Reversible Conjugation of Ethacrynic Acid with Glutathione and Human Glutathione S-Transferase P1-1

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ABSTRACT

The reversibility of the conjugation reaction of the diuretic drug ethacrynic acid (EA), an α,β-unsaturated ketone, with glutathione and glutathione S-transferase P1-1 (GST P1-1) has been studied. When the glutathione conjugate of EA was incubated with a 5-fold molar excess of N-acetyl-L-cysteine or GST P1-1, a time-dependent transfer of EA to N-acetyl-cysteine or GST P1-1 was observed. With increasing pH, the pseudo first order rate constants of transfer of EA to N-acetyl-cysteine increased from 0.010 h⁻¹ (pH 6.4) to 0.040 h⁻¹ (pH 7.4) and 0.076 h⁻¹ (pH 8.4).

From the fact that preincubation of GST P1-1 with 1-chloro-2,4-dinitrobenzene reduced the incorporation of [¹⁴C]EA from 0.04 ± 0.21 (SD) to 0.16 ± 0.02 mol EA/mol subunit and from automated Edman degradation of the major radioactive peptide isolated after pepsin digestion of the [¹⁴C]EA-labeled enzyme, it was concluded that the reaction of EA takes place with cysteine 47 of GST P1-1.

When GST P1-1 was inactivated with a 5-fold molar excess of EA, adding an excess of glutathione resulted in full restoration of the catalytic activity in about 120 h.

These findings may have several implications. Under normal physiological conditions the inhibition of GST P1-1 by covalent binding of EA would be reversed by glutathione, leaving reversible inhibition by the glutathione conjugate of EA and by EA itself as the main mechanism of inhibition; however, when glutathione levels are low the covalent inhibition might be predominant, resulting in a completely different time course for the inhibition.

INTRODUCTION

Conjugation with the tripeptide glutathione is considered to be an important detoxification reaction for electrophilic xenobiotics, including numerous cytostatic agents. In general this reaction is catalyzed by GST and includes GST in cytosol and belongs to one of four multigene families, termed alpha, mu, pi, and theta. The effectivity of many clinically useful anticancer drugs is severely limited by drug resistance. Several findings suggest the involvement of GST, besides other mechanisms, especially with respect to resistance to alkylating agents such as chlorambucil and melphalan (5-7). These findings include GST overexpression of especially the pi class in tumors (8, 9), the direct conjugation of alkylating agents by GST (10, 11), and the overexpression of GST in yeast and mammalian cell lines by genetic engineering which confers to these organisms resistance to alkylating agents (6, 12). A promising strategy to overcome this alkylator resistance phenotype may be based on inhibition of GST.

The diuretic drug ethacrynic acid, an α,β-unsaturated ketone, is a potent reversible inhibitor of GST isoenzymes (13-15) and has been used to study the role of GST in drug resistance in vitro, using cells lines (16), and colon tumor xenographs (17) and in a phase I clinical study with the cytostatic agent thiopeta (18). Moreover, a concentration-dependent inhibition by ethacrynic acid of the enzyme-catalyzed conjugation of glutathione with the clinically important alkylating agent chlorambucil has been reported (11). The reversible inhibition would further be enhanced by the formation of the glutathione conjugate of ethacrynic acid, which is an even stronger inhibitor for all GSTs but the pi class (14).

Since ethacrynic acid also contains an α,β-unsaturated ketone moiety, the present study was designed to investigate the reversible covalent interaction of ethacrynic acid with glutathione as well as with GST P1-1. The interaction of ethacrynic acid with this enzyme was included, since the inactivation of the GST of the pi class in several cases has been shown to be the result of the modification of a highly reactive cysteine residue (25, 26) and since pi class, next to alpha, is one of the primary GST classes that are involved in drug resistance (5).

MATERIALS AND METHODS

Chemicals and Enzymes. Ethacrynic acid [2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxyl]acetic acid, S-hexylglutathione, N-acetyl-cysteine, and Tris were from Sigma Chemical Co., St. Louis, MO. Epoxy-activated Sepharose 6B was purchased from Pharmacia, Uppsala, Sweden. [¹⁴C]Ethacrynic acid (15 mCi/mmol) was purchased from Amersham, Buckinghamshire, United Kingdom. Trifluoroacetic acid was from J. T. Baker Chemical Co., Inc., Philipsburg, NJ. Pepsin from porcine gastric mucosa was obtained from Boehringer Mannheim, Mannheim, Germany.

