Active Site-directed Irreversible Inhibition of Glutathione S-Transferases by the Glutathione Conjugate of Tetrachloro-1,4-benzoquinone*

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Purified glutathione S-transferases from rat liver cytosol are irreversibly inhibited by the glutathione conjugate of tetrachloro-1,4-benzoquinone, 2-S-gluathionyl-3,5,6-trichloro-1,4-benzoquinone. The inhibition is due to covalent binding in or near the active site, resulting in modification of a single amino acid residue/subunit, presumably a cysteine residue. The amount of inhibition is related to the molar ratio of the inhibitor and the enzyme and is independent of the enzyme concentration. A 70-80% inhibition is obtained on incubating the enzyme with a 5-fold molar excess of the conjugate. Complete 100% inhibition is never reached. The derivative bound to the enzyme still possesses a quinone structure and is able to react with thiol-containing compounds. Reduction of the enzyme-bound quinone abolishes its reactivity but does not decrease the inhibition. At 0 °C, the glutathione conjugate of tetrachloro-1,4-benzoquinone inhibits the glutathione S-transferases at a much higher rate than the corresponding β-mercaptoethanol conjugate, indicating a distinct targetting effect of the glutathione moiety. However, the parent compound, tetrachloro-1,4-benzoquinone, also has a considerable affinity for the enzymes. Although it does not react as fast as the glutathione conjugate, it reacts with the same amino acid residue. Protection from inhibition by the substrate analog S-hexylglutathione also indicates an active site-directed modification. Small but significant differences exist between the different rat liver transferase isoenzymes; using a 20-fold molar excess the inhibition ranges from 78 to 98% for the conjugate, and from 72 to 93% for the quione, with isoenzyme 1-1 being the most and isoenzyme 2-2 the least inhibited forms.

The glutathione S-transferases (EC 2.5.1.18) are a group of dimeric isoenzymes involved in the conjugation of glutathione to a large number of electrophilic xenobiotics (1). Although detected in many tissues, they are mainly present in the liver (1). A variety of inhibitors of these enzymes is known, but very few systematic studies have been performed (2-4). Several quinones are known to inhibit glutathione S-transferases (5-7). Among these are several naturally occurring compounds such as juglone and vitamin K, as well as synthetic ones such as tetrachloro-1,2-benzoquinone. The nature of this inactivation, however, is not clear. Quinones as a rule react efficiently with sulphydryl groups, as has been described both for the reaction with protein-SH groups as well as with low molecular weight compounds such as cysteine (8). Evidence exists for the presence of a sulphydryl group in the active site of the glutathione S-transferases (10, 11).

We have previously shown that tetrachloro-1,4-benzoquinone (1,4-TCBQ)1 reacts very efficiently with protein-SH groups and with glutathione (9). This compound, in contrast to quinones with one or more carbon-hydrogen bonds, retains its oxidized structure after conjugation with glutathione (Fig. 1) and in fact will continue to react with glutathione molecules until all four chlorine atoms have been substituted (9). Thus initially a reactive conjugate is formed which might selectively bind at and react with the active site of the glutathione S-transferases. The combination of a reactive group and a substrate moiety could lead to a preferential covalent binding to glutathione S-transferases. In the present paper the occurrence of such binding and the subsequent inhibition of the glutathione S-transferases is presented.

MATERIALS AND METHODS

1H-Labeled glutathione (1 Ci/mmol) was purchased from Du Pont-New England Nuclear; tetrachloro-1,4-benzoquinone was from Merck. The 2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone (GS-1,4-TCBQ) was prepared by adding, with vigorous shaking, 2.7 mg of glutathione in 1 ml of water to 20 mg of 1,4-TCBQ in a volume of 25 ml of methanol. After evaporating the solvents, 2 ml of water was added, and the remaining 1,4-TCBQ was removed by repeated extractions with ethyl acetate. The identity and purity of the conjugate was established by field desorption mass spectrometry (m/z = 518) and HPLC (RP18 column, elution with 37% methanol and 63% 50 mM Tris phosphate buffer, pH 2.5). The radioactive conjugate was prepared similarly. The β-mercaptoethanol conjugate of tetrachloro-1,4-benzoquinone was prepared by slowly adding a 1.7 molar excess of β-mercaptoethanol (10-fold diluted in water) to 1,4-TCBQ in methanol and evaporating the solvents as well as the excess of mercaptoethanol. Under these conditions more than 95% of the 1,4-TCBQ reacted, while field desorption mass spectrometry and HPLC revealed no diconjugates (m/z = 286; RP18 elution with 55% of the above mentioned buffer in methanol). For both conjugates the oxidized (quinone) structure was also confirmed by HPLC analysis; after reduction with ascorbic acid K+ values shifted from 4.2 to 3.8 for the glutathione conjugate and from 6.8 to 4.6 for the mercaptoethanol conjugate. Oxidation with dichlorodicyanoquinone did not alter the elution.

