxides, its own metabolism is of interest, since it is mutagenic (15,31,45) and may be the major DNA-binding species formed from benzo(a)anthracene in vivo and in vitro (9,30).

**MATERIALS AND METHODS**

**Chemicals.** BA-8,9-diol-10,11-oxide and BA 5,6-oxide were synthesized as described (28,34), crystallized, and re-crystallized, and their purity was examined by chromatography (37). Glutathione, which was obtained from Serva (Heidelberg, Federal Republic of Germany), was dissolved in H2O immediately before use, and the solution was adjusted to pH 7.4 with NaOH.

**Enzymes.** The glutathione transferases A, B, and C and a form that is distinguishable from all described forms, and which has been provisionally termed glutathione transferase X, were purified from liver cytosol of control rats and of rats that had been pretreated with 2 i.p. injections of Aroclor 1254 (400 mg/kg body weight) 14 and 7 days, respectively, before killing. A detailed report on the purification and characterization of the enzymes has been published elsewhere (12). The purity of the preparations was established by isoelectric focusing and by sodium dodecyl sulfate-gel electrophoresis, where single protein bands were obtained with all 4 glutathione transferases except B, which showed, in agreement with the literature (19,26), 2 subunits with molecular weights of 22,000 and 25,000 by sodium dodecyl sulfate-gel electrophoresis. The enzymes purified from animals induced with Aroclor 1254 were not distinguishable from the glutathione transferases purified from control animals by Ouchterlony double immunodiffusion tests, by immunoelectrophoresis, and by isoelectric focusing in analytical agarose slab gels.

The glutathione transferase assays were performed as described (19) at 25° except for the assay with menaphthyl sulfate which was carried out at 37° as described by Gilham (13).

The isoelectric points, some catalytic properties, and the subunit composition of the purified enzymes are shown in Table 1.

**Mutagenicity Assay.** Mutagenicity in Salmonella typhimurium TA100 (1) was studied as described (15) with the modification that albumin (0.4 mg) was added to each incubation in order to protect the diluted enzyme. Briefly, the test compound was preincubated for 20 min with the metabolizing system, before the bacteria were added. After a further incubation for 20 min in the presence of bacteria, the mixture was added to histidine-poor agar plates. Revertant colonies were counted after incubation for 3 days at 37°.

**RESULTS**

Chart 1 shows the mutagenicity of the epoxides as a function of their concentration in experiments that were carried out without the addition of enzymes. The experiments were performed according to the standard protocol (see "Materials and Methods"), i.e., preincubating the epoxide in the test solution before addition of the bacteria. Simultaneous addition of test compound and bacteria led to virtually the same mutagenicity (data not shown) indicating that the test compounds were chemically stable under the test conditions used and in the absence of glutathione and enzymes. Chart 1 also shows the effect of the presence of a constant concentration of glutathione (2 mM) at various concentrations of the mutagens. Results of experiments carried out with constant concentrations of the mutagens and variable concentrations of glutathione are presented in Chart 2. These experiments demonstrated a marked difference between the 2 epoxides. Glutathione clearly reduced the mutagenicity of the K-region epoxide but had little if any effect upon that of the diol-epoxide.

In order to study the effects of the purified enzymes, we used the test compounds at concentrations where the concentration-mutagenicity curve was approximately linear. When the epoxides were incubated in the absence of glutathione but in the presence of increasing amounts of glutathione transferases A, C, and X, the mutagenicity of the epoxides was not significantly decreased (Chart 3) which indicates that covalent reaction of these epoxides with the proteins of the enzymes does not occur to any appreciable extent. When glutathione was added, the glutathione...
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Chart 2. Effect of varying the concentration of glutathione, in the absence of enzymes, on the mutagenicity of BA 5,6-oxide (O) and of BA-8,9-diol-10,11-oxide (C). The number of mutants above solvent control that were induced by 1 µg of BA 5,6-oxide or by 3 µg of BA-8,9-diol-10,11-oxide in the presence of glutathione is expressed as the percentage of the corresponding value obtained in the absence of glutathione. The absolute numbers of colonies obtained in the absence of glutathione were 84 for the solvent control, 320 for BA 5,6-oxide, and 1,250 for BA-8,9-diol-10,11-oxide. Triplicate incubations were performed, and the variation in the numbers of colonies on replicate plates was less than 10%.