The radioactive conjugate of ethacrynic acid was prepared by adding 6 μmol of glutathione in 180 μl of 0.1 m potassium phosphate buffer, pH 8, with 50% ethanol, to 1.3 μmol of [¹⁴C]ethacrynic acid. After overnight incubation, the glutathione conjugate of ethacrynic acid was purified by preparative RP-HPLC using Zorbax ODS (Dupont; 21.2 x 250 mm), eluted at a flow rate of 4 ml/min with 0.01% formic acid (solvent I) and methanol (solvent II), with a linear gradient of 40-100% solvent II in 60 min (k' = 2.0 and 3.1 for the conjugate and ethacrynic acid, respectively). About 70% conversion of ethacrynic acid to the glutathione conjugate was obtained. Methanol was removed under N₂ after which a stock solution (of 136 μM) of the glutathione conjugate...
was stored at -30°C. A product of 95% purity was obtained as judged with RP-HPLC analysis, with a retention time identical to that of the nonradioactive conjugate (14).

The N-acetyl-L-cysteine conjugate of ethacrylic acid was prepared, in analogy to the synthesis of the glutathione conjugate (14). The H nuclear magnetic resonance (400 MHz, D2O) spectra of the N-acetyl-L-cysteine conjugate are consistent with the expected structure (19), with the following information for the ethacrylic acid part of δ 7.81-7.79 (dd, 1H, J = 8.8 Hz), δ 7.23 (d, 1H, J = 8.8 Hz), 4.99 (s, 2H), 3.8 (m, 1H), 1.95/1.81 (dm, 2H), and for the N-acetyl-L-cysteine part of δ 2.19 (s, 3H), -CH3. The proton signals of the carboxyl group overlap with δ 4.7, while the signals of the protons next to the sulfonamide (of the ethacrylic acid moiety) were found in the region of δ 2.5-3.1, as a complicated multiplet pattern.

GST P1-1 was purified from human placenta as described (19). Protein was determined by the method of Lowry et al. (27), using bovine serum albumin as standard.

Incubations. The glutathione conjugate of ethacrylic acid (0.5 mm) was incubated at 20°C with N-acetyl-L-cysteine (2.5 mm) in 0.4 ml of 0.1 m potassium phosphate buffer with 0.1 mm EDTA at three pH levels (6.4, 7.4, and 8.4). For each pH, 22 independent samples were prepared. At each time point, 20 µl were injected on RP-HPLC, using a Zorbax ODS (250 * 4.6 mm; LC Service, the Netherlands) column and eluted at a flow rate of 1 ml/min with 0.1% trifluoroacetic acid in deionized water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent C), with a linear gradient of 30-95% solvent B in 18 min, followed by 2 min at 95% solvent B. The main radioactive peak was identified, which contained >80% of the incorporated label.

Coevalent binding of [14C]ethacrylic acid was studied in a volume of 75 µl with 0.1 m potassium phosphate buffer (pH 7.4) with 0.1 mm EDTA (buffer A), after preincubation of 25 µM GST P1-1 for 75 min at room temperature with (n = 3) or without (n = 2) 1 mM CDNB, whereafter the enzyme was incubated for 110 min with [14C]ethacrylic acid (final concentration, 100 µM). Enzyme-bound ethacrylic acid was separated from ethacrylic acid by RP-HPLC (Vydac TPS column; Chrompack, the Netherlands; 200 * 3 mm). Elution was performed with a flow of 0.6 ml/min, with solvent A (see above) and 0.1% trifluoroacetic acid in acetonitrile (solvent C), with a linear gradient of 30-60% solvent C in 30 min (k' = 4.0, and 6.3 for ethacrylic acid and enzyme with bound ethacrylic acid, respectively). UV detection (at 214 nm) was used to identify the enzyme peak, while simultaneously the radioactivity was measured using an on-line radiochemical detector.

The [14C]ethacrylic acid-labeled GST P1-1 (0.25 mg) was digested with pepsin [enzyme/protein ratio, 1/20 (w/w)] in 0.05 m Tris/H3PO4 (pH 1.8) for 18 h at 37°C. The pepsin-peptide mixture was purified on a RP column (C18, Vydac Protein & Peptide USA; 250 * 4.6 mm) and eluted with solvents A and C (see above), 5 min isocratically at 100% solvent A, followed by a linear gradient from 0-60% solvent C in 70 min (flow rate, 1 ml/min). The main radioactive peak was repeatedly purified on the same column. The peptide was degraded using automated Edman degradation on an Applied Biosystems Model 475 peptide sequencer on-line connected to a Model 120A PTH analyzer.