1 The abbreviations used are: 1,4-TCBQ, tetrachloro-1,4-benzoquinone; GS-1,4-TCBQ, 2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone; CDNB, 1-chloro-2,4-dinitrobenzene; HPLC, high performance liquid chromatography.

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Unbound conjugates by gel filtration, the inhibition of CDNB a 50-pmol enzyme sample was transferred to a cuvette containing 1 mM EDTA and 0.2 mM dithiothreitol. Both methods resulted in the same amount of binding. Glutathione S-transferases were purified from livers of phenobarbital-induced rats using S-hexylglutathione affinity chromatography (12) to a purity of 45.5 units/mg protein. The separation of the different isoenzymes was achieved by chromatofocusing, using a Pharmacia fast protein liquid chromatograph equipped with a mono-P HRS/20 column, which was eluted with Servalyte ampholytes, as described elsewhere (13). Purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and isoelectric focusing was performed according to Radola (14). The specific activities with CDNB as second substrate were: 1-1, 45.4 units/mg; 1-2, 29.3 units/mg; 2-2, 17.1 units/mg; 3-3, 37.3 units/mg; 3-4, 31.5 units/mg, and 4-4, 12.9 units/mg.

Incubations were performed at 25 or 0 °C in a 1-ml volume of 100 mM potassium phosphate buffer, pH 6.5, using enzyme concentrations of 0.05, 1, or 10 μM. Incubations with 50 nM enzyme were performed in 1-ml cuvettes in which the inhibitor was added to the enzyme solution 5 min before glutathione was added (final concentration, 1 mM). By the addition of such an excess of glutathione the remaining 1,4-TCBQ is converted into a complete (4-fold) conjugate (9). This conjugate does not bind to the enzyme and neither does it inhibit the conjugation of CDNB competitively, when using low enzyme concentrations. Incubations with 1 and 10 μM enzyme concentrations were performed in tubes from which, 5 min after addition of the inhibitor, a 50-μmol enzyme sample was transferred to a cuvette containing 1 mM glutathione. CDNB-conjugation was measured according to Habig et al. (15) using a CDNB concentration of 1.0 mM.

Covalent binding of [3H]GS-1,4-TCBQ was studied using the above mentioned incubation conditions either by extensive washing of methanol-precipitated glutathione S-transferases (4 × 4 ml of methanol, 1 × 1.5 ml of diethyl ether) and solubilization of the protein in Soluene 550 (Packard) or by separation of the glutathione S-transferases and the unbound conjugate on a Bio-Gel P-6DG column (1.5 × 10 cm) eluted with 25 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.2 mM diethiothreitol. Both methods resulted in the same amount of binding.

RESULTS

The glutathione conjugate of tetrachloro-1,4-benzoquinone (GS-1,4-TCBQ) displayed a strong inhibitory effect toward glutathione S-transferases. About 70% inhibition was reached at a 5-fold molar excess of GS-1,4-TCBQ to the monomeric enzyme concentration (Fig. 2a). This effect was very rapid; at 25 °C, the inhibition was complete within 30 s.

Radiolabeled GS-1,4-TCBQ was used to establish whether the inhibition was of a covalent nature. Covalent binding was detected to a maximal extent of 0.85 nmol/nmol of glutathione S-transferase monomer at a 25-fold molar excess of conjugate (Fig. 3). A striking similarity was observed between the inhibition curve and the covalent binding, as measured at different molar ratios of conjugate to glutathione S-transferase monomers (Fig. 3). The fact that the inhibition is related to the ratio between the enzyme and inhibitor (see Fig. 2a) similar curves for 50 nM and 10 μM enzyme concentration) rather than to the enzyme concentration also indicates a covalent modification. After separation of the transferases from the unbound conjugates by gel filtration, the inhibition of CDNB conjugation was equal to that observed before separation.