Chart 3. Effect of different forms of glutathione transferase (GST), purified from untreated rats, on the mutagenicity of BA 5,6-oxide in the presence (O) and absence (C) of glutathione (2 µm) and of BA-8,9-diol-10,11-oxide in the presence (C) and absence (O) of glutathione (2 µm). The number of mutants above solvent control induced by 1 µg of BA 5,6-oxide or by 3 µg of BA-8,9-diol-10,11-oxide in the presence of various amounts of the purified glutathione transferases are expressed as the percentage of the corresponding value obtained in the absence of enzyme. The absolute numbers of colonies obtained in the absence of enzyme were 74 and 65 for the solvent control, 730 and 1,040 for BA 5,6-oxide, and 1,350 and 1,540 for BA-8,9-diol-10,11-oxide, in the presence and absence of glutathione, respectively. Triplicate incubations were performed. The variation in the numbers of colonies on replicate plates was less than 10%.

transfases A, C, and X inactivated the K-region epoxide and the diol-epoxide (Chart 3). In addition, about 1,000-fold higher concentrations of glutathione transferase were required for inactivation of the diol-epoxide than for inactivation of the K-region epoxide, and this was independent of the enzyme form used. These similarities in the ratio of inactivation of the 2 epoxides by the 3 enzymes were remarkable because the enzymes differed substantially from each other in the efficiency with which they inactivated the epoxides. Form X was much more efficient than Form C, which in turn was clearly more active than Form A. In order to verify this striking finding, we repeated the experiment using different preparations of purified enzymes (Chart 4). Moreover, in contrast to the previous experiment, these enzymes were purified from the livers of Aroclor 1254-treated animals, in which the enzymes are present in higher concentrations, and glutathione transferase B was also included in these mutagenicity experiments. The experiment confirmed the difference in inactivation of the 2 epoxides by the glutathione transferases A, C, and X and their relative efficiencies. Glutathione transferase B also inactivated the K-region epoxide more efficiently than it inactivated the diol-epoxide; its activity towards the diol-epoxide was similar to that of Enzyme A, which was the least active of the other glutathione transferases, and its efficiency towards the K-region epoxide was even lower (Chart 4). This low efficiency of glutathione transferase B was confirmed in a repeat experiment with a separate preparation of this enzyme (data not shown).

Table 2 summarizes the results obtained in comparisons of the amounts of enzyme required for a 50% reduction in the mutagenicity with the amounts of enzyme present in the liver. Results that were previously obtained with microsomal and cytosolic epoxide hydrolase and with dihydrodiol dehydrogenase (15) have also been included. The data show that, when intrinsic enzyme activities and the relative amounts of enzymes present in the liver are considered (but disregarding subcellular compartmentalization, see "Discussion"), the glutathione transferases can play a more important role in the inactivation of BA 5,6-oxide and of BA-8,9-diol-10,11-oxide than do the epoxide hydrolases and dihydrodiol dehydrogenase. In rat liver, Forms C and X appear to be able to contribute most to the inactivation of the 2 epoxides examined here, Form C because of its quantitative abundance and Form X because it is able to inactivate these epoxides more efficiently.