The catalytic activity of GST P1-1, inactivated with ethacrylic acid, was monitored after the addition of glutathione. GST P1-1 (1 µM) was preincubated in buffer A (see above) with or without 10 µM ethacrylic acid (final volume, 200 µl), after which glutathione was added (final concentrations, 0, 10, 100, and 1000 µM). These incubations were performed in triplicate, at room temperature. At various time points, 20-nmol enzyme samples were transferred to cuvetes, after which the catalytic activity towards CDNB was measured (28). A time series was stopped when the remaining catalytic activity in the corresponding blank incubation was less than 70%.

To study the interaction of GST P1-1 with the glutathione conjugate of [14C]ethacrylic acid, seven independent samples of 10 µM GST P1-1 were incubated at room temperature with 2 µM radioactive glutathione conjugate in buffer A (see above) (final volume, 50 µl). To separate the glutathione conjugate and free ethacrylic acid from the enzyme with bound ethacrylic acid, 30 µl were injected on the Vydac TPS column (see above).

RESULTS

The occurrence of the retro Michael cleavage of the glutathione conjugate of ethacrylic acid was studied by incubation of the glutathione conjugate of ethacrylic acid with an excess of N-acetyl-L-cysteine (Fig. 1). The transfer of the ethacrylic acid moiety to N-acetyl-L-cysteine was followed with time by quantification of the glutathione and N-acetyl-L-cysteine conjugates of ethacrylic acid on RP-HPLC. The rate of transfer increased with increasing pH, with pseudo first order rates of 0.010, 0.040, and 0.076 h⁻¹ for pH 6.4, 7.4, and 8.4, respectively. After 180 h of incubation at pH 8.4, an equilibrium was reached between the glutathione and the N-acetyl-L-cysteine conjugate, suggesting that the distribution of ethacrylic acid over glutathione and N-acetyl-L-cysteine is mainly determined by their relative concentrations.

Retro Michael cleavage can also occur with GST P1-1-bound ethacrylic acid, if the assumption is right that ethacrylic acid reacts with a cysteine residue of GST P1-1 (19). In order to check this assumption, the incorporation of [14C]ethacrylic acid in GST P1-1 after preincubation with CDNB was studied, which is known to inactivate GST P1-1 by modification of cysteine 47 (25). Identical to an earlier study (19), 0.94 ± 0.21 nmol label/nmol GST P1-1 could be incorporated in blank incubations. As expected, CDNB protects against incorporation of ethacrylic acid; 0.16 ± 0.02 (SD) nmol label/nmol GST P1-1 could be incorporated after preincubation with CDNB, supporting the hypothesis that ethacrylic acid reacts with cysteine 47 of GST P1-1. In order to definitely identify the amino acid involved in the reaction, the GST P1-1 with bound ethacrylic acid was digested with pepsin and the resulting peptides were separated on HPLC. A main radioactive peak was identified, which contained >80% of the radioactivity, eluting at 45 min (Fig. 2). The amino acid sequence of this peptide indicated that it spans residues 44-46 in the primary amino acid sequence of GST P1-1 (Lys-Ala-Ser) (29-31), while an unknown residue was observed in the 4th cycle (presumably the cysteine-ethacrylic acid adduct). Thus it was again concluded that cysteine 47 is the main target site.
Then, GST P1-1 (1 μM) was incubated with ethacrynic acid (10 μM), resulting in 90% loss of activity towards CDNB, and glutathione was added. The catalytic activity toward CDNB was measured over a 120-h period (Fig. 3). Full restoration of the catalytic activity occurs with 0.1 and 1 mM glutathione (Fig. 3, inset). The 10 μM incubation initially shows partial restoration of catalytic activity, but after prolonged incubation loss of catalytic activity is observed, probably due to oxidation. This is also observed in the corresponding control incubation (a 30% loss of activity in about 30 h; result not shown). Without a trapping agent for free ethacrynic acid, no restoration of activity was observed (Fig. 3).

In order to investigate whether retro Michael cleavage of the glutathione conjugate of ethacrynic acid concomitant with incorporation of ethacrynic acid in GST P1-1 occurs, a 5-fold molar excess of the enzyme was incubated with the glutathione conjugate of [3H]ethacrynic acid. A time-dependent increase of enzyme-bound label was observed (Fig. 4), in accordance with the reversible nature of the reactions.