Reduction of the quinone structure of the conjugate with ascorbic acid, before addition of the transferase, resulted in loss of inhibitory action (Table I). Incubation of [3H]glutathione (2 mM) with glutathione S-transferase (10 μM) which was preincubated with the GS-1,4-TCBQ resulted in covalent binding of radioactivity to the protein, indicating the quinone structure to be still intact. Addition of ascorbic acid (2 mM) (9) or β-mercaptoethanol (2 mM) to the modified protein prior to the addition of [3H]glutathione completely prevented the labeling of the protein.

In order to estimate the contribution of the glutathione moiety to the "targeting" of the inhibitor, the rate of inhibition of glutathione S-transferase by GS-1,4-TCBQ was compared with the rate of inhibition by the β-mercaptoethanol conjugate of 1,4-TCBQ. The experiments were performed at 0 °C because at 25 °C complete inhibition was reached within 30 s after mixing. As shown in Fig. 4a, a large difference existed in the rate of inhibition between the two trichloro compounds; the glutathione conjugate inhibited the transferases at a much higher rate than the β-mercaptoethanol conjugate. The inhibition of only 20% after 12 min of incubation with the mercaptoethanol conjugate is due to an incomplete reaction, since both addition of a 20-fold surplus of GS-1,4-TCBQ after the incubation with the mercaptoethanol conjugate, as well as raising the temperature to 25 °C for 10 min, resulted in a 90% inhibition. The "parent" compound, 1,4-TCBQ, was also tested for its inhibitory action of glutathione S-transferases. Fig. 4a also shows the time course of inactivation by 1,4-TCBQ. The conjugate reacts faster than the quinone itself at 0 °C, but at 20 °C no difference could be observed (complete inhibition within 30 s in both cases). As is shown in Fig. 2b, there is only a small difference between the quinone and its glutathione conjugate, when looking at the ratio between inhibitor and enzyme, after 5 min of incubation with the inhibitors at 25 °C.

Reduction of the bound 1,4-TCBQ (addition of ascorbic acid after 5 min of incubation), before the addition of glutathione, did not result in a weaker inhibition, demonstrating that the bound glutathione does not play a major role in the inhibition once the quinone is bound to the protein (Table I). The fact that reduction of the bound conjugate results in a stronger inhibition than reduction of the bound quinone (Table I) also suggests a binding in the vicinity of the glutathione binding site. When radiolabeled GS-1,4-TCBQ was added to glutathione S-transferase which was preincubated with 1,4-TCBQ, no covalent binding of radioactivity was observed, indicating that 1,4-TCBQ and GS-1,4-TCBQ bind at a common site (Table II).

Additional evidence for an active site-directed modification was obtained by investigating the effect of S-hexylglutathione on the time course of the inhibition. This compound inhibits glutathione S-transferases competitively with regard to glutathione and noncompetitively with regard to CDNB (1). S-Hexylglutathione markedly delayed the rate of inhibition by GS-1,4-TCBQ (Fig. 4b). Raising the temperature to 25 °C for 10 min resulted in the same amount of inhibition with and without S-hexylglutathione.

Incubation of the major purified isoenzymes present in rat liver, at a 50 nM concentration with a 20-fold molar excess of both GS-1,4-TCBQ and 1,4-TCBQ, resulted in a remaining activity varying from 2.1 to 21.7% for the conjugate and 6.6 to 27.5% for the quinone (Table III). For both compounds, isoenzyme 1-1 gave the lowest and isoenzyme 2-2 showed the highest remaining activity after inhibition. Increasing the
Ascorbic acid added before 95.4
Ascorbic acid added after 5.1
Ascorbic acid incubated with 102.8
Ascorbic acid added after

Table. This concentration of ascorbic acid is known to completely reduce 1,4-TCBQ and its conjugate (9). Values are the average of 4-6 incubations.

Table I

| Incubation of glutathione S-transferase with | Remaining activity |
|--------------------------------------------|--------------------|
| GS-1,4-TCBQ | 1,4-TCBQ |
| Inhibitor | 8.6 ± 1.4 | 21.4 ± 2.0 |
| Ascorbic acid added after 5 min prior to GSH | 1.5 ± 0.8 | 16.1 ± 1.1 |
| Ascorbic acid added before the incubation with enzyme | 95.4 ± 5.2 | 90.8 ± 0.4 |
| Ascorbic acid added after termination with GSH | 5.1 ± 0.8 | 21.6 ± 0.9 |
| Ascorbic acid incubated with inhibitor before incubation with enzyme | 102.8 ± 5.2 | 99.8 ± 10.4 |