DISCUSSION

In this study, we have shown that the mutagens BA 5,6-oxide and BA-8,9-diol-10,11-oxide are inactivated by each of the 3 major rat liver glutathione transferases, enzymes A, B, and C, as well as by a newly found enzyme, called glutathione transfer-
Table 2
Quantitative comparison of the inactivation of BA 5,6-oxide and BA-8,9-diol-10,11-oxide by different enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>BA 5,6-oxide</th>
<th>BA-8,9-diol-10,11-oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/incubation</td>
<td>mg liver equivalents</td>
</tr>
<tr>
<td>Experiment shown in Chart 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione transferase A</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Glutathione transferase C</td>
<td>1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Glutathione transferase X</td>
<td>0.25</td>
<td>&lt;=0.01b</td>
</tr>
<tr>
<td>Experiment shown in Chart 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione transferase A</td>
<td>0.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Glutathione transferase B</td>
<td>2.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Glutathione transferase C</td>
<td>1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Glutathione transferase X</td>
<td>0.25</td>
<td>0.003</td>
</tr>
<tr>
<td>Experiments using other enzymesc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal epoxide hydrolase</td>
<td>0.5</td>
<td>0.7</td>
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<tr>
<td>Cytosolic epoxide hydrolase</td>
<td>0.16</td>
<td>4</td>
</tr>
<tr>
<td>Dihydriodiol dehydrogenase</td>
<td>0.45</td>
<td>Inactive (170) &lt;&lt;1000</td>
</tr>
</tbody>
</table>

* Values refer to untreated, adult males of the species from which the enzyme was purified.

b Determinable only as an upper limit from the experiment.

c Data taken from Ref. 15. These experiments were performed in the same way as those using glutathione transferases.

use X. Interestingly, the K-region oxide was appreciably inactivated by glutathione even in the absence of enzyme, and very small amounts of enzyme strongly enhanced this inactivation. In contrast, inactivation of the diol-epoxide was negligible with glutathione alone and required relatively large amounts of enzyme. When taken together with the observation that other potential inactivation mechanisms such as those involving epoxide hydrolases and dihydriodiol dehydrogenase are efficient towards the K-region epoxides but are less active towards the diol-epoxide (15), this may explain why BA-8,9-diol-10,11-oxide wards the K-region epoxides but are less active towards the glutathione alone and required relatively large amounts of enzyme. In contrast, inactivation of the diol-epoxide was negligible with small amounts of enzyme strongly enhanced this inactivation. In this regard, however, it was surprising that less than one-half the amount of Form X, compared to Form C, was required in each experiment for equivalent detoxification. It is noteworthy that the relative efficiency of the different glutathione transferases was similar (X > C > A ≥ B) with both substrates in spite of the structural differences in the epoxides and the large differences (about 1000-fold) in the amount of enzyme required for the inactivation of the 2 epoxides. This suggests that glutathione transferase X could also be particularly efficient for the detoxification of other epoxides derived from polycyclic aromatic hydrocarbons.

K-region epoxides and diol-epoxides are substrates not only for glutathione transferases but also for more specialized enzymes. K-region epoxides for epoxide hydrolases (4, 25) and diol-epoxides for dihydriodiol dehydrogenase (15). In order to obtain a first approximation of the relative roles of the different enzymes, we have calculated the quantity of liver tissue that contains the amount of enzyme required for a 50% reduction in the mutagenicity of the epoxides (Table 2). Obviously, such an estimate is very crude when different types of enzymes are compared, because differences in cofactor concentration, in pH optima, and in other environmental factors may lead to substantial differences between enzyme activity both in vivo and under our experimental conditions. For example, microsomal epoxide hydrolase was tested in the mutagenicity experiments as the free pure enzyme, whereas in vivo it is situated in the endoplasmic reticulum and in other membranes (40). This may be of advantage in comparison with cytosolic enzymes, since it increases the opportunities for reaction with epoxides, which are themselves generated in these membranes and tend to stay there because of their relative lipophilicity (16). In the case of dihydriodiol dehydrogenase, one should bear in mind that it may not only inactivate diol-epoxides but may also sequester precursor dihydriodiol, an effect that has not been taken into account in the experimental model used. In spite of these limitations, the data presented in this paper on the more efficient in vivo inactivation of epoxides by glutathione transferases than by the other enzymes indicate (a) that it is quite likely that glutathione transferases play an important role in the in vivo inactivation of epoxides derived from polycyclic aromatic hydrocarbons and (b) that, at least for some of these epoxides, glutathione transferase X is of significance even in tissues where it is a minor form, due to its high efficiency.

ACKNOWLEDGMENTS

We thank Dr. B. N. Ames for a gift of S. typhimurium TA100 and U. Brewi for excellent technical assistance.

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