**DISCUSSION**

α,β-unsaturated aldehydes and ketones have long been known to form conjugates with glutathione, both spontaneously and enzyme catalyzed (32). The extent to which the enzyme plays a role differs widely among members of this class of compounds (33). The chemical reaction involved in the conjugation of ethacrynic acid and structurally related compounds, a Michael addition, is reversible. In the present study, it was shown that this retro Michael cleavage of ethacrynic acid and glutathione indeed occurs. Thus, ethacrynic acid may be transferred from one low molecular weight compound to another or to reactive and accessible cysteines in proteins, e.g., cysteine 47 of GST P1-1 as observed. Transfer from the glutathione conjugate of ethacrynic acid might take place in one of two ways, namely a release from the glutathione conjugate in the active site of the enzyme and/or reconjugation of released ethacrynic acid from the free glutathione conjugate in the incubation medium, followed by binding to the enzyme. This transfer phenomenon is probably a common feature of α,β-unsaturated aldehydes and ketones; transport via a thiol conjugate and subsequent regeneration of the reactive agent thus may be involved in the biological activity of such adducts (23).
The nature of the inhibition of GST by ethacrynic acid has been studied in detail, since it was reported that ethacrynic acid in vivo bound covalently to rat GST 3-4 (34). Previously we showed that ethacrynic acid and its glutathione conjugate were potent reversible inhibitors of GST isoenzymes, with 50% inhibitory values in the range of 1–10 μM (14). This reversible inhibition was suggested to be the predominant inhibitory mechanism in vivo, since the incorporation of ethacrynic acid in GST 3-4 did not appear to inactivate the enzyme (14, 19). In the case of GST P1-1, it was shown recently that the mechanisms of the reversible inhibition by ethacrynic acid and its glutathione conjugate were distinct (noncompetitive and competitive, respectively) (35). In the alpha and mu class the conjugate of ethacrynic acid was a more potent reversible inhibitor than the parent compound, while for the pi class some conflicting results have been reported (14, 35).

GSTs of the pi class are inhibited by covalent binding of α,β-unsaturated aldehydes and ketones; acrolein, a toxic aldehyde that occurs as an environmental pollutant (36), and also ethacrynic acid (19), specifically inactivated GST of the pi class. It is now clear that the inhibition is only transitory, since the chemical reaction is reversible. Full restoration of the catalytic activity can be achieved by prolonged incubation with an excess of glutathione. In an earlier study, only marginal inactivation of GST 7-7 by ethacrynic acid was observed using overnight dialysis experiments (14). Presumably the reversibility of the chemical reaction contributed to the failure of this dialysis experiment to detect time-dependent inhibition of GST P1-1 by ethacrynic acid.

The implications of the present findings may be several. Firstly, this mode of action, reversible covalent binding to GST P1-1 and glutathione, may have some significance for the use of ethacrynic acid as an in vivo inhibitor for GST P1-1 in drug resistance. Under normal physiological conditions [glutathione concentration 1–10 mM (1)], glutathione may be expected to reverse any covalent binding of ethacrynic acid to GST P1-1, and the inhibition of GST would occur only reversibly, through the glutathione conjugate of ethacrynic acid and of ethacrynic acid itself. However, in those cells with high levels of GST P1-1 and/or low levels of glutathione, covalent inhibition of GST P1-1 might be predominant. The time course of inhibition would be completely different in these two cases.

Recently, it was shown that chronic exposure of human colon carcinoma cells to ethacrynic acid led to a 2–3-fold increase of GST P1-1 activity, by an induction of the enzyme at the transcriptional level (37). This phenomenon has been proposed by Talalay et al. (38) to be a general one; compounds that contain a Michael acceptor or from which a Michael acceptor can be formed during metabolism are usually inducing agents for GST. The contrasting effects observed for Michael acceptors, i.e., inhibition of GST by covalent modification and induction of GST, deserve further attention.

Furthermore, some α,β-unsaturated aldehydes are established inhibitors of GST. In the case of 4-hydroxynonenal and related compounds, it was shown that inhibition of DNA synthesis was involved, presumably as a result of a reaction with a functional sulfhydryl group of DNA polymerase (23). More recently, another type of growth inhibition has been reported, which involves the α,β-unsaturated ketone 1,1′-prostaglandin J2, a cyclopentenone prostaglandin which readily forms glutathione conjugates (39). These conjugations should in principle also be able to undergo retro Michael cleavage to reform the parent compounds. Interestingly, it has been shown that ethacrynic acid, along with other inhibitors of GST, also has antiproliferative effects on cell lines (40), which seem to be reversible.

REFERENCES


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