**Fig. 2.** a, inhibition of glutathione S-transferases (GST) by the glutathione conjugate of tetrachloro-1,4-benzoquinone, 2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone, at two different enzyme concentrations. b, comparison of tetrachloro-1,4-benzoquinone (●) and its glutathione conjugate (○) in the inactivation of glutathione S-transferases. For both panels, the concentration of the inhibitor is expressed in relation to the amount of monomeric enzyme (i.e. subunit) used. The activity was determined as rate of CDNB conjugation and expressed as percentage of activity as compared to enzyme without inhibitor. Each point represents at least two incubations.

**Fig. 3.** Covalent binding to and inhibition of glutathione S-transferases by the glutathione conjugate of tetrachloro-1,4-benzoquinone, the 2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone. Binding was determined by using the tritiated conjugate and separation of bound and free label by gel filtration chromatography. Activity was determined as the rate of CDNB conjugation and expressed as percentage of activity as compared to enzyme without inhibitor. 1 μM enzyme was incubated for 5 min with different amounts of inhibitor (expressed as related to the amount of monomeric enzyme).

**Fig. 4.** a, time course of inhibition of glutathione S-transferases by tetrachloro-1,4-benzoquinone (●), its glutathione conjugate (○), and its β-mercaptoethanol conjugate (+). Inhibitor (5 μM) and enzyme (0.5 μM) were mixed at 0 °C, and 50-nmol enzyme samples were transferred into cuvettes containing 1 mM glutathione, thus terminating the reaction at different time intervals. The remaining activity was determined after addition of 1 mM CDNB as second substrate. b, effect of S-hexylglutathione (100 μM) on the time course of inhibition of glutathione S-transferases, the glutathione conjugate of tetrachloro-1,4-benzoquinone (● = incubation in the presence of S-hexy1glutathione, ○ = without S-hexy1glutathione). The same experimental procedure was used as described for a.

**Table II**

| Incubation | nmol bound/nmol GST monomer |
|------------|----------------------------|
| GSTs +[^3]H]GS-1,4-TCBQ | 0.70 ± 0.055 |
| GSTs, preincubated with 1,4-TCBQ +[^3]H]GS-1,4-TCBQ | 0.08 ± 0.003 |

ratio of inhibitor/enzyme did not markedly influence these figures.

**DISCUSSION**

Glutathione S-transferases are strongly inhibited as a result of covalent modification by the glutathione conjugate of t-
irreversible inhibition of glutathione S-transferase isoenzymes by 2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone and tetrachloro-1,4-benzoquinone

50 nM enzyme was incubated with 1 μM inhibitor for 5 min at 25°C, after which the reaction was terminated by addition of glutathione to a final concentration of 1 mM. The activity is expressed as a percentage of CDNB-conjugating activity without inhibitor. Mean ± S.D. is presented for 4–6 incubations.

| Isoenzyme | GS-1,4-TCBQ Remaining activity | 1,4-TCBQ Remaining activity |
|-----------|-------------------------------|-----------------------------|
| 1-1       | 2.1 ± 0.6                     | 6.8 ± 2.6                   |
| 1-2       | 16.4 ± 1.7                    | 11.2 ± 0.7                  |
| 2-2       | 21.7 ± 2.1                    | 27.5 ± 3.1                  |
| 3-3       | 7.2 ± 0.4                     | 14.8 ± 1.1                  |
| 3-4       | 4.0 ± 1.0                     | 16.3 ± 1.0                  |
| 4-4       | 2.4 ± 0.8                     | 8.2 ± 1.9                   |

glutathione for its binding site to be involved. The conjugate thus forms a unique combination of targetting and covalent modification.

A number of quinones, among which is 1,2-TCBQ, have been described to inhibit glutathione S-transferase. K_i values in the 10^{-5}–10^{-4} M range were presented (5–7). GS-1,4-TCBQ is by far the most potent covalently binding inhibitor of purified glutathione S-transferase available. Other covalently binding inhibitors described are, for example, ethylene dibromide (2) and ethacrynic acid, which react in the millimolar range (2, 3), and certain metabolically activated metabolites (e.g. 1,1-dichloroethylene) (4).

GS-1,4-TCBQ thus promises to be a powerful tool in studying the mechanism of action of the glutathione S-transferases as well as a starting point for the development of effective in vivo inhibitors of these enzymes.